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Role of cyclic GMP in the modulation by endothelium of the adrenolytic action of prazosin in the rat isolated aorta

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1 The effect of endothelium on the adrenolytic action of prazosin was studied in the rat isolated aorta.

2 Prazosin showed a non-competitive type of antagonism in preparations with intact endothelium while in preparations where endothelium had been removed, prazosin at concentrations between 0.3 nM–10 nM acted as a competitive antagonist.

3 Methylene blue, used to decrease tissue levels of guanosine 3′:5′-cyclic monophosphate (cyclic GMP), converted prazosin from a non-competitive antagonist into an apparently competitive antagonist in the presence of endothelium.

4 Increasing tissue levels of cyclic GMP by incubation with 8-bromo-cyclic GMP converted prazosin from an apparently competitive antagonist into a non-competitive antagonist in the absence of endothelium.

5 Analysis of concentration-response curves for noradrenaline in the presence and absence of endothelium showed that the affinity for noradrenaline was the same but the efficacy, measured by estimating the receptor reserve, was not; it was lower in the presence than in the absence of endothelium.

6 It was concluded that the change in the mode of antagonism of prazosin after endothelium removal could be related to an alteration in the efficacy of the agonist, brought about by a change in the tissue levels of cyclic GMP.

Introduction

It is now well established that vascular endothelium mediates wholly or partially the relaxant response to many agents via the release of an endothelium-derived relaxant factor (EDRF) (reviewed by Furchgott, 1984). The mechanism by which EDRF is thought to mediate vascular relaxation is by the activation of guanylate cyclase leading to an elevation of smooth muscle cyclic guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Holzmann, 1982; Diamond & Chu, 1983; Rapoport *et al.*, 1983).

Recently, the role of endothelium as a modulator of contractile agonist effects in the dog and pig coronary artery (Cocks & Angus, 1983) and in the rat aorta (Allan *et al.*, 1983; Konishi & Su, 1983; Zuleica *et al.*, Eglème *et al.*, 1984; Miller *et al.*, 1984; Bigaud *et al.*, 1984) has been demonstrated. In particular, we have shown that the removal of endothelium enhances the contractile response of the rat isolated aorta to α-adrenoceptor agonists, especially to partial agonists that have a weak effect in intact preparations (Godfraind *et al.*, 1985).

The present experiments were undertaken to investigate whether the endothelium plays a role in modifying the effects of prazosin, a well known α-adrenoceptor antagonist. The antagonistic effect of prazosin in the rat isolated aorta has been a matter of controversy, since it has been found to be a competitive antagonist by some authors and a non-competitive antagonist by others (Cohen *et al.*, 1979; Digges & Summers, 1983; Hamed *et al.*, 1983; Downing *et al.*, 1983). We have compared the inhibitory effects of prazosin on noradrenaline-evoked responses in the rat isolated aorta in the presence and absence of endothelium. The results show that prazosin acts as a non-competitive antagonist in the presence of endothelium and as a competitive antagonist in the absence of endothelium.

It seemed of interest to find out whether this

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modulation of the adrenolytic effect of prazosin by endothelium was associated with changes in tissue cyclic GMP levels. Our results show that pretreatment with methylene blue, an inhibitor of soluble guanylate cyclase (Ignarro & Kadowitz, 1985), resulted in competitive antagonism by prazosin of noradrenaline responses in preparations with intact endothelium. On the other hand, pretreatment with 8-bromo-cyclic GMP (8-Br-cyclic GMP) resulted in a non-competitive antagonism of noradrenaline responses by prazosin in the absence of endothelium.

Analysis of the concentration-response curves for noradrenaline showed that the effect of EDRF could possibly be explained by a reduction in the intrinsic efficacy of the agonist.

A preliminary communication of these results was made to the Belgian Society for Fundamental and Clinical Physiology and Pharmacology (Alosachie & Godfraind, 1985).

