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Selective inhibition by gossypol of endothelium-dependent relaxations augments relaxations to glyceryl trinitrate in rabbit coeliac artery

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1 Acetylcholine, substance P, prostaglandin E_1 and the nitrovasodilator glyceryl trinitrate induced concentration-dependent relaxations of endothelium-intact strips of rabbit coeliac artery precontracted with noradrenaline.

2 Endothelium-denuded strip preparations contracted to acetylcholine and showed no response to substance P. The relaxant response to prostaglandin E_1 was unimpaired after removal of endothelium, whereas the response to glyceryl trinitrate was increased.

3 A 20 min exposure of endothelium-intact strips to gossypol, an irreversible inhibitor of the production and/or release of endothelium-derived relaxing factor, abolished vasodilatation in response to the endothelium-dependent agents acetylcholine and substance P, did not change relaxations to prostaglandin E_1, but significantly enhanced relaxations in response to glyceryl trinitrate.

4 In view of the assumed common mechanism of action of endothelium-derived relaxing factor and nitrovasodilators, these results suggest an interference of the two active principles at the level of the vascular smooth muscle cell.

Introduction

Acetylcholine, substance P and several other vasodilators require an intact endothelium to elicit their relaxant effect (for review see Furchgott, 1984; Peach *et al.*, 1985; Förstermann, 1986). These agents induce the formation and release of endothelium-derived relaxing factor (EDRF). EDRF is a humoral agent with a short half life in the range of seconds (Griffith *et al.*, 1984; Förstermann *et al.*, 1984). Relaxations mediated by EDRF were found to be associated with increased levels of cyclic guanosine monophosphate (cyclic GMP) in vascular smooth muscle cells (for review see Ignarro & Kadowitz, 1985; Murad, 1986), and EDRF is probably a direct activator of soluble guanylate cyclase (Förstermann *et al.*, 1986b). Relaxation of vascular preparations with nitric oxide (NO)-containing vasodilators like organic nitrates and sodium nitroprusside is also associated with guanylate cyclase activation and cyclic GMP accumulation. These 'nitrovasodilators' (Murad, 1986), or their metabolites, stimulate soluble guanylate cyclase by interacting directly with the enzyme (Böhme *et al.*, 1984; Ignarro & Kadowitz, 1985; Murad, 1986).

Recently, reports have appeared showing that the relaxant response to several nitrovasodilators was augmented after removal of the endothelium (Shirasaki & Su, 1985; Shirasaki *et al.*, 1986; White *et al.*, 1986). In view of the similar mechanism of action of nitrovasodilators and EDRF we have now investigated whether the ability of endothelial cells to produce EDRF might interfere with the effect of the nitrovasodilator glyceryl trinitrate.

Methods

Preparation of vascular strips

Rabbits of either sex (2.5–3.5 kg bodyweight) were killed by stunning and exