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Atriopeptin II-induced relaxation of rabbit aorta is potentiated by M&B 22,948 but not blocked by haemoglobin

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1 We examined the effects of haemoglobin (which inhibits the vascular responses to stimulation of soluble guanylate cyclase) and of M&B 22,948 (which selectively inhibits cyclic GMP phosphodiesterase) on the relaxation induced in rabbit aorta by the atrial natriuretic peptide, atriopeptin II (which stimulates particulate guanylate cyclase).

2 Pretreatment with M&B 22,948 (100 μM) produced a 2.3 fold potentiation of atriopeptin II-induced relaxation of endothelium-denuded rings of rabbit aorta.

3 Pretreatment with haemoglobin (10 μM) had no effect on the relaxation or the 10.9 fold increase in cyclic GMP content induced by atriopeptin II in endothelium-denuded rings of rabbit aorta.

4 The potentiation by M&B 22,948 suggests a causal role for cyclic GMP in mediating atriopeptin II-induced vasodilatation of rabbit aorta.

5 The inability of haemoglobin to block the atriopeptin II-induced rise in cyclic GMP suggests that it does not block stimulation of particulate guanylate cyclase. Thus, it is unlikely that a ferrous haem-containing receptor site is involved in the activation of the particulate form of guanylate cyclase as it is with soluble guanylate cyclase.

Introduction

Smooth muscle relaxation induced by the endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980), the bovine retractor penis inhibitory factor (IF) (Ambache *et al.*, 1975; Gillespie & Martin, 1980), ultraviolet light (Furchgott *et al.*, 1961), and the nitrovasodilators is associated with an increase in cyclic GMP content (Schultz *et al.*, 1977; Rapoport & Murad, 1983; Bowman & Drummond, 1984; Karlsson *et al.*, 1984; Furchgott *et al.*, 1984b). For the nitrovasodilators, acting via nitric oxide (Arnold *et al.*, 1977; Katsuki *et al.*, 1977), and for EDRF and ultraviolet light, these responses are mediated through activation of the soluble form of the enzyme guanylate cyclase (Rapoport *et al.*, 1983; Karlsson *et al.*, 1984; Busse *et al.*, 1985), though the ability of IF to activate this form of the enzyme has not yet been tested. Soluble guanylate cyclase has a ferrous haem-containing receptor site with which stimulants interact to induce enzyme activation (Craven & De Rubertis, 1978). Exogenously added haemoglobin inhibits the smooth muscle relaxation and concomitant increases in guanosine 3′:5′-cyclic monophosphate (cyclic GMP) induced by EDRF, the nitrovasodilators, IF and ultraviolet light (Bowman & Gillespie, 1982; Bowman & Drummond, 1984; Martin *et al.*, 1985, Furchgott *et al.*, 1984b). It has been proposed that this inhibition results from haemoglobin competing with the haem moiety of guanylate cyclase for the active principles (Martin & Smith, 1985; Martin *et al.*, 1986b).

Another class of substances, the atrial natriuretic peptides (ANPs) which appear to be involved in fluid volume regulation (Currie *et al.*, 1983), likewise induce smooth muscle relaxation in association with a rise in cyclic GMP (Fiscus *et al.*, 1985). ANPs do not activate soluble guanylate cyclase, but a particulate form of this enzyme (Waldman *et al.*, 1984; Winquist *et al.*, 1984; Mittal, 1985).

It is not known if particulate guanylate cyclase, like the soluble isoenzyme, has a ferrous haem-containing receptor site, although some indirect evidence against this has been obtained (Rapoport *et al.*, 1985). We investigated this possibility by examining the effects of haemoglobin on vascular relaxation and increases in cyclic GMP content induced by the ANP, atriopeptin II, in endothelium-denuded rings of rabbit aorta.

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Methods

Organ bath studies

The preparation of aortic rings was similar to that described by Furchgott & Zawadzki (1980). Briefly, male New Zealand white rabbits weighing 2 to 3 kg were killed by stunning and exsanguination. The aorta was removed, cleaned of adhering fat and connective tissue and cut into 2.5 mm wide transverse rings with a razor blade slicing device. Endothelial cells were removed by gently rubbing the intimal surface with a wooden stick for 30 to 60 s. Successful removal of endothelial cells was confirmed later by the inability of acetylcholine (1 μM) to induce relaxation. Rings were then mounted under 2 g resting tension on stainless steel hooks in 20 ml organ baths, and bathed at 37°C in Krebs solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11 and disodium EDTA 0.03, and gassed with 95% O_2 and 5% CO_2. Tension was recorded isometrically using Ormed UF1 transducers, and displayed on an Ormed Multitrace 4 chart recorder. Tissues were allowed to equilibrate for 90 min before experiments were begun, during which time the resting tension was maintained at 2 g.