Methods

Experimental protocol

Pairs of rings (2 mm long) were cut from the thoracic aorta of male Wistar rats (300–350 g). One ring of each pair was left intact, while the other was stripped of its endothelium by mechanical rubbing. Each ring was carefully suspended under a tension of 2 g in a 50 ml organ bath containing a physiological solution (composition, mmol l^{-1}: NaCl 112, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1.0, $MgSO_4$ 1.2, $CaCl_2$ 1.25 and glucose 11.5) at 37°C gassed with a mixture of 95% O_2 and 5% CO_2.

Contractile responses were measured with isometric strain gauges coupled to potentiometric pen recorders. After an equilibration period of 60 min, the artery preparations were contracted maximally with 1 μM noradrenaline. Preparations were then washed and allowed a further 60 min period of equilibration. Absence of endothelial cells was demonstrated by the failure of the preparation to relax to acetylcholine (1 μM), which was applied when the initially evoked contraction was stable, and by histological examination.

Cumulative concentration-response curves (CRCs) to noradrenaline were obtained by increasing the organ bath concentration of the agonist in steps of approximately 3 fold, allowing equilibrium to be attained at each concentration. When the maximum effect was attained, preparations were washed and allowed a further 60 min period of equilibration, after which time the tissue was incubated with prazosin for 30 min. A second cumulative CRC was then constructed in the presence of the antagonist. When methylene blue and 8-Br-cyclic GMP were used, each was added to the tissue bath 30 min before CRCs to noradrenaline were obtained.

A pair of rings taken from the same aorta, one with and one without endothelium, were treated simultaneously in exactly the same way as described above but without prazosin; these served as controls to which all subsequent results were compared.

Preliminary experiments were carried out to determine the optimal doses of 8-Br-cyclic GMP and methylene blue to be used.

Analysis of the concentration-effect curves

The affinity of noradrenaline for its receptors (K_A value) was calculated using the irreversible antagonist phenoxybenzamine (Pbz) to produce a reduction in the number of available receptors. In these experiments. Pbz (10 nM) was added to the organ bath 60 min after the first CRC to noradrenaline and was allowed to act for 10 min. The preparations were then repeatedly washed over a period of 30 min before a further concentration-response curve was constructed. The K_A values for noradrenaline were obtained using the graphical analysis developed by Furchgott & Bursztyn, based on the law of mass action (Furchgott & Bursztyn, 1967) and described by the equation

$$\frac{1}{(A)} = \frac{1-q}{qK_A} + \frac{1}{q(A')} \qquad (1)$$

where (A) and (A′) are equieffective concentrations of the agonist respectively before and after partial inactivation of a part of the receptor population, and q is the fraction of receptors remaining (receptors not alkylated).

A plot of the reciprocals of equieffective concentrations of agonist before and after partial irreversible blockade produces a straight line. The slope and the intercept of this line were used to calculate the agonist dissociation constant K_A according to equation (2)

$$K_A = \frac{\text{Slope} - 1}{\text{Intercept}} \qquad (2)$$

The K_A value thus determined was used in the following equation (3) to calculate the fraction (R_A/R_t) of receptors occupied at each concentration of noradrenaline in the intact and denuded preparations

$$R_A/R_t = \frac{(A)}{K_A + (A)} \qquad (3)$$

where (R_A) is the concentration of receptor-agonist complex and (R_t) is the total receptor concentration. The control concentration-response curves for noradrenaline in the presence and absence of endothelium were then plotted as a function of the negative logarithm of the fractional receptor occupation (R_A/R_t) and the appropriate curves were constructed.

The relative intrinsic efficacy of noradrenaline was calculated as the antilog of the distance between the receptor-occupancy response curves for noradrenaline in the presence and absence of endothelium, for more details see Furchgott & Bursztyn (1967).

Schild plots were constructed as log (dose-ratio − 1) against log (antagonist concentration). Dose-ratios were calculated at the EC_{50} level (Arunlakshana & Schild, 1959). Antagonism was considered to be competitive if there was not more than a 10% reduction in the maximum response and if the 95% confidence limits for the slope of the Schild plot, drawn by linear regression, overlapped unity.

