Br. J. Pharmac. (1985), **85**, 481–487

Insensitivity of calcium-dependent endothelial stimulation in rat isolated aorta to the calcium entry blocker, flunarizine

R.C. Miller[1], P. Schoeffter & J.C. Stoclet

Université Louis Pasteur, Laboratoire de Pharmacodynamie (INSERM U 243, CNRS ERA 787), B.P. 10, 67048 Strasbourg Cedex, France

1 In rat aortic segments complete with endothelium, acetylcholine (1 μM) relaxed noradrenaline, phenylephrine and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced contractions of various magnitudes. Maximal 1 μM phenylephrine-induced contractions were relaxed to a greater extent than were maximal contractions induced by the other two agonists.

2 Contractions elicited by various concentrations of phenylephrine and $PGF_{2\alpha}$ in the presence of a maximal effective concentration of the calcium entry blocker flunarizine (3 μM) were relaxed by acetylcholine to about the same residual tension as were contractions elicited in the absence of flunarizine.

3 Acetylcholine (1 μM) and phenylephrine (1 μM) increased tissue levels of guanosine cyclic 3′5′-monophosphate (cyclic GMP) by about 37 fold and 2 fold respectively. Preincubation of tissues in the absence of calcium abolished these agonist-induced increases in cyclic GMP levels, but preincubation with flunarizine had no significant effect on the increase in cyclic GMP level induced by the agonists. Pretreatment with flunarizine had no significant effect on the basal tissue level of cyclic GMP, but pretreatment in calcium-free solution reduced the basal tissue level of the cyclic nucleotide by about half.

4 It is concluded that in rat aorta, endothelium-dependent acetylcholine-induced relaxation and endothelium-dependent acetylcholine and phenylephrine-induced increases in tissue levels of cyclic GMP, are dependent on extracellular calcium, but are not antagonized by flunarizine. This may indicate that if calcium channels of endothelial cells are activated by these agonists, their characteristics are not identical with those of the calcium channels of the smooth muscle cells.

Introduction

Relaxant responses of vascular tissues evoked by many compounds are completely or in part dependent on the presence of the endothelium and it has been shown that endothelial stimulation, by acetylcholine for example, leads to the liberation of a factor, endothelium-derived relaxing factor, which acts on the smooth muscle of the vessel to produce a relaxation (see review by Furchgott, 1983). Liberation of this factor has been shown to be partially dependent on extracellular calcium in rabbit aorta (Singer & Peach, 1982).

Endothelium-dependent modulation of agonist-induced contractions of *in vitro* vascular preparations has also been described (Allan *et al.*, 1983; Konishi & Su, 1983; Zuleica *et al.*, 1983; Cocks & Angus, 1983; Eglème *et al.*, 1984a,b; Miller *et al.*, 1984; Bigaud *et al.*, 1984; Miller & Stoclet, 1984). In general, this modulation comprises an inhibition of responses to α_2-adrenoceptor agonists and a shift to the right of concentration-effect curves to other types of agonists with relatively little or no change in the maximal response.

It is not known if the same endothelium-derived factor (or factors) is involved both in relaxant responses and in modulation of α-adrenoceptor agonist-induced contractions, but both of these endothelium-mediated effects have been associated with an increase in the total tissue level of guanosine cyclic 3′,5′-monophosphate (cyclic GMP) (Holzmann, 1982; Diamond & Chu, 1983; Rapoport & Murad, 1983; Bigaud *et al.*, 1984; Miller *et al.*, 1984).

[1] Author for correspondence.

Secretion of a factor by the endothelium subsequent to stimulation is likely to be dependent on extracellular calcium (Rubin, 1970) and therefore liable to modulation by drugs that change cellular calcium metabolism.

Calcium entry blockers antagonize agonist-induced contractions of vascular tissues and exhibit tissue selectivity (Towart, 1981; Godfraind & Miller, 1983) and it is therefore interesting to know if endothelium-mediated effects in vascular tissue are also sensitive to this type of antagonist. We have investigated the effects of the calcium entry blocker, flunarizine (Godfraind & Dieu, 1981; Godfraind & Miller, 1982) on acetylcholine-induced relaxation of rat isolated aorta and on phenylephrine- and acetylcholine-induced increases in tissue cyclic GMP levels.

The results demonstrate that although endothelium-mediated responses in this artery are dependent on extracellular calcium, they are not antagonized by flunarizine.

