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Modulation by endothelium of contractile responses in rat aorta in absence and presence of flunarizine

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1 The possible modulation by endothelium of phenylephreine- and prostaglandin $F_{2\alpha}$-induced mobilization of calcium for contraction in the rat aorta has been investigated. Contractions elicited by these and other agonists are inhibited in the presence of endothelium.

2 For any single concentration of phenylephrine in the presence of endothelium, the initial phasic components of contractions were significantly greater, the maximal contractions were achieved sooner and were less well maintained as compared to contractions elicited in the absence of endothelium.

3 The kinetic characteristics of contractions stimulated by single concentrations of $PGF_{2\alpha}$ were similar in the presence and absence of endothelium and did not exhibit initial phasic components of contraction.

4 Sub-maximal contractions elicited by both $PGF_{2\alpha}$ and phenylephrine in the absence of endothelium were inhibited to a greater extent by flunarizine 3 μM than equieffective contractions elicited in the presence of endothelium.

5 Maximal contractions elicited by phenylephrine (1 μM) were inhibited to a similar extent by flunarizine in the presence and absence of endothelium, but maximal contractions elicited by $PGF_{2\alpha}$ (30 μM) were inhibited by flunarizine to a greater extent in the presence than in the absence of endothelium.

6 It is concluded that an endothelium-derived factor, perhaps distinct from endothelium-derived relaxing factor, can modulate the ability of both phenylephrine and $PGF_{2\alpha}$ to mobilize calcium for contraction. This modulatory effect is associated with an enhanced mobilization of intracellular calcium. Thus, submaximal concentrations of both agonists were less dependent on extracellular calcium than on intracellular calcium to elicit contractions in the presence of endothelium, as compared to contractions elicited in the absence of endothelium.

Introduction

It is now well established that relaxant responses of vascular tissues evoked by many compounds are wholly or partially dependent on the presence of the endothelium (see review by Furchgott, 1983). It has been proposed that stimulation of the endothelium, by acetylcholine for example, leads to the liberation of a factor, endothelium-derived relaxing factor (EDRF), which acts on the smooth muscle of the vessel to produce a relaxation. EDRF has a half-life of about 6 s (Griffith *et al.*, 1984) and its liberation is dependent on extracellular calcium in rat aorta (Miller *et al.*, 1985) and partially dependent on extracellular calcium in rabbit aorta (Singer & Peach, 1982).

Agonist-induced contractions of *in vitro* vascular preparations are also modified by the presence or absence of endothelium in dog and pig coronary arteries (Cocks & Angus 1983) and in rat aorta (Allan *et al.*, 1983; Konishi & Su, 1983; Zuleica *et al.*, 1983; Eglème *et al.*, 1984a,b; Miller *et al.*, 1984; Bigaud *et al.*, 1984; Godfraind *et al.*, 1985). In rat aorta, concentration-effect curves elicited by noradrenaline, phenylephrine, guanfacine, prostaglandin $F_{2\alpha}$. ($PGF_{2\alpha}$) and 5-hydroxytryptamine (5-HT), are displaced to the left in the absence of endothelium (agonists of this type will be referred to as group 1 agonists). In contrast maximal contractions evoked by the α_2-adrenoceptor agonists oxymetazoline, clonidine and B-HT 920 (group 2 agonists) are only about 10% of those elicited by noradrenaline in the presence of endothelium, but

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are comparable to those evoked by noradrenaline in the absence of endothelium.

One of the notable differences between these two groups of agonists in the rat aorta is that while the members of group 2 are almost totally dependent on extracellular calcium to elicit contractions, group 1 agonists elicit contractions that are partially independent of extracellular calcium (Godfraind *et al.*, 1982; Nghiem *et al.*, 1982).

Therefore, among the many possible reasons for the different effect of endothelium on contractions induced by these two groups of agonists, is the possibility that an endothelial derived factor (or factors) modulates the ability of agonists to mobilize extracellular and/or intracellular pools of calcium to maintain contractions.

The effects of endothelium on the mobilization of intracellular calcium has been studied in two ways. Firstly, the kinetics of phenylephrine- and $PGF_{2\alpha}$-induced contractions in rat aorta in the presence and absence of endothelium have been examined. It is known that in this tissue the initial phasic component of phenylephrine-induced contractions is mostly due to the liberation of intracellular calcium (Godfraind *et al.*, 1982). Secondly, by investigating the inhibitory effects of the calcium entry blocker, flunarizine. Flunarizine has been shown to inhibit agonist-induced calcium influx in rat arterial tissue without a marked effect on intracellular calcium release (Godfraind & Dieu, 1981; Godfraind & Miller, 1982).

