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Endothelium and the vasodilator action of rat calcitonin gene-related peptide (CGRP)

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1 Acetylcholine and rat calcitonin gene-related peptide (CGRP) produced a relaxation in rat isolated aortic rings which was entirely dependent on the presence of the endothelium.

2 In the absence of any exogenous vasodilator agent, the cyclic guanosine monophosphate (cyclic GMP) content was higher in rings with endothelium than in those without.

3 The vasorelaxation produced by acetylcholine and sodium nitroprusside was accompanied by increases in cyclic GMP in the smooth muscle, whereas that produced by CGRP was not accompanied by cyclic GMP accumulation.

4 Therefore, it appears unlikely that CGRP releases an endothelium-derived relaxing factor similar to that released by acetylcholine.

Introduction

Calcitonin gene-related peptides (CGRPs) are a family of novel peptides found in the brain and in nerve fibres associated with blood vessels in the periphery (Rosenfeld *et al.*, 1983). Rat and human CGRP increase blood flow in many vascular beds including skin, coronary, and mesenteric circulations (Brain *et al.*, 1985; Marshall *et al.*, 1986a, b), and are potent dilators of large distributing vessels including the rat aorta and the coronary arteries of several species (Kubota *et al.*, 1985; Thom *et al.*, 1986). However, the relaxation of vascular smooth muscle is thought to be mediated indirectly as it is abolished by removal of the endothelium *in vitro* (Brain *et al.*, 1985; Kubota *et al.*, 1985). Thus CGRP could be one of a growing number of endogenous vasodilators (including acetylcholine, ATP, bradykinin and substance P; Furchgott, 1983) that act by releasing an endothelium-derived relaxing factor (EDRF).

In a previous study we showed that rat CGRP has a dose-dependent stimulatory effect on adenylate cyclase in rat aortic smooth muslce, but does not directly affect accumulation of cylic guanosine monophosphate (cyclic GMP; Kubota *et al.*, 1985). In contrast, the EDRF released by acetylcholine appears to act on vascular smooth muscle by stimulating guanylate cyclase, and does not affect adenylate cyclase (Rapoport & Murad, 1983). Therefore in the present study we have examined whether CGRP also induces endothelium-dependent dilatation by releasing an EDRF that, in turn, elevates cyclic GMP in aortic smooth muscle.

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Methods

Relaxation of rat aorta

Male Sprague-Dawley rats (200–300 g) were heparinized (200 u, i.v.) and killed by a blow to the head after inhalation of diethyl ether. The thoracic aorta was quickly removed, cleaned of connective tissue and fat, and cut into rings, 3–4 mm in length. Care was taken to avoid unintentional rubbing of the intimal surface so as to maintain the integrity of the endothelial layer. In some rings, the intimal surface was rubbed gently with a wooden taper to remove the endothelium. The rings were mounted on wire hooks in 5 ml organ baths containing oxygenated (95% O_2: 5% CO_2) Krebs solution of the following composition (mM); NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2 and glucose 11. A resting tension of 2.0 g was maintained for a 30 min equilibration period. The rings were contracted with KCl (40 mM for 2 min) and washed for a further 30 min. Rings not contracting to KCl were rejected from the experiment. After this second equilibration period all rings were contracted by submaximal concentration of phenylephrine (0.1 or 0.3 μM) producing 60–80% of a maximal contraction. Contractions were allowed to reach a plateau before the addition of sodium nitroprusside,

acetylcholine or rat CGRP. Cumulative concentration-response curves were studied initially to determine concentrations of each dilator agent which produced 60–100% of the maximal relaxation response to each agent. This concentration and a lower one of acetylcholine were then given to separate rings (contracted with phenylephrine, 0.1 μM) as a single dose, and at various times after exposure to the agent the tissues were quickly removed from the organ bath and snap frozen in liquid nitrogen.

Determination of cyclic GMP

The frozen rings were individually ground in a mortar and pestle, transferred to acidified ethanol (95% solution adjusted to pH 3 with HCl) and left overnight for extraction of the cyclic nucleotides. Samples were then centrifuged and the supernatant fraction evaporated to dryness. This was reconstituted in a sodium acetate buffer (50 mM at pH 5) containing theophylline (1 mM), acetylated and assayed for cyclic GMP as previously described (Hunt *et al.*, 1980). The tissue residue was dissolved in 2 M NaOH and protein content was determined by the Biorad assay (Bradford, 1976) technique, with bovine serum albumin as the standard.