Drugs

Noradrenaline bitartrate (Flücka) was dissolved in distilled water containing 7.9 mM Na_2SO_3 and 34 mM HCl as a stock solution of 10 mM. Prazosin (Pfizer) and phenoxybenzamine (gift from Janssen Pharmaceutica) were dissolved in ethanol and diluted in distilled water to 10 mM (final ethanol concentration 15%). Methylene blue (Sigma) and 8-bromo-guanosine 3':5'-cyclic monophosphate (Boehringer Mannheim) were dissolved in distilled water.

Statistical analysis

The data are expressed as mean ± s.e. mean. Tests for significance were made by means of Student's *t* test, *P* values less than 0.05 being considered significant. A least squares linear regression analysis was used to fit straight lines to data when appropriate.

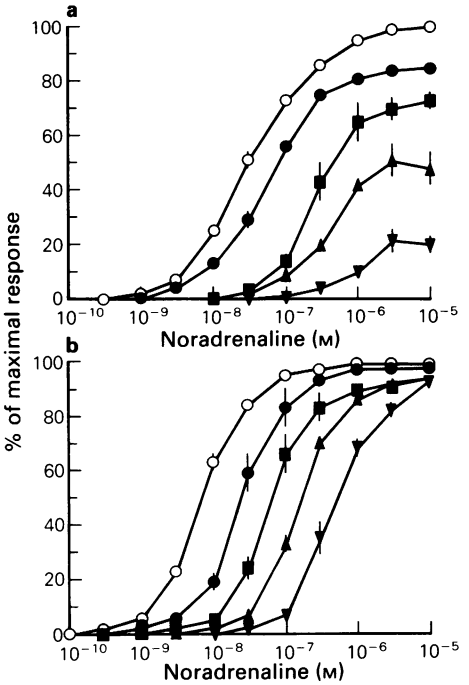

Figure 1 Effect of prazosin on cumulative concentration-response curves to noradrenaline in the rat isolated aorta (a) in preparations with endothelium present and (b) in preparations with endothelium removed. (○) Control responses and responses in the presence of prazosin, (●) 0.3 nM, (■) 1 nM, (▲) 3 nM and (▼) 10 nM are shown. Each point represents the mean and vertical lines show s.e. mean ($n > 5$).

Results

Comparison of the effect of prazosin on the concentration-response curves of noradrenaline in the presence and the absence of endothelium

Concentration-effect curves elicited by noradrenaline in rings of rat isolated aorta were displaced to the left in the absence of endothelium as compared to those obtained in the presence of endothelium. EC_{50} values were $3.1 \pm 0.7 \times 10^{-8}$ M and $4.1 \pm 0.3 \times 10^{-9}$ M ($n = 6$), maximal responses were 2153 ± 126 mg and 2650 ± 103 mg ($n = 6$) respectively in the presence and absence of endothelium. These values are significantly different.

In preparations with intact endothelium, prazosin (0.3 nM–10 nM) evoked a concentration-dependent, non-parallel shift to the right of noradrenaline CRCs with a progressive depression of the maximum response (Figure 1a). The slope of the Schild plot ($n = 1.2$) calculated from these data was significantly different from unity, an indication of non-competitive antagonism.

In preparations without endothelium, prazosin (0.3–10 nM) displaced noradrenaline CRCs to the right in a parallel manner and did not significantly affect the maximum response (Figure 1b). The slope of the Schild plot, ($n = 0.98$), was close to unity indicating competitive antagonism. However, when prazosin was used at concentrations higher than 10 nM, a significant depression of the maximum response to noradrenaline was observed (data not shown).

The effect of methylene blue

The vital stain methylene blue, shown to inhibit the guanylate cyclase enzyme (Ignarro & Kadowitz, 1985), was employed in these experiments to prevent the production of cyclic GMP, in order to determine whether there was an association between the endothelium modulation of prazosin antagonism and of cyclic GMP production.