The results show that the absence of the endothelium altered the kinetics of phenylephrine-induced contractions and decreased the dependence of both phenylephrine- and $PGF_{2\alpha}$-induced submaximal contractions on intracellular calcium. This latter effect was not consistently observed with maximal concentrations of the agonists. It is concluded that there may be a single factor liberated from the endothelium having more than one action on the smooth muscle or there may be more than one factor liberated by the endothelium. A preliminary account of this work has been published (Miller & Stoclet, 1984).

Methods

Contractile experiments

Female Wistar rats of 12–14 weeks of age (280–300 g) were killed, the thorax opened and the aorta flushed gently with warm oxygenated physiological solution (composition, mM: NaCl 112, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1, $MgSO_4$ 1.2, $CaCl_2$ 1.25 and glucose 11.5) and carefully cleaned of all loosely adherent tissue before it was removed. Pairs of rings of aorta about 2 mm wide were cut from the proximal end; not more than 2 pairs were obtained from each aorta. The interior of one ring of each pair was rubbed gently with a wooden match stick. Rings were suspended between parallel hooks in organ baths containing physiological solution aerated with a mixture of 95% O_2 and 5% CO_2, under 2 g tension. Fifteen minutes later tissues were rinsed, and the tension readjusted to 2 g; tissues were then equilibrated for a further 45 min before a maximal contraction was elicited with 1 μM noradrenaline. After 30 min, when the noradrenaline contraction had stabilized, 1 μM acetylcholine was added for 10 min before rinsing with physiological solution. One hour later, during which time tissues were rinsed 3 times, contractions were elicited by increasing the concentration of phenylephrine cumulatively in approximately 3 fold steps, until a maximal response was attained. Acetylcholine 1 μM was then added for 10 min before tissues were rinsed with physiological solution. Two hours later (during which time tissues were rinsed 5 times) a second maximal cumulative concentration-effect curve was elicited followed by acetylcholine 1 μM for 10 min. The response elicited by each addition of agonist was allowed to stabilize before each increase in concentration. The same protocol was followed, substituting 30 min exposures, to single concentrations of either phenylephrine or $PGF_{2\alpha}$ in place of the cumulative additions of agonist. In the case of phenylephrine, responses measured 10 s after the initiation of contraction are referred to as phasic (Godfraind *et al.*, 1982). When necessary flunarizine 3 μM was included in the bath for 90 min before the second contraction was elicited in the presence of flunarizine. Contractions were recorded using Statham UC2 isometric force transducers linked to a Beckman dynograph type 811A.

Drugs

Noradrenaline bitartrate (Sigma) was dissolved in distilled water containing 7.9 mM Na_2SO_3 and 34 mM HCl as a stock solution of 10 mM. Acetylcholine chloride (Sigma) was prepared as a stock solution of 10 mM in NaH_2PO_4 (0.1 M). Phenylephrine HCl (Sigma) was dissolved in distilled water, $PGF_{2\alpha}$ (Dinolytic, a gift from Upjohn France) was used as supplied. Dilutions were prepared in physiological solution. Flunarizine (a gift from Janssen Pharmaceutica Belgium) was dissolved in an aqueous solution of 100 mM tartaric acid (pH 3.1) to a concentration of 1 mM and further diluted as required with distilled water. All drug concentrations are expressed in terms of the base.

Statistical analysis

The data are expressed as means ± s.e.means. Tests of significance have been made using Student's *t* test or paired *t* test. *P* values less than 0.05 were considered significant.

Results

Single maximal concentrations of noradrenaline (1 μM) induced contractions of rat aortic segments of similar magnitude in intact tissues (1.1 ± 0.2 g) and in internally rubbed tissues (1.3 ± 0.1 g). To verify the presence or absence of endothelium, acetylcholine (1 μM) was added when contractions had stabilized for 30 min (Furchgott, 1983) and it produced a relaxation of about 70% in intact tissues and no detectable effect on internally rubbed tissues. Cumulative additions of phenylephrine (1 nM to 10 μM) elicited concentration-dependent contractile responses of both intact and internally rubbed tissues. Maximal responses were not significantly different from those produced by noradrenaline in either case but concentration-effect curves in the absence of endothelium (EC_{50} $2.4 \pm 0.4 \times 10^{-8}$ M) were displaced to the left about 5 fold with respect to those in the presence of endothelium (EC_{50} $11.8 \pm 1.3 \times 10^{-8}$ M).