Chemicals

The following chemicals were used: acetylcholine perchlorate (Sigma), phenylephrine (Sigma), sodium nitroprusside dihydrate (Roche). Synthetic rat CGRP was purchased from Bachem, Palo Alto, CA, U.S.A. and stored at −20°C in 0.01 M acetic acid aliquots. Drugs were diluted or dissolved in Krebs solution immediately before use. [^{125}I]-cyclic GMP tyrosine methyl ester was purchased from the Radiochemical Centre, Amersham, UK, and cyclic GMP from Boehringer Mannheim (Australia). Antiserum to cyclic GMP was provided by Dr N.H. Hunt (Canberra, Australia).

Statistical analysis

Results shown in the text and figures are expressed as the means ± s.e.means. For statistical evaluation, data were analysed by Student's *t* test.

Results

Relaxant responses to sodium nitroprusside, acetylcholine and rat CGRP

All three vasodilators elicited concentration-dependent relaxation of phenylephrine-contracted rings when functional endothelium was present (Figure 1).

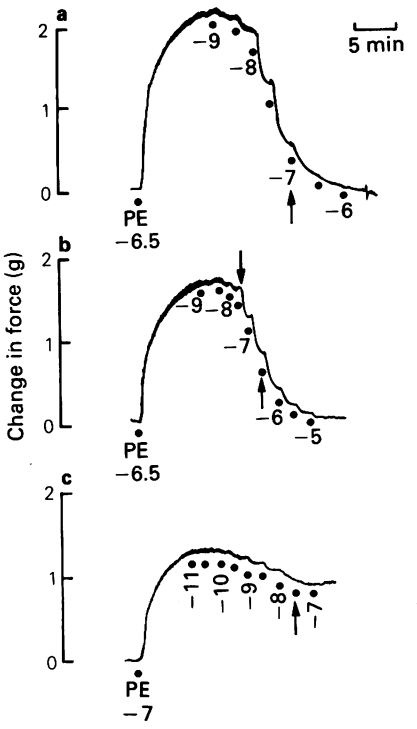

Figure 1 Relaxation of rat aortic rings by (a) sodium nitroprusside, (b) acetycholine, and (c) rat CGRP. All rings were preconstricted by phenylephrine (PE), and the dilators were added at the indicated log-molar concentrations. The concentrations used in subsequent studies of cyclic GMP levels are indicated by the arrows.

Only nitroprusside was capable of relaxing rings from which the endothelium had been removed, and this was not significantly different from that induced in rings with preserved endothelium. In endothelium-containing rings, nitroprusside and acetylcholine produced maximal relaxations exceeding 80% of the initial contractions, but rat CGRP caused only a weak, dose-dependent relaxation to a maximum of 20–25% of initial contractions (Figure 1).

For further studies of cyclic GMP and relaxation responses, concentrations of nitroprusside (0.1 μM), acetylcholine (0.3 μM) and CGRP (0.03 μM) were chosen that elicited 70–100% of the maximal relaxations obtainable with each agent in endothelium-containing rings. A lower concentration of acetylcholine (0.03 μM) that produced relaxation equivalent to CGRP (0.03 μM) was also studied.

Time course of relaxation and cyclic GMP response

In rings with endothelium, the time course of relaxation induced by acetylcholine (0.3 μM) was similar to

that described by others (Furchgott & Zawadzki, 1980; Rapoport & Murad, 1983). On addition of acetylcholine the smooth muscle content of cyclic GMP increased rapidly, concomitant with the decline in tension, the former reaching a maximum between 20 and 30 s. However, the cyclic GMP content declined from this point, whereas the smooth muscle continued to relax further for at least 5 min in the continued presence of acetylcholine (Figure 2). In nine other sets of rings with endothelium, the time courses of cyclic GMP elevation and relaxation induced by nitroprusside were similar to acetylcholine (Figure 3). Therefore, for further study of the vasodilators, 30 s was chosen as the optimal time for measurement of the maximum cyclic GMP response.