Methods

Contractile experiments

Female Wistar rats of 12–14 weeks of age (280–300 g) were used. The rat was killed, the thorax opened, 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. Rings of aorta about 2 mm long were cut from the proximal end. When necessary, endothelium was removed by gently rubbing the interior with a wooden stick. Rings were suspended between parallel hooks in organ baths containing physiological solution maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2, under 2 g tension. Fifteen minutes later tissues were rinsed, readjusted to 2 g tension then equilibrated for a further 45 min before a maximal contraction to noradrenaline (1 μM) was elicited. After 30 min, when the noradrenaline contraction had stabilized, acetylcholine (1 μM) 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 was again added for 10 min. Two hours later (during which time tissues were rinsed 5 times) a second cumulative concentration-effect curve was elicited, which included a maximal response and was also followed by acetylcholine (1 μM) for 10 min. The same protocol was followed substituting single 30 min exposures to single concentrations of either phenylephrine or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in place of the cumulative additions of agonist. When necessary tissues were preincubated with flunarizine (3 μM) for 90 min before the second contraction to phenylephrine or $PGF_{2\alpha}$ was elicited in the presence of flunarizine. Contractions were recorded using Statham UC2 isometric force transducers linked to a Beckman dynograph type 811A.

Determination of cyclic nucleotide levels

Rat thoracic aortae, prepared as described above were divided into 4 or 5 segments of about 5 mg each. Each segment was then incubated in 2 ml of physiological solution at 37°C bubbled with a mixture of 95% O_2 and 5% CO_2 for 120 min during which time the physiological solution was changed periodically. Phenylephrine (1 μM) or acetylcholine (1 μM) was then added and after 1 min the aortic segments were frozen using aluminium tongs precooled in liquid nitrogen. When necessary, the tissues were pre-incubated for 90 min with flunarizine (3 μM) before the addition of agonist. Control tissues treated only with solvents were included in all treatment groups. In other experiments tissues were incubated for the last 15 min before addition of agonist in calcium-free solution, prepared by omitting calcium. During this time the calcium-free solution was changed 3 times. Control tissues were washed with normal physiological solution at the same times. Tissues were thawed in 400 μl of perchloric acid (1N), then homogenized with a Potter glass/glass homogenizer followed by sonication (Ultrasons-Annemasse, type 75TS) for 15 s. The homogenate was centrifuged at 10,000 *g* for 5 min and the cyclic GMP content of the supernatant assayed by a radio-immunological method (Cailla *et al.*, 1976). The DNA content of each tissue segment was assayed by a fluorometric method (Schoeffter & Stoclet, 1982). Cyclic nucleotide levels were expressed as fmol μg^{-1} DNA.

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, Upjohn) was used as supplied. Dilutions were prepared in physiological solution. Flunarizine (Janssen Pharmaceutica) 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. Cyclic GMP specific antibodies were the generous gift of Drs Cailla and Delaage of the Centre d'Immunologie, Marseille-, Luminy, France.

Cyclic GMP and DNA (from calf thymus, Type I) were obtained from Sigma. Labelled antigens were prepared by iodination of the succinyl-cyclic nucleotide tyrosylmethylester (Sigma). All drug concentrations are expressed in terms of the base.

Statistical analysis

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

Results

Contractile experiments

Single 1 μM concentrations of noradrenaline induced contractions of rat aortic segments of 1139 ± 125 mg ($n = 6$). Acetylcholine (1 μM), added after 30 min when contractions to noradrenaline had stabilized, produced a relaxation of 67.9 ± 5.8%. Cumulative additions of phenylephrine induced maximal responses of 1463 ± 191 mg. These maximal contractions were not significantly different from those induced by noradrenaline ($0.1 < P < 0.2$, $n = 13$). Acetylcholine (1 μM) relaxed these maximal phenylephrine-induced contractions by 80.3 ± 3.8%. This acetylcholine-induced relaxation was significantly greater than that elicited when the same tissues were contracted by 1 μM noradrenaline ($0.001 < P < 0.005$, paired *t* test). In separate experiments, acetylcholine (1 μM) relaxed single phenylephrine (1 μM)-induced contractions by 69.9 ± 9.9% and noradrenaline (1 μM)-induced contractions by 41.9 ± 3.6% in the same tissues. These values are significantly different ($0.001 < P < 0.005$, $n = 6$, paired *t* test).