Single 1 μM concentrations of phenylephrine induced time-dependent increases in tension which were characterized by two phases in tissues with and without endothelium (Figure 1). An initial rapid (phasic) increase in tension over the first 10 s was followed by a secondary slow (tonic) increase in tension which, in the presence of endothelium, was maximal after about 6 min, maintained for the next 4 to 5 min and slowly declined over the next 20 min of observation. In the absence of endothelium the maximal increase in tension was observed after about 20 min and was then maintained (Figure 1). The initial phasic increase in tension in the presence of endothelium of 47.2 ± 3.9% of the eventual maximal contraction was significantly greater ($0.05 < P < 0.025$, $n = 6$) than the phasic component of contraction of 36.3 ± 2.0% induced in the absence of endothelium. The maximal degree of contraction induced by 1 μM phenylephrine, 1.3 ± 0.1 g and 1.4 ± 0.2 g in the presence or absence of endothelium respectively, was not significantly different from the maximal response of the tissue elicited by phenylephrine.

In the presence of flunarizine 3 μM, a concentration sufficient to inhibit maximally α-adrenoceptor agonist-induced influx of calcium in rat aorta (Godraind & Dieu, 1981), phenylephrine (1 μM) induced contractions were inhibited by about 50% (Figure 1). The phasic component of the contraction, shown to be mostly due to liberation of intracellular calcium and not to be dependent on extracellular calcium in this tissue (Godfraind & Kaba, 1972; Godfraind *et al.*, 1982), was only slightly affected by flunarizine.

To investigate further the different kinetics of phenylephrine-induced responses, contractions were elicited in tissues complete with endothelium by 100 nM phenylephrine and in tissues without endothelium by 30 nM phenylephrine; i.e. by concentrations of phenylephrine which would be expected to produce contractions of comparable magnitude, of about 50% of the maximal response (see above), in the presence or absence of endothelium respectively. These contractions, which amounted to 0.61 ± 0.07 g and 0.52 ± 0.04 g in the presence or absence of endothelium respectively, are represented in Figure 2. Again, the kinetics of the contractions elicited in the presence or absence of endothelium were different and the two phases of contraction could be distinguished in each

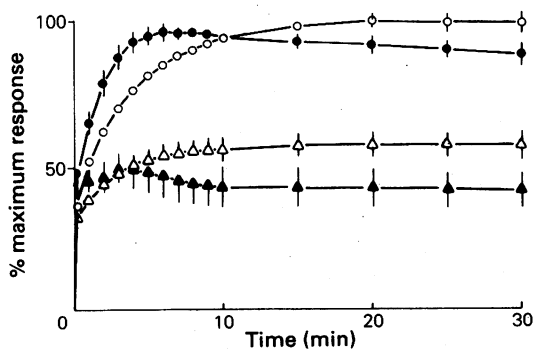

Figure 1 Concentration-time curves evoked by 1 μM phenylephrine in rat aorta in the presence (filled symbols) and absence (open symbols) of endothelium and in the absence (circles) and presence (triangles) of flunarizine 3 μM. Responses are expressed as a percentage of the maximal responses attained in the absence of flunarizine. Each curve is the mean of at least 6 observations. Vertical bars represent s.e.mean when it exceeds the size of the symbol.

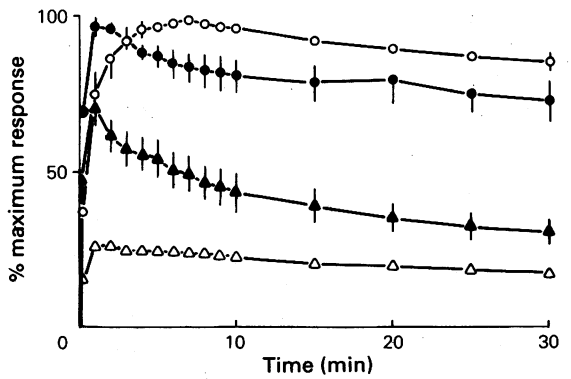

Figure 2 Concentration-time curves evoked by equieffective concentrations of phenylephrine in the absence (30 nM, open symbols) and presence (100 nM, solid symbols) of endothelium in the rat aorta in the absence (circles) and presence (triangles) of flunarizine 3 μM Responses are expressed as a percentage of the maximal response attained in the absence of flunarizine. Each curve is the mean of at least 7 observations. Vertical bars represent s.e.mean when it exceeds the size of the symbol.