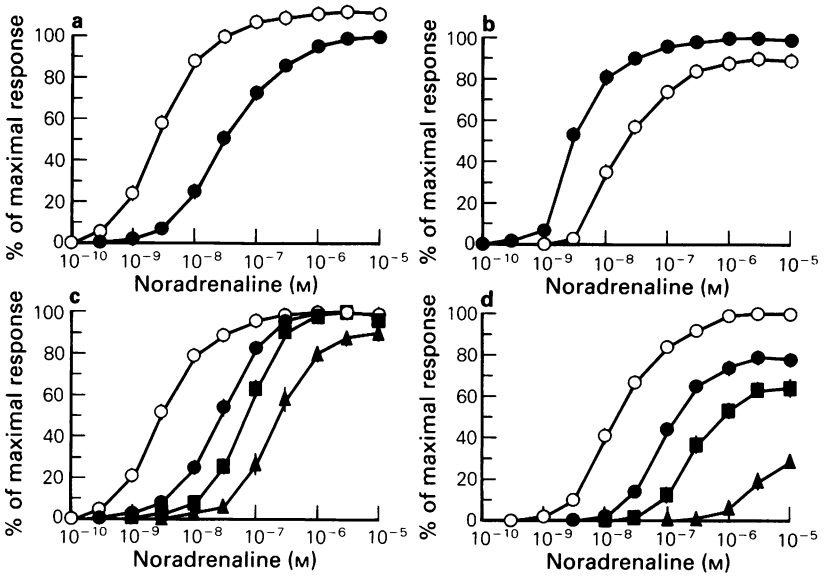

Figure 2 Effect of prazosin on the concentration-response curves to noradrenaline, and the role of cyclic GMP, in the presence (a, c) and absence (b, d) of endothelium. (a) Effect of methylene blue (○) and (b) effect of cyclic GMP (○) on control responses to noradrenaline (●) respectively in the presence and absence of endothelium. (c) Effect of prazosin in aortae with endothelium treated by methylene blue and (d) in aortae without endothelium treated with 8-bromo-cyclic GMP. (c and d) Control (○), prazosin (●) 1 nM, (■) 3 nM and (▲) 10 nM. Each point represents the mean and vertical lines show s.e. mean ($n > 4$).

Methylene blue (3 μM) treatment resulted in a shift to the left of the control CRCs for noradrenaline in the presence of endothelium with a significant enhancement of the maximum response (Figure 2a) comparable to that observed on removal of the endothelium.

A comparison of Figures 1b and 2c shows that methylene blue changed the non-competitive antagonism of prazosin into a competitive antagonism. In the presence of methylene blue, the maximal responses were not significantly altered by prazosin 1, 3 or 10 nM and the slope of the Schild plot ($n = 1.01$) was not significantly different from unity.

The effect of 8-bromo-cyclic GMP

These experiments were designed to mimic an increase in tissue content of cyclic GMP, by pre-incubation with the lipophilic analogue of cyclic GMP, 8-Br-cyclic GMP. This analogue was selected rather than cyclic GMP and its other analogues because of its higher stability and resistance to hydrolysis by cyclic nucleotide-phosphodiesterase, also it is better at activating cyclic GMP-dependent protein kinase and it has a potent relaxant effect. Furthermore, it penetrates the cell membrane more readily than cyclic GMP and its other analogues (Schultz *et al.*, 1979; Napoli *et al.*, 1980; Lincoln, 1983).

In the absence of endothelium, 8-Br-cyclic GMP (0.1 mM) caused a rightward shift of the CRCs to noradrenaline with a depression of the maximal response (Figure 2b) similar to that obtained with noradrenaline in the presence of endothelium. Moreover, it changed the competitive antagonism by prazosin of CRCs to noradrenaline in the absence of endothelium (see Figure 1b) into a non-competitive type (Figure 2d).

Effect of partial receptor inactivation by phenoxybenzamine on the action of prazosin

The possible relationship between the modulation of prazosin antagonism by endothelium and a change in receptor availability was tested using phenoxybenzamine to produce an irreversible inactivation of a fraction of the α_1-adrenoceptor population.