Preparation of haemoglobin

Bovine haemoglobin Type 1 (Sigma) contains a mixture of oxyhaemoglobin and its oxidized derivative methaemoglobin. Pure haemoglobin (oxyhaemoglobin) was prepared by adding to a 1 mM solution of Sigma haemoglobin in distilled water, a 10 fold molar excess of the reducing agent sodium dithionite ($Na_2S_2O_4$). Sodium dithionite was then removed by dialysis against 100 volumes of distilled water for 2 h at 4°C. The purity of the solutions of haemoglobin was determined spectrophotometrically, and the solutions were frozen in aliquots at −20°C and stored for up to 14 days.

Measurement of cyclic GMP levels

Endothelium-denuded rings of rabbit aorta were incubated in 50 ml baths containing Krebs solution at 37°C gassed with 95% O_2 and 5% CO_2. At any time in an experiment these tissues could be removed and plunged immediately into liquid N_2. Frozen tissues were homogenized in 2 ml ice-cold 6% trichloroacetic acid (TCA), centrifuged at 2,000 *g* for 10 min at 4°C and the pellet and supernatant separated. TCA was removed from the supernatant by adding 4 ml of 0.5M tri-n-octylamine in freon (1, 1, 2trichlorotrifluoroethane) and vortex mixing until the aqueous phase was pH 5.5–6. The aqueous phase was removed and its cyclic GMP content determined by radioimmunoassay using New England Nuclear kits as previously described (Furchgott *et al.*, 1984a). The tissue pellet was resuspended in 1M NaOH, incubated for 30 min at 70°C and protein content determined by the colourimetric method of Bradford (1976).

Drugs

Rat synthetic atriopeptin II and phenylephrine were obtained from Sigma and dissolved in double-distilled water. M&B 22,948 (2-0-propoxyphenyl-8-azapurine-6-one) was a generous gift from May and Baker Ltd., and was prepared as a 10^{-1}M stock solution in 10% triethanolamine. The final bath concentration of triethanolamine was 0.01% which did not affect tone in control rings.

Statistical analysis

Results are expressed as the mean ± s.e.mean and comparisions were made by means of Student's *t* test. A probability of 0.05 or less was considered significant.

Results

The relaxation of rabbit aortic rings by atriopeptin II and its potentiation by M&B 22,948

Following contraction of endothelium-denuded rings of rabbit aorta with phenylephrine (30–300 nM), exposure to atriopeptin II (30 pM–10nM) induced a dose-dependent relaxation (Figure 1): EC_{50} for relaxation was $8.9 \pm 1.3 \times 10^{-10}$M ($n = 8$). The selective cyclic GMP phosphodiesterase inhibitor, M&B 22,948 (100 μM), induced a 20.2 ± 1.8% ($n = 8$) relaxation of phenylephrine-contracted endothelium-denuded aortic rings. When tone was re-established to the original level with additional phenylephrine, subsequent relaxation to atriopeptin II (30 pM–10nM) was significantly ($P < 0.005$) potentiated 2.3 fold (Figure 1): EC_{50} for relaxation was $3.8 \pm 0.6 \times 10^{-10}$M ($n = 8$).

The effect of haemoglobin on atriopeptin II-induced relaxation of rabbit aortic rings

Exposure of phenylephrine (30–300 nM)-contracted rings to haemoglobin (10 μM) for 10–20 min produced a 9.5 ± 1.7% ($n = 8$) further increase in tone. Following haemoglobin pretreatment atriopeptin II-induced relaxation was not significantly different from that obtained on control rings (Figure 2).

The effect of haemoglobin on the atriopeptin II-induced increase in cyclic GMP content of rabbit aortic rings

Exposure of aortic rings to atriopeptin II (10 nM) for

3 min induced a 10.9 fold increase in the cyclic GMP content (Table 1). Pretreatment of aortic rings for 20 min with haemoglobin (10 μM) reduced the resting level of cyclic GMP, but the increase induced by atriopeptin II (10 nM) was unaffected (Table 1).