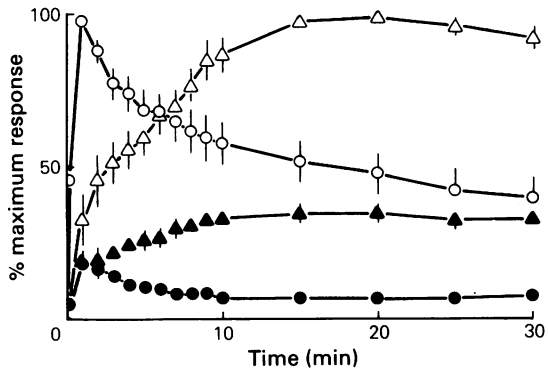

Figure 3 Contraction-time curves evoked by approximately equi-effective concentrations of phenylephrine 30 nM (circles) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 3 μM (triangles) in rat aorta in the presence of endothelium and in the absence (open symbols) and presence (solid symbols) of flunarizine 3 μM. Responses are expressed as a percentage of the maximal response attained in the absence of flunarizine. Each curve is the mean of at least 5 observations. Vertical bars represent s.e.mean when it exceeds the size of the symbol.

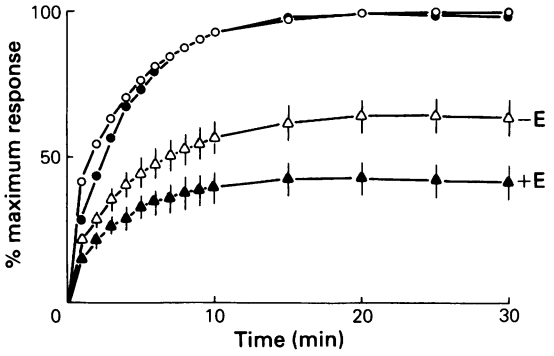

Figure 4 Contraction-time curves evoked by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 30 μM in rat aorta in the presence (solid symbols) and absence (open symbols) of endothelium and in the absence (circles) and presence (triangles) of flunarizine 3 μM. Responses are expressed as a percentage of the maximal responses attained in the absence of flunarizine. Each curve is the mean of at least 5 observations. Vertical bars represent s.e.mean when it exceeds the size of the symbol.

case. In the presence of endothelium the phasic component was pronounced and amounted to 72.3 ± 1.5% ($n = 7$) of the maximal contraction which was achieved after about 1 to 2 min and declined slightly thereafter. In the absence of endothelium the phasic component was significantly ($P < 0.001$) smaller, 37.3 ± 5.6% ($n = 6$) of the maximal response, and the maximal contraction was reached after about 5 min, maintained for the next 5 min and then declined over the succeeding 20 min.

In the presence of flunarizine 3 μM these submaximal contractions were strongly inhibited (Figure 2). Flunarizine had a greater inhibitory effect in the absence than in the presence of endothelium (maximal inhibition of about 79% and 59% respectively) and the initial phasic component of contraction was significantly ($P < 0.001$, paired t test) more resistant to flunarizine in tissues complete with endothelium.

To verify that these markedly different kinetics of submaximal contractions induced by phenylephrine were indeed due to the presence of endothelium and not related to the level of contraction, responses were also elicited by 30 nM (about EC_{20}) phenylephrine in arteries complete with endothelium. The time course of this contractile response (Figure 3) was similar to that produced by 100 nM phenylephrine in the presence of endothelium (Figure 2). The maximal response was attained after about 1 min and then declined markedly over the next 29 min. The kinetics of this response obviously differed from those elicited by the same concentration of phenylephrine (30 nM) in the absence of endothelium (Figure 2). This relatively small contraction (of 0.32 ± 0.02 g, $n = 7$) was inhibited by flunarizine 3 μM by about 85% (Figure 3).