In preparations without endothelium pretreated with phenoxybenzamine (10 nM for 10 min), prazosin (1 nM) induced a non-parallel shift to the right, with a significant depression of the maximum response, of the CRC to noradrenaline (Figure 3), thus resulting in a non-competitive antagonism.

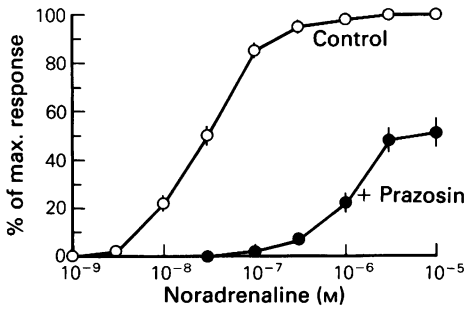

Figure 3 Effect of prazosin on the concentration-response curve to noradrenaline in the rat isolated aorta pretreated with phenoxybenzamine (10 nM for 10 min) in the absence of endothelium. Control (○), prazosin 1 nM (●). Each point represents the mean and vertical lines show s.e. mean ($n = 4$).

A comparison of the K_A values and the receptor reserve for noradrenaline in the presence and the absence of endothelium

The technique of Furchgott & Bursztyn (1967), which employs phenoxybenzamine to inactivate a part of the α-adrenoceptor pool, was used to determine the dissociation constant (K_A) of noradrenaline in preparations of rat isolated aorta with and without endothelium. Results of these experiments are presented in Figure 4. Phenoxybenzamine treatment (10 nM for 10 min) produced a non-parallel shift to the right of the CRC to noradrenaline with a depression of the maximal response that was more prominent in the presence (about 60%) than in the absence (about 20%) of endothelium.

The slopes and the intercepts of plots of the reciprocals of equieffective concentrations of

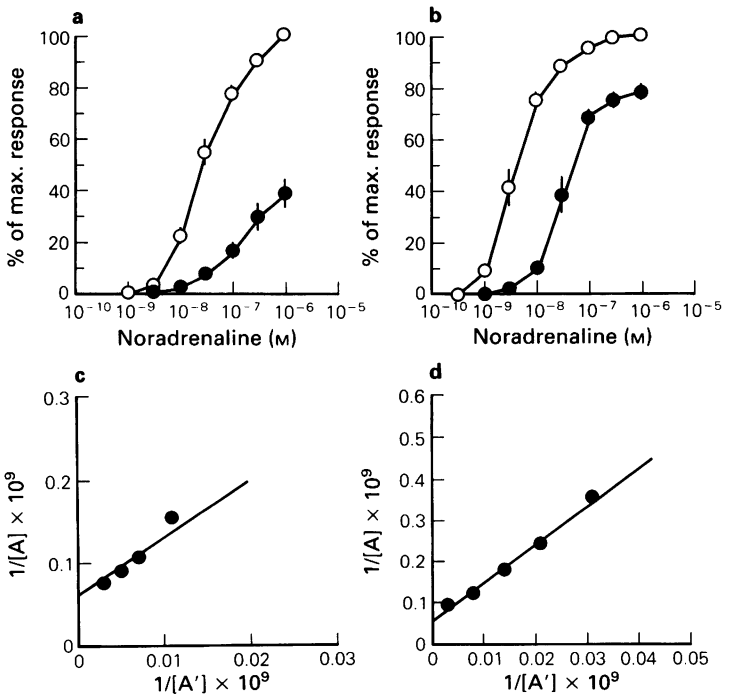

Figure 4 Estimation of K_A values for noradrenaline in the rat isolated aorta. (a and b) The effect of phenoxybenzamine (Pbz, 10^{-8} M for 10 min) on concentration-response curves to noradrenaline; (a) in preparations with endothelium, (b) in preparations without endothelium: (○) control responses; (●) after Pbz treatment. Each point represents the mean and vertical lines show s.e. mean ($n > 4$). (c and d) Double reciprocal plots are shown of equieffective concentrations of noradrenaline before (1/(A)) and after (1/(A')) treatment with phenoxybenzamine in the absence (d) and presence (c) of endothelium.