Acetylcholine (1 μM) relaxed successive maximal (cumulative) phenylephrine-induced contractions by 72.7 ± 8.0% and by 75.3 ± 7.7% respectively ($n = 5$), demonstrating the reproducibility of the acetylcholine-induced response.

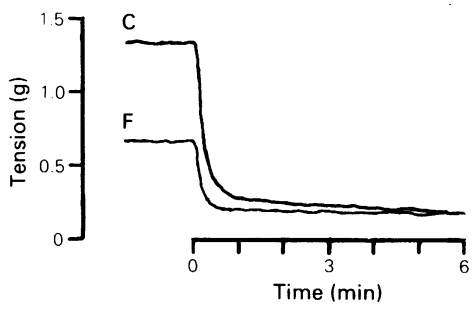

Figure 1 Superimposed experimental records of acetylcholine (1 μM)-induced relaxations of contractions elicited by phenylephrine (1 μM) in rat isolated aorta, in the absence (trace C) and presence (trace F) of 3 μM flunarizine.

Table 1 Effect of flunarizine (3 μM) on contractions induced by single concentrations of phenylephrine (Phen) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in rat isolated aorta

Agonist		*100% tension (mg)*		*% tension after 30 min* *Control*	*Flunarizine*
Phen	1 μM	1 303 ± 86	(6)	87.7 ± 3.7	41.0 ± 6.0
Phen	100 nM	612 ± 68	(7)	73.3 ± 7.3	31.0 ± 4.8
Phen	30 nM	324 ± 17	(7)	39.8 ± 7.0	7.5 ± 1.6
PGF2α	30 μM	1 295 ± 198	(5)	98.2 ± 1.2	41.4 ± 6.3
PGF2α	10 μM	690 ± 110	(10)	98.5 ± 0.9	42.8 ± 3.7
PGF2α	3 μM	422 ± 135	(5)	91.4 ± 4.0	31.1 ± 2.5

Values in the Control and Flunarizine columns are the mean (± s.e.mean) contraction (in the absence and presence of flunarizine respectively) measured after 30 min exposure to the agonists. The values are expressed as a percentage of the maximal contraction (expressed in mg in column 1) elicited by the indicated concentrations of the agonists in the absence of flunarizine. Numbers in parentheses represent number of rats.

Maximal contractions induced by 30 μM $PGF_{2\alpha}$ (1295 ± 198 mg, $n = 5$) and by noradrenaline (1 μM) were also of a similar magnitude and were relaxed to the same extent by 1 μM acetylcholine (61.3 ± 4.0% and 55.8 ± 7.8% respectively, $P > 0.3$, paired *t* test, $n = 5$).

Single submaximal contractions induced by 30 nM and 100 nM concentrations of phenylephrine (that induce about 20% and 50% respectively of the maximum response) and by 3 μM and 10 μM concentrations of $PGF_{2\alpha}$ (about 25% and 60% respectively of the maximum response) were not always maintained, but after 30 min of contact the residual contractions (Table 1) were relaxed by 1 μM acetylcholine (Table 2).

In the presence of flunarizine (3 μM), phenylephrine and $PGF_{2\alpha}$-induced contractions were partially inhibited (Figure 1; Table 1). However, these reduced contractions were also relaxed by 1 μM acetylcholine and the residual acetylcholine-resistant component of contraction in the presence of flunarizine was of about the same magnitude as that seen in the absence of flunarizine (Figure 1, Table 2).

Determination of cyclic nucleotide levels

As shown in Figure 2, phenylephrine (1 μM) increased tissue levels of cyclic GMP by about 2 fold ($P < 0.001$). Preincubation of tissues in the absence of

Table 2 Effect of acetylcholine (1 μM) on phenylephrine and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced contractions of rat isolated aorta

Agonist		*Pretreatment* *None*	*Flunarizine*
Phenylephrine	1 μM	27.2 ± 9.5 (6)	18.6 ± 6.1
Phenylephrine	0.1 μM	7.3 ± 5.1 (7)	5.4 ± 0.8
Phenylephrine	30 nM	3.5 ± 2.5 (7)	4.3 ± 1.7
$PGF_{2\alpha}$	30 μM	38.1 ± 3.7 (5)	24.9 ± 6.0
$PGF_{2\alpha}$	10 μM	10.7 ± 2.3 (10)	14.0 ± 1.4
$PGF_{2\alpha}$	3 μM	16.9 ± 4.7 (5)	19.0 ± 5.8

Values are the mean residual contraction (expressed as % of the control contraction before addition of acetylcholine, ± s.e.mean) after relaxation induced by acetylcholine in the absence and presence of flunarizine (3 μM). Numbers in parentheses represent number of rats.