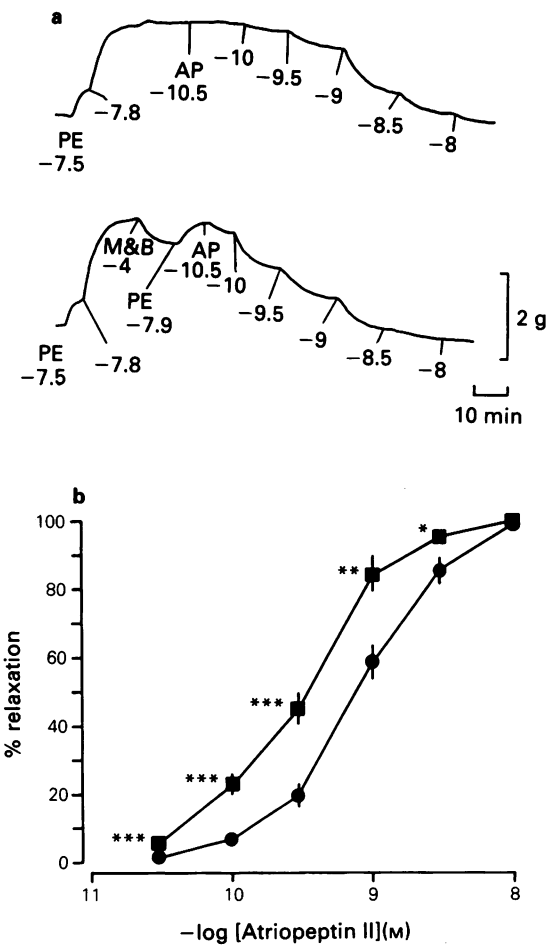

Figure 1 The effect of the selective cyclic GMP phosphodiesterase inhibitor, M&B 22,948, on the atriopeptin II-induced relaxation of endothelium-denuded rings of rabbit aorta. (a) Top panel: relaxation of a phenylephrine (PE)-contracted aortic ring by atriopeptin II (AP). Bottom panel: relaxation induced by atriopeptin II following pretreatment with M&B 22,948 (M&B, 100 μM). Cumulative molar concentrations are given in log units. (b) Dose-response curves showing control responses to atriopeptin II (●) and responses to atriopeptin II following pretreatment for 10–20 min with M&B 22,948 (100 μM) (■). Each point is the mean of 8 observations and vertical lines show s.e.mean. $*P < 0.05$; $**P < 0.005$; $***P < 0.001$.

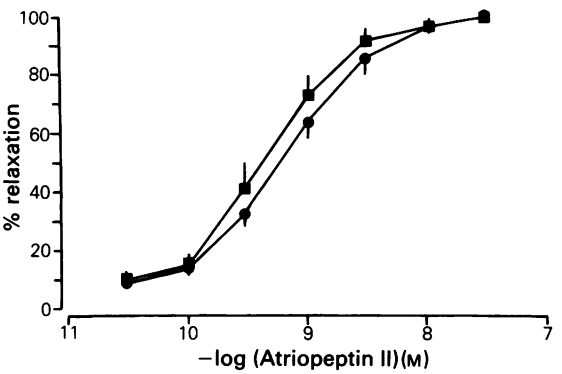

Figure 2 Dose-response curves showing the effects of haemoglobin on the atriopeptin II-induced relaxation of endothelium-denuded rings of rabbit aorta. Control responses to atriopeptin II (●) and responses to atriopeptin II following pretreatment for 10–20 min with haemoglobin (10 μM) (■) are shown. Each point is the mean of 8–10 observations and vertical lines show s.e.mean.

Discussion

We found that the relaxation of endothelium-denuded rings of rabbit aorta induced by the atrial natriuretic peptide, atriopeptin II, was potentiated significantly by the selective cyclic GMP phosphodiesterase inhibitor, M&B 22,948. This potentiation was of similar magnitude to the potentiation by M&B 22,948 of relaxation induced by the nitrovasodilators (Kukovetz *et al.*, 1979; Martin *et al.*, 1986a), which also elevate cyclic GMP levels. It suggests a causal role for cyclic GMP in atriopeptin II-induced vasodilatation.

Table 1 Effect of haemoglobin on the atriopeptin II-induced increase in cyclic GMP content of endothelium-denuded rings of rabbit aorta

Stimulus	*Pretreatment*	*Cyclic GMP* (pmol g^{-1} protein
None (control)	None	71 ± 5 (12)
None	Hb, 10 μM	37 ± 3 ***(10)
APII, 10 nM	None	774 ± 64***(6)
APII, 10 nM	Hb, 10 μM	770 ± 76***(6)

The effects of pretreatment for 20 min with haemoglobin (Hb) were determined on both the basal content of cyclic GMP and on the increased content stimulated by a 3 min exposure to atriopeptin II (APII). The cyclic GMP content is expressed in pmol g^{-1} protein. Results are expressed as the mean ± s.e.mean and the numbers of observations are shown in parentheses. $***P > 0.001$, significantly different from control.

It is not known whether the particulate form of guanylate cyclase has, like the soluble isoenzyme (Craven & De Rubertis, 1978), a ferrous haem-containing receptor site. Methylene blue and cyanide, which inhibit soluble guanylate cyclase, probably by oxidizing or by binding to the haem moiety of the enzyme, respectively (Rapoport & Murad, 1984; Martin *et al.*, 1985), do not inhibit atriopeptin II-induced increases in cyclic GMP content in rat aortic rings (Rapoport *et al.*, 1985). This has been taken as evidence against the presence of haem moieties on particulate guanylate cyclase.

We found that haemoglobin, in concentrations which inhibit smooth muscle relaxation and increases in cyclic GMP induced by EDRF, IF, nitrovasodilators and ultraviolet light (Bowman & Drummond, 1984; Furchgott *et al.*, 1984b; Martin *et al.*, 1985), had no effect on the atriopeptin II-induced relaxation and associated increases in cyclic GMP in endothelium-denuded rings of rabbit aorta. Our results provide further evidence that a ferrous haem-containing receptor site is not involved in activation of the particulate form of guanylate cyclase.

In conclusion, potentiation by M&B 22,948 is consistent with the view that cyclic GMP mediates the vasodilator action of atriopeptin II. The inability of haemoglobin to block atriopeptin II-induced vasodilatation and associated increases in cyclic GMP in rabbit aorta, suggests that a ferrous haem-containing receptor site is not involved in the activation of particulate guanylate cyclase.

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