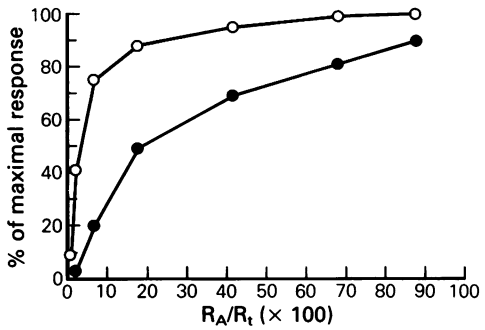

Figure 5 Contractile responses to noradrenaline in rat isolated aorta as a function of receptor occupation in tissues with (●) and without (○) endothelium. R_A/R_t is the proportion of receptors occupied.

noradrenaline estimated before and after treatment with phenoxybenzamine in preparations with or without endothelium (Figure 4c, d), were used to calculate the dissociation constants of noradrenaline in the presence and absence of endothelium as described in the Methods section. The K_A values of noradrenaline in preparations with and without endothelium were not significantly different ($0.137 \pm 0.02\,\mu$M and $0.136 \pm 0.08\,\mu$M) respectively.

The K_A values thus determined were used to calculate the fraction of receptors occupied (R_A/R_t, equation 3) at each concentration of noradrenaline, in the presence and absence of endothelium (see Methods). The responses from control CRCs to noradrenaline in the presence and absence of endothelium were then replotted as a function of the fractional receptor occupation R_A/R_t. This is shown in Figure 5, where a marked shift to the left of the curve representing the contractile response of the rat aorta as a function of receptor occupation after endothelium removal is evident, indicating the presence of a greater receptor reserve in preparations without endothelium. In preparations with endothelium present 19% of the receptors were needed to produce 50% of the maximum contraction while in preparations without endothelium only 3% of the receptors were needed to produce the same effect.

The relative intrinsic efficacy of noradrenaline was calculated as the antilog of the distance between the negative log of the receptor occupancy versus response curves for noradrenaline in the presence and absence of endothelium. In the absence of endothelium the relative intrinsic efficacy of noradrenaline was 7 times that in the presence of endothelium.

Discussion

Recent observations have demonstrated that vascular endothelial cells play an important role in the modification of the actions of contractile agonists in the rat aorta (Eglème *et al.*, 1984; Lues & Schumann, 1984; Carrier & White, 1985; Godfraind *et al.*, 1985).

The results presented here clearly demonstrate that the vascular endothelium also modifies the α-adrenoceptor antagonistic action of prazosin in the rat isolated aorta: prazosin from the threshold active concentration (0.3 nM) up to 10 nM acted as a non-competitive antagonist in the presence of endothelium and apparently as a competitive antagonist in preparations where the endothelium was removed (Figure 1). However, even in the absence of endothelium, prazosin, at doses higher than 10 nM, resulted in a significant depression of the maximum, hence resulting in a non-competitive antagonism. The discrepancies between the reports of the type of antagonistic action of prazosin in the rat aorta (see Introduction) may well be due to the variable amount of endothelium present in the preparations used.

Our analysis of the CRCs for noradrenaline by the method of Furchgott & Bursztyn (1967), in the presence and absence of endothelium, showed that the affinity of noradrenaline for its receptor, expressed as K_A values, was the same in the two preparations. However, the efficacy of noradrenaline was calculated to be about 7 times lower in the presence than in the absence of endothelium, expressed as a decrease in the size of receptor reserve. The results of this analysis are in agreement with the suggestion that the effect of endothelium on the agonist-induced contraction could possibly be explained by a reduction in the relative intrinsic efficacy of the agonist (Malta *et al.*, 1986a). They are also consistent with the observation by Carman-Krzan (1985) that removal of endothelium resulted in an increase in the binding sites for prazosin in microsomal preparations of bovine aorta without changing the affinity of the ligand for its receptor.