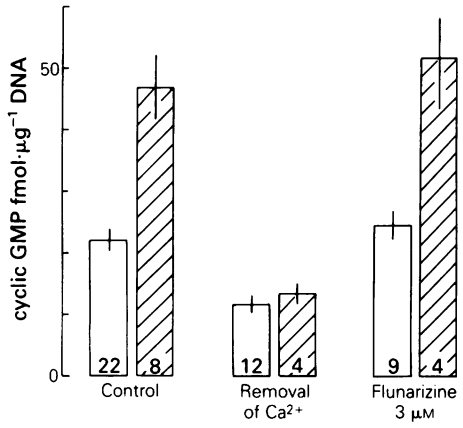

Figure 2 Effect of phenylephrine (1 μM; 1 min contact, hatched columns) on rat aortic content of cyclic GMP (fmol μg^{-1} DNA) in the absence and in the presence of Ca^{2+} and flunarizine (3 μM); open columns = controls, without phentephrine. Numbers in columns represent number of determinations. Vertical bars represent s.e.mean.

calcium reduced significantly the basal tissue levels of cyclic GMP ($P<0.001$) and abolished the phenylephrine-induced increase. Preincubation with flunarizine (3 μM) had no significant inhibitory effect on either basal levels of the cyclic nucleotide, or on the stimulatory effect of phenylephrine ($P>0.2$).

Removal of the endothelium reduced basal tissue levels of cyclic GMP from 18.4 ± 4.5 fmol μg^{-1} DNA to 7.5 ± 2.7 fmol μg^{-1} DNA. This reduced level of cyclic GMP in the absence of endothelium was not significantly different from the basal level of cyclic GMP (10.8 ± 3.1 fmol μg^{-1} DNA, $P>0.4$, $n=4$) in tissues complete with endothelium in the absence of calcium. Removal of both endothelium and calcium reduced tissue cyclic GMP levels to 7.5 ± 1.9 fmol μg^{-1} DNA. This value was not significantly different ($P>0.3$) from that measured either after removal of endothelium or after removal of calcium in the presence of endothelium.

In normal physiological solution, 1 μM acetylcholine increased tissue cyclic GMP levels by about 37 fold (Table 3). Removal of calcium dramatically reduced the stimulatory effect of acetylcholine to about 2.5 fold. In the presence of flunarizine, acetylcholine increased tissue cyclic GMP content to about the same level ($0.05<P<0.1$) as in the absence of flunarizine (Table 3).

Discussion

Stimulation of the endothelium of numerous blood vessels by many vasodilator compounds has been shown to be essential to their vasodilator activity (Furchgott, 1983). The maximal endothelial-dependent vasodilator activities of acetylcholine, histamine and the calcium ionophore A23187 have been associated with increased tissue levels of cyclic GMP of 10 to 30 fold (Holzmann, 1982; Diamond & Chu, 1983; Rapoport & Murad, 1983). Endothelium also modulates the contractile activity of many α-adrenoceptor agonists (Eglème *et al.*, 1984a,b) and also of such compounds as 5-hydroxytryptamine and $PGF_{2\alpha}$ (Cocks & Angus, 1983; Eglème *et al.*, 1984b; Miller & Stoclet, 1984). It has further been shown that in the

Table 3 Effect of acetylcholine (1 μM; 1 min contact) on cyclic GMP levels in rat aorta, in the presence and absence of Ca^{2+} and of flunarizine (3 μM)

	Pretreatment *None*	*Removal of Ca^{2+}*	*Flunarizine*
Control	22.8 ± 2.7 (10)	14.0 ± 2.5 (4)	22.6 ± 3.2 (5)
Acetylcholine	855 ± 129 (10)	34.2 ± 8.7 (4)	596 ± 61 (6)

Values are the mean ± s.e.mean expressed in fmol μg^{-1} DNA. Numbers in parentheses represent number of determinations.