Preconstricted rings without endothelium had basal levels of cyclic GMP that were less than 30% of those of rings with endothelium (0.18 ± 0.02 and 0.78 ± 0.08 fmol ug^{-1} protein, respectively) before addition of any vasodilator agent. Interestingly, these cyclic GMP levels in rings stripped of endothelium increased about 2 fold after addition of acetylcholine (0.3 μM), compared with the approximately 7 fold increase that occurred in rings with endothelium (Figure 2). There was no relaxation associated with the small cyclic GMP response in the rings without endothelium.

Effect of nitroprusside, acetylcholine and CGRP on cyclic GMP levels

In both rubbed and unrubbed rings, nitroprusside (0.1 μM) caused a significant elevation of cyclic GMP levels above the corresponding controls (Figure 4). There was no significant difference between the cyclic GMP levels or relaxation attained with nitroprusside in rings with intact endothelium as compared to rings without endothelium.

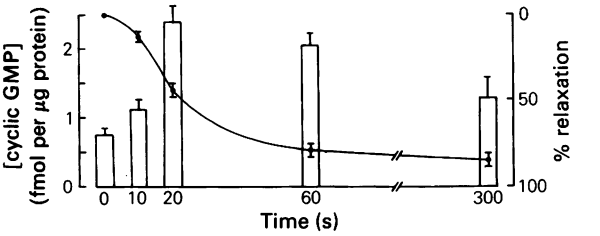

Figure 3 Time courses of relaxation (●) and change in cyclic GMP content (histograms) induced by sodium nitroprusside (0.1 μM) added at 0 s. Data are for 8 sets of rings with endothelium. Vertical lines represent s.e.means.

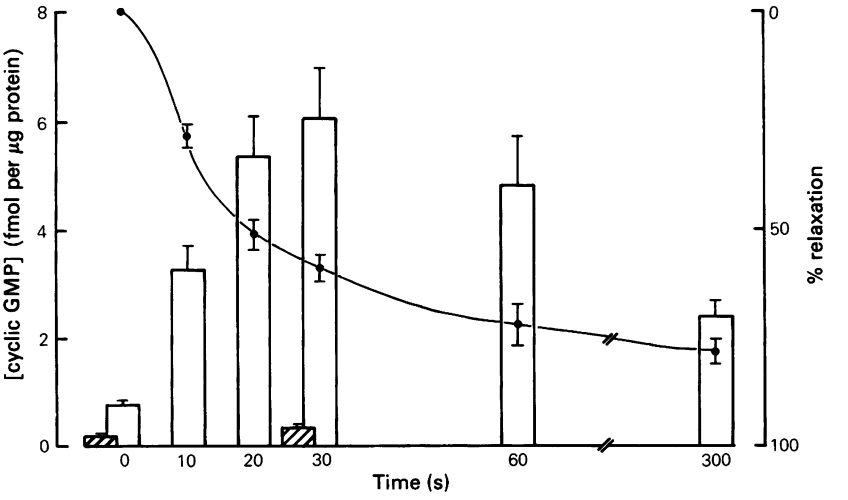

Figure 2 Time courses of relaxation (●) and change in cyclic GMP content (histograms) induced by acetylcholine (0.3 μM) added at 0 s. Open columns represent means for 8 sets of rings with endothelium, and hatched columns for 10 pairs of rings without endothelium. Rings without endothelium did not relax. Vertical lines represent s.e.means.

Acetylcholine (0.03 and 0.3 μM) caused marked elevation of cyclic GMP levels in rings with endothelium, but, as noted above, only a slight response in endothelium-denuded rings. The maximum levels attained with acetylcholine (0.3 μM) were 17 fold higher in endothelium-containing rings (Figure 4).

The effects of CGRP were markedly different from those of both nitroprusside and acetylcholine. CGRP (0.03 μM) did not significantly alter cyclic GMP levels in rings with or without endothelium, but had a relaxant effect in rings with endothelium and no effect in rings without endothelium (Figure 4).