In a second series of experiments noradrenaline (1 μM) elicited maximal contractions of 1.3 ± 0.2 g in the presence of endothelium and of 1.5 ± 0.1 g in the absence of endothelium. These contractions were not significantly different. In the presence of endothelium, acetylcholine relaxed these contractions by about 56%. Subsequent contractions elicited by $PGF_{2\alpha}$ 30 μM were not significantly different from those induced by noradrenaline and were equal to 120.1 ± 16.6% ($P < 0.3$, $n = 5$, paired t test) of the noradrenaline response in the presence and to 102.1 ± 2.9% ($P < 0.5$) in the absence of endothelium. Smaller concentrations of $PGF_{2\alpha}$ produced contractions of greater magnitude in the absence than in the presence of endothelium, consistent with the reported displacement of concentration-effect curves to the left in the absence of endothelium (Eglème *et al.*, 1984b). For all concentrations of $PGF_{2\alpha}$ studied, greater variations in responses were noted than were seen with phenylephrine.

$PGF_{2\alpha}$ (30 μM)-induced contractions did not exhibit an obvious initial phasic component of contraction (Figure 4) but rather a steady increase in tension that reached a maximum after about 15 to 20 min and was maintained thereafter. Flunarizine (3 μM) inhibited these $PGF_{2\alpha}$-induced contractions to a significantly greater extent in the presence of endothelium than in its absence (Figure 4), except during the initial 4 to 5 min of the contraction.

Concentrations of $PGF_{2\alpha}$ that produced similar

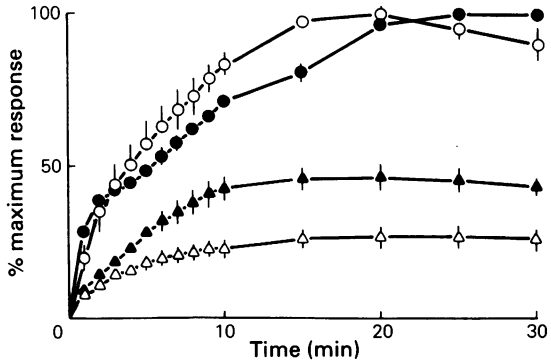

Figure 5 Contraction-time curves evoked by approximately equi-effective concentrations of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the absence (3 μM, open symbols) and presence (10 μM, solid symbols) of endothelium in rat aorta in the absence (circles) and presence (triangles) of flunarizine 3 μM. Responses are expressed as a percentage of the maximal response attained in the absence of flunarizine. Maximal contractions amounted to 0.69 ± 0.01 g and 0.65 ± 0.01 g in the presence and absence of endothelium respectively. Each curve is the mean of at least 5 observations. Vertical bars represent s.e.mean when it exceeds the size of the symbol.

submaximal contractions (about 60% of maximum) in the presence or absence of endothelium (10 μM and 3 μM respectively) exhibited similar kinetics (Figure 5). These contractions were also inhibited by flunarizine 3 μM and, like submaximal contractions evoked by phenylephrine, flunarizine had a more pronounced inhibitory effect in the absence than in the presence of endothelium (Figure 5). The maximal degree of inhibition by flunarizine of these intermediate contractions evoked by $PGF_{2\alpha}$ (about 60% of the maximal response) was similar to the degree of inhibition of intermediate concentrations of phenylephrine. $PGF_{2\alpha}$ 3 μM elicited a contraction of about 25% of maximum (0.42 ± 0.01 g) in the presence of endothelium. These contractions exhibited similar kinetic features to those seen with larger concentrations of $PGF_{2\alpha}$ in the absence or presence of endothelum and were also inhibited by about 60% by flunarizine (Figure 3).

Discussion

This study confirms that contractile responses elicited by phenylephrine and $PGF_{2\alpha}$ in the rat aorta are influenced by the presence of the endothelium (Eglème *et al.*, 1984b; Miller & Stoclet, 1984).

The time courses of contractions induced by various single concentrations of phenylephrine in the presence of endothelium were obviously different from those induced in the absence of endothelium whether compared at the same concentration (Figure 1 and Figures 2 and 3), or by comparing responses induced by approximately equi-effective concentrations of phenylephrine in the presence and absence of endothelium (Figures 1 and 2). This difference consisted of a more pronounced phasic component of contraction and a maximal response achieved in a shorter time in the presence of endothelium. The phasic component of contractions elicited by α-adrenoceptor stimulation in this tissue has been associated with intracellular calcium release and the tonic contraction with influx of extracellular calcium (Godfraind & Kaba, 1972; Godfraind *et al.*, 1982; Soares de Moura & Socrates Lima, 1983). No obvious phasic component of contraction was induced by the agonist $PGF_{2\alpha}$ and similar time courses of contraction were seen when contractions were induced with both maximal and submaximal concentrations in the presence and absence of endothelium (Figures 4 and 5). Thus phenylephrine, unlike $PGF_{2\alpha}$ induces an early, transient, contractile response that is markedly enhanced in the presence of endothelium.