case of the α-adrenoceptor agonists B-HT 920, clonidine and methoxamine, stimulation of the rat aorta in the presence of the endothelium, but not in its absence, evokes an increase in tissue levels of cyclic GMP of about 2 to 3 fold (Bigaud *et al.*, 1984; Miller *et al.*, 1984). This study demonstrates that phenylephrine also increases tissue levels of cyclic GMP in the presence of endothelium by about the same order of magnitude as other α-adrenoceptor agonists and confirms the effect of acetylcholine. In the absence of endothelium neither α-adrenoceptor agonists nor acetylcholine significantly alter the basal level of cyclic GMP in rat aorta (Rapoport & Murad, 1983; Miller *et al.*, 1984; Bigaud *et al.*, 1984). Therefore, an increase in tissue cyclic GMP content can be taken as evidence of the presence of endothelium, at least when α-adrenoceptor agonists and acetylcholine are used as stimulants.

The physical location of this cyclic GMP in the tissue has not yet been directly demonstrated but in cultured endothelial cells acetylcholine, phenylephrine and noradrenaline evoke an increase in cyclic GMP content of 2 to 5 fold (Buonassisi & Venter, 1976). An increase in cyclic GMP content of this magnitude in the monocellular layer of endothelial cells could not account for an increase in the total tissue level of even 2 fold. Since smooth muscle cells constitute the predominant cell type found in the tissue, it might be assumed that they account for the increase in cyclic GMP and that this is associated with the relaxant response. However, it should be emphasized that a causal connection between increased tissue cyclic GMP levels and inhibition of maximal contraction has not been conclusively demonstrated. A modest increase in tissue cyclic GMP levels of about 2 fold evoked by the α-adrenoceptor agonists B-HT 920 and clonidine in the presence of endothelium (Bigaud *et al.*, 1984; Miller *et al.*, 1984) is associated with an inhibition of contraction of about 90%. On the other hand, maximal contractions evoked by methoxamine are not markedly different in the presence or absence of endothelium but are also associated with about a 2 fold increase in tissue cyclic GMP levels in the presence of endothelium (Bigaud *et al.*, 1984). In comparison, relaxation of precontracted arterial preparations by about 80% by acetylcholine is associated with an increase of tissue cyclic GMP levels of about 25 to 35 fold (Table 3; Rapoport & Murad, 1983). These large differences in the measured tissue levels of cyclic GMP evoked by acetylcholine and by the α-adrenoceptor agonists in the presence of endothelium may mean that different endothelial factors are liberated by the different stimuli or that the cyclic GMP generated in response to the stimuli is differently compartmentalized.

Stimulated increases in tissue cyclic GMP levels were not significantly affected by preincubation with 3 μM of the calcium entry blocker flunarizine, a concentration that is sufficient to inhibit maximally agonist-induced influx of calcium in rat arteries (Godfraind & Dieu, 1981; Godfraind & Miller, 1982; 1983). Also, 1 μM acetylcholine relaxed rat isolated aorta preparations contracted by maximal and submaximal concentrations of phenylephrine and $PGF_{2\alpha}$ to a similar residual tension in the absence and in the presence of flunarizine (Table 2). It might therefore be assumed that stimulated secretion of an endothelial factor is insensitive to flunarizine.

Secretion is generally considered to be a calcium-dependent process (Rubin, 1970) and there is no reason to suppose that secretion of a factor from the endothelial cells is an exception. Some evidence for such a dependence on extracellular calcium has been presented here. Firstly, in unstimulated preparations the basal tissue level of cyclic GMP fell when the endothelium was removed, as has also been previously described (Holzmann, 1982; Rapoport & Murad, 1983; Miller *et al.*, 1984; Bigaud *et al.*, 1984) and also when calcium was removed from the incubating solution in the presence of endothelium. There is therefore probably a continuous basal release of an endothelium-derived factor. Secondly, increases in tissue cyclic GMP levels stimulated by phenylephrine were abolished (Figure 2) and those stimulated by acetylcholine dramatically reduced (Table 3), after preincubation in calcium-free solution.

Interpretation of the effects of calcium removal is complicated by the fact that a reduction in extracellular calcium may affect not only the endothelial cells but also the smooth muscle cells, the presumed site of cyclic GMP production, and calcium is reported to be essential for the functioning of guanylate cyclase in some smooth muscles (Schultz *et al.*, 1973; Takayanagi *et al.*, 1981). In the rat aorta, increased sarcoplasmic calcium levels alone are evidently not a sufficient condition for activation of guanylate cyclase, since no increase in cyclic GMP levels was detected in the absence of endothelium during stimulation with phenylephrine, which is known to induce an influx of calcium in this tissue. Further, even in the absence of extracellular calcium or in the presence of flunarizine, phenylephrine mobilizes intracellular calcium sufficiently to induce contractions (Godfraind *et al.*, 1982).