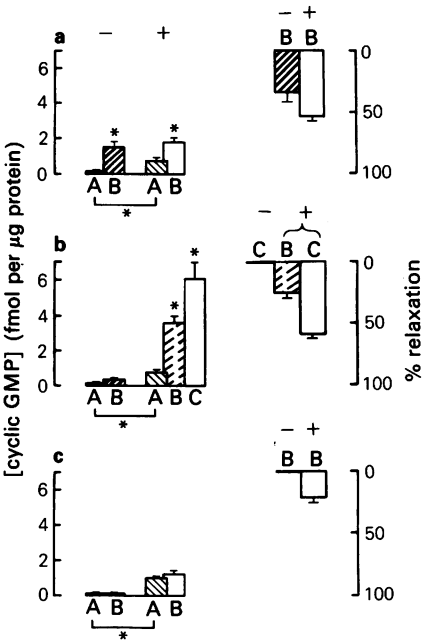

Figure 4 Cyclic GMP content (left panels) and relaxation responses (right panels) in rings with (+) and without (−) endothelium. Cyclic GMP measurements were made in paired rings before (A) or 30 s after (B, C), addition of (a) sodium nitroprusside (0.1 μM; B, 9 sets of rings), acetylcholine (0.03 (B) and 0.3 (C) μM, 11 and 8 sets of rings, respectively) or rat CGRP (0.03 μM; B, 8 sets of rings). Relaxations shown were measured at 30 s. Cyclic GMP levels that are significantly different from rings with endothelium intact before the addition of the dilators are indicated (* $P < 0.05$, Student's t test).

Discussion

The present experiments have confirmed the previous observations that rat CGRP, at low concentrations, causes vasorelaxation of rat thoracic aorta, which is entirely dependent on the integrity of the endothelium (Brain *et al.*, 1985; Kubota *et al.*, 1985). However, the maximal relaxation induced by CGRP was considerably less than that observed with the endothelium-dependent dilator acetylcholine, or the directly acting dilator sodium nitroprusside.

Endothelium-dependent dilatation induced by acetylcholine, histamine, ATP and the calcium ionophore A23187 has been strongly linked to activation of soluble guanylate cyclase in the smooth muscle (Rapoport & Murad, 1983). However, in contrast to acetylcholine and nitroprusside, CGRP did not elevate cyclic GMP either in rings with endothelium or in rings denuded of endothelium. Thus the mechanism of dilatation induced by CGRP is clearly different from that of either acetylcholine or nitroprusside.

Although CGRP has no effect on cyclic GMP accumulation, basal levels of cyclic GMP in aortic rings with endothelium are 4–5 fold higher than in similar rings without endothelium. This could indicate that in rat aortic rings under these conditions there is a basal release of EDRF sufficient to provide a modest stimulation of guanylate cyclase, as recently suggested by Martin *et al.* (1986). In addition, this difference in basal cyclic GMP levels may explain the endothelium-dependence of the vasodilator action of CGRP. Cyclic GMP accumulation leads to smooth muscle relaxation through activation of a cyclic GMP-dependent protein kinase which, in turn, activates a calcium ATPase and lowers the cytosolic calcium ion concentration (Popescu *et al.*, 1985; Lincoln *et al.*, 1986). A related series of events is thought to occur following stimulation of adenylate cyclase in smooth muscle (Kam & Stull 1985). The precise biochemical details of how these two cyclic nucleotide systems interact co-operatively in control of smooth muscle tone is not yet clear (Ignarro & Kadowitz, 1985). In our previous study we showed that CGRP is a potent activator of adenylate cyclase in rat aortic smooth muscle (Kubota *et al.*, 1985). Thus, although activation of adenylate cyclase alone does not alter smooth muscle tone in endothelium-denuded aortic rings, it does so when superimposed upon a modest accumulation of cyclic GMP in endothelium-containing tissues.

Whatever the mechanism involved, it is clear that the effects of endothelium removal reported in many studies on vascular tissues should be interpreted with caution. In particular, the demonstration that the vasodilator action of an agent is attenuated by removal of the endothelium is not sufficient evidence to conclude that it releases EDRF. A similar point has been made in relation to the augmentation of vasoconstrictor responses to α-adrenoceptor agonists following endothelium removal (Martin *et al.*, 1986).

In conclusion, although the vasodilator action of rat CGRP in rat aorta is totally dependent on the presence

of endothelium, it is not associated with accumulation of cyclic GMP. Therefore, either CGRP does not release EDRF, or it releases a relaxing factor different from that released by acetylcholine.

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