However, contractions elicited by various concentrations of both phenylephrine and $PGF_{2\alpha}$ in the presence and absence of endothelium were inhibited incompletely by the calcium entry blocker, flunarizine (3 μM). This concentration of flunarizine has been shown to inhibit maximally α-adrenoceptor agonist and $PGF_{2\alpha}$-stimulated influx of calcium (Godfraind & Dieu, 1981; Godfraind & Miller, 1982) and not to affect either basal activity or agonist stimulation of the endothelium (Miller *et al.*, 1985) in rat blood vessels. Therefore, at all concentrations of phenylephrine and $PGF_{2\alpha}$ tested, the flunarizine-resistant component of contraction can be considered to be dependent on intracellular calcium.

Comparing approximately equi-effective submaximal concentrations of phenylephrine and $PGF_{2\alpha}$, flunarizine was less effective in the presence than in the absence of endothelium. In the case of phenylephrine this difference was mostly due to the marked increase in the phasic component of contraction which was relatively resistant to flunarizine (Figure 2). In the case of $PGF_{2\alpha}$, contractions elicited in the presence and absence of endothelium were similar over the first 3 min (Figure 5) but thereafter differed considerably.

These observations, taken together, suggest that in the presence of endothelium, submaximal contractions are more dependent on intracellular calcium than on extracellular calcium. They also suggest that while the two agonists, phenylephrine and $PGF_{2\alpha}$ are both capable of releasing intracellular calcium, the time course of this release is quite different. Nevertheless, in both cases it is the early part of the contractile response that is primarily dependent on liberation of intracellular calcium.

Maximal contractions induced by phenylephrine

can also be interpreted to be more dependent on intracellular than extracellular calcium in the presence of endothelium, since the phasic component of contraction was potentiated in the presence of endothelium, as was the total contraction in the first 6 or 7 min (Figure 1), although thereafter no marked difference was evident. However, maximal contractions induced by $PGF_{2\alpha}$ in both the presence and absence of endothelium exhibited similar features, and contractions elicited in the presence of endothelium were inhibited to a greater extent by flunarizine than contractions elicited in the absence of endothelium. This may be due to a slowly developing endothelial-dependent increase in the ability of $PGF_{2\alpha}$ to augment intracellular calcium release.

Phenylephrine and $PGF_{2\alpha}$ elicited concentration-effect curves are similarly affected by the presence of the endothelium (group 1 agonists, Introduction) and both are capable of activating the release of intracellular calcium. However, the differences between the time courses of contractions elicited by phenylephrine and $PGF_{2\alpha}$, and the lack of effect of endothelium on the kinetics of contractions induced by $PGF_{2\alpha}$, indicates that the group 1 agonists are not homogeneously affected by the endothelium. This might reflect differences in their ability to stimulate the endothelium.

In conclusion, the results clearly demonstrate that the kinetics of phenylephrine-induced contractions in rat aorta are modified in the presence of endothelium and that submaximal contractions elicited in the presence of endothelium are more dependent on the mobilization of intracellular calcium than are contractions of similar magnitude elicited in the absence of endothelium. Although the kinetics of $PGF_{2\alpha}$-induced contractions were not visibly modified by the endothelium, the mobilization of intracellular calcium was altered. Therefore, there are at least two actions of endothelium-derived factor(s) on the smooth muscle of rat aorta, one that antagonizes contractions evoked by α-adrenoceptor and other agonists, particularly group 2 agonists (introduction) and another that enhances the mobilization of intracellular calcium by agonists that have this property to a marked degree – group 1 agonists. It remains to be investigated whether these two effects are due to one or more endothelium-derived factors. These results also imply that flunarizine (and possibly other calcium entry blockers) would be more effective in antagonizing contractions of arterial tissue initiated at sites of endothelial damage or modification, which may account for their usefulness in treatment of vascular spasm.

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