This indicates that the pattern of antagonism exhibited by prazosin is dependent on the size of the receptor reserve. This is consistent with our observation that in denuded aortae the competitive antagonism of prazosin was turned to a non-competitive type when a fraction of the α_1-adrenoceptors were inactivated by phenoxybenzamine. It has been shown that non-competitive antagonists produce greater inhibition in systems lacking a receptor reserve (Ariens & van Rossum, 1957). Since the action of prazosin seems to be related to the size of the receptor reserve in the rat aorta, this indicates that prazosin could not be considered as a pure competitive α_1-adrenoceptor antagonist in this tissue.

The action of prazosin is rather a complex one: apart from its selective action on α_1-adrenocep-

tors further effects have been described. Downing *et al.* (1983) have shown an effect of prazosin on the phasic component of the noradrenaline contractile effect, attributed to Ca^{2+} release from the intracellular compartment. Godfraind (1983) noted an inhibitory effect of prazosin on the slow residual inward Ca^{2+} influx which escaped blockade by nifedipine in K^+-depolarized rat aortae. Constantine *et al.* (1973) suggested that part of the action of prazosin might be distal to the postsynaptic α_1-adrenoceptors. Prazosin has also been found to inhibit the phosphodiesterase enzyme (Hess, 1974). Such effects could contribute to the non-competitive antagonism observed with prazosin.

The change in intrinsic efficacy of noradrenaline observed here seems to be related to the tissue levels of cyclic GMP. When the enzyme guanylate cyclase was inhibited using methylene blue, in the presence of endothelium, prazosin behaved more like a competitive antagonist of the noradrenaline-induced responses. In experiments designed to mimic an increase in the tissue content of cyclic GMP, by pre-incubation with 8-Br-cyclic GMP, it was observed that in the absence of endothelium prazosin now behaved as a non-competitive antagonist of noradrenaline-induced responses. That is, reducing or increasing tissue levels of cyclic GMP converted prazosin from an apparently competitive antagonist into a non-competitive antagonist and vice versa, as did the absence or presence of endothelium.

There are several possible mechanisms whereby cyclic GMP could alter the apparent efficacy of noradrenaline. In the rat aorta, the contraction evoked by noradrenaline is supported by two pools of calcium, one is intracellular and the other is extracellular. The mobilization of the extracellular pool is achieved by the opening of Ca^{2+} channels (Godfraind, 1985). It has been shown in cardiac cells that intracellular injection of cyclic GMP blocks slow action potentials generally attributed to Ca^{2+} entry through Ca^{2+} channels (Bkaily & Sperelakis, 1985). This suggests that cyclic GMP might also regulate the opening of the Ca^{2+} channel in smooth muscle thereby modulating the Ca^{2+} gating mechanism activated by noradrenaline. This interpretation is in agreement with observations on the role of endothelium in Ca^{2+} fluxes where it has been shown that the presence of endothelium reduces noradrenaline-dependent Ca^{2+} influx in rat and rabbit aortae (Godfraind *et al.*, 1985; Collins *et al.*, 1985; Malta *et al.*, 1986b).

On the other hand, Lincoln (1983) suggested that increased levels of cyclic GMP might enhance Ca^{2+} uptake into sarcoplasmic reticulum, an assumption based on contractile experiments. Suematsu *et al.* (1984) have shown that cyclic GMP-dependent protein kinase phosphorylates a sarcolemmal protein, thereby stimulating Ca^{2+} uptake by sarcolemmal vesicles which are responsible for Ca^{2+} extrusion from the cell (Morel *et al.*, 1981).

All such effects could affect free cytoplasmic Ca^{2+} concentrations subsequent to receptor stimulation and hence the apparent efficacy of noradrenaline, which could also be altered by a direct effect of cyclic GMP on the contractile apparatus (Pfitzer *et al.*, 1984).

In conclusion, the increase in cyclic GMP levels in vascular smooth muscle cells of the rat aorta, evoked by the release of EDRF, could modulate the efficacy of noradrenaline, hence resulting in modulation of prazosin antagonism.

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