The absence of an inhibitory effect of flunarizine on the basal tissue levels of cyclic GMP (Table 3, Figure 2) is to be expected since it is known to inhibit stimulated but not unstimulated calcium entry into smooth muscle cells (Godfraind & Dieu, 1981; Godfraind & Miller, 1982).

Overall therefore, calcium influx into endothelial cells in resting conditions and on stimulation by the agonists is probably a prerequisite for the secretion of endothelium-derived relaxing factor but this secretion

is not significantly inhibited by flunarizine. The apparent lack of effect of flunarizine suggests that if calcium channels are associated with the endothelial cells they are not identical to those of the adjacent vascular smooth muscle cells, or that perhaps calcium entry is via a mechanism other than an ion channel.

Acetylcholine was more effective as an antagonist of maximal contractions induced by phenylephrine rather than those produced by noradrenaline or $PGF_{2\alpha}$. The reason for this difference is not clear, but some evidence has been put forward showing that phenylephrine is less effective than noradrenaline at stimulating calcium flux into the smooth muscle cells of the rat aorta in certain conditions (Godfraind *et al.*, 1982). Also, in the experiments reported here, contractions elicited by submaximal concentrations of phenylephrine were not well maintained in comparison with those induced by $PGF_{2\alpha}$ (Table 1) or noradrenaline (unpublished data). The maintenance of contractions is thought to be due to influx of Ca^{2+}. In this regard, it is interesting to note that α_2-adrenoceptor agonists, which are mostly dependent on extracellular calcium to elicit contractions in the rat aorta (Godfraind *et al.*, 1982; Nghiem *et al.*, 1982), elicit no, or very small, contractions in the presence of endothelium, even though they produce contractions comparable to those of noradrenaline in the absence of endothelium (Eglème *et al.*, 1984a,b; Miller *et al.*, 1984). It is possible therefore, that the endothelium-derived factor affects stimulated plasmalemmal calcium movements and that those induced by phenylephrine and α_2-adrenoceptor agonists are more easily modulated than those induced by noradrenaline and $PGF_{2\alpha}$.

In conclusion, these results indicate that although stimulated liberation of an endothelium-derived factor(s) is dependent on extracellular calcium in this tissue, the stimulated calcium entry into the endothelial cells is apparently not inhibited to any great extent by flunarizine.

The authors wish to thank Dr M. Spedding for his helpful comments on this manuscript.

References

ALLAN, G., BROOK, C.D., CAMBRIDGE, D. & HLADKIWISKYJ, J. (1983). Enhanced responsiveness of vascular smooth muscle to vasoactive agents after removal of endothelial cells. *Br. J. Pharmac.*, **79**, 334P.

BUONASSISI, V. & VENTER, J.C. (1976). Hormone and neurotransmitter receptors in an established vascular cell line. *Proc. natn. Acad. Sci. U.S.A.*, **73**, 1612–1616.

BIGAUD, M., SCHOEFFTER, P., STOCLET, J.C. & MILLER, R.C. (1984). Dissociation between endothelial mediated increases in tissue cGMP levels and modulation of aortic contractile responses. *Naunyn-Schmiedebergs Arch. Pharmac.*, **328**, 221–223.

CAILLA, H.L., VANNIER, C.T. & DELAAGE, M.A. (1976). Guanosine 3',5'-cyclic monophosphate assay at 10^{-15} mole level. *Anal. Biochem.*, 70, **195–202.**

COCKS, T.M. & ANGUS, J.A. (1983). Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature*, **305**, 627–630.

DIAMOND, J. & CHU, E.B. (1983). Possible role for cyclic GMP in endothelium-dependent relaxation of rabbit aorta by acetylcholine. Comparison with nitroglycerin. *Res. Commun. Path. Pharmac.*, **41**, 369–372.

EGLÈME, C., GODFRAIND, T. & MILLER, R.C. (1984a). Enhanced responsiveness of rat isolated aorta to clonidine after removal of the endothelial cells. *Br. J. Pharmac.*, **81**, 16–18.

EGLÈME, C., GODFRAIND, T. & MILLER, R.C. (1984b). Role of endothelium in the rat aorta response to vasoconstrictors. *Archs int. Pharmacodyn.*, **268**, 116.

FURCHGOTT, R.F. (1983). Role of endothelium in responses of vascular smooth muscle. *Circulation Res.*, **53**, 557–573.

GODFRAIND, T. & DIEU, D. (1981). The inhibition by flunarizine of the norepinephrine-evoked contraction and calcium influx in rat aorta and mesenteric arteries. *J. Pharmac. exp. Ther.*, **217**, 510–515.

GODFRAIND, T. & MILLER, R.C. (1982). Actions of prostaglandin $F_{2\alpha}$ and noradrenaline on calcium exchange and contraction in rat mesenteric arteries and their sensitivity to calcium entry blockers. *Br. J. Pharmac.*, **75**, 229–236.

GODFRAIND, T. & MILLER, R.C. (1983). Specificity of action of Ca^{++} entry blockers. A comparison of their actions in rat arteries and in human coronary arteries. *Circulation Res.*, **52**, (Supplement I), 81–91.

GODFRAIND, T., MILLER, R.C. & SOCRATES LIMA, J. (1982). Selective α_1- and α_2-adrenoceptor agonist induced contractions and ^{45}Ca fluxes in the rat isolated aorta. *Br. J. Pharmac.*, **77**, 597–604.

HOLZMANN, S. (1982). Endothelium-induced relaxation by acetylcholine associated with larger rises in cyclic GMP in coronary arterial strips. *J. Cyclic Nucleotide Res.*, **8**, 409–419.

KONISHI, M. & SU, C. (1983). Role of endothelium in dilator responses of spontaneously hypertensive rat arteries. *Hypertension*, **5**, 881–886.

MILLER, R.C., MONY, M.C., SCHINI, V., SCHOEFFTER, P. & STOCLET, J.C. (1984). Endothelial mediated inhibition of contraction and increase in cGMP levels evoked by the α-adrenoceptor agonist B-HT 920 in rat isolated aorta. *Br. J. Pharmac.*, **83**, 903–908.

MILLER, R.C. & STOCLET, J.C. (1984). Influence de l'endothelium sur la sensibilité de l'aorte isolée de rat à la flunarizine. *J. Pharmac.* (Paris), **15**, 494–495.

NGHIEM, C., SWAMY, V.C. & TRIGGLE, D.J. (1982). Inhibition by D600 of norepinephrine and clonidine-induced responses of the aorta from normotensive and spontaneously hypertensive rats. *Life Sci.*, **30**, 45–49.

RAPOPORT, R.M. & MURAD, F. (1983). Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cGMP. *Circulation Res.*, **52**, 352–357.

RUBIN, R.P. (1970). The role of calcium in the release of neurotransmitter substances and hormones. *Pharmac. Rev.*, **22**, 389–428.

SCHOEFFTER, P. & STOCLET, J.C. (1982). Age-related decrease of in vitro isoproterenol-induced cyclic AMP accumulation in rat aorta. *Eur. J. Pharmac.*, **77**, 183–186.

SCHULTZ, G., HARDMAN, J.G., SCHULTZ, K., BAIRD, C.E. & SUTHERLAND, E.W. (1973). The importance of calcium ions for the regulation of guanosine 3′:5′-cyclic monophosphate levels. *Proc. natn. Acad. Sci. U.S.A.*, **70**, 3889–3893.

SINGER, H.A. & PEACH, M.J. (1982). Calcium- and endothelial-mediated vascular smooth muscle relaxation in rabbit aorta. *Hypertension*, **4** (Supplement II), 19–25.

TAKAYANAGI, I., HISAYAMA, T. & KOTSUGAI, T. (1981). Activation of guanylate cyclase as secondary reaction following increase in intracellular Ca ion concentrations. *Jap. J. Pharmac.*, **31**, 831–834.

TOWART, R. (1981). The selective inhibition of serotonin-induced contractions of rabbit cerebral vascular smooth muscle by calcium-antagonistic dihydropyridines. An investigation of the mechanism of action of nimodipine. *Circulation Res.*, **48**, 650–657.

ZULEICA, B., FORTES, J., LEME, G. & SCIVOLETTO, R. (1983). Vascular reactivity in diabetes mellitus: role of the endothelial cell. *Br. J. Pharmac.*, **79**, 771–781.

(Received October 25, 1984.
Revised January 23, 1985.
Accepted February 4, 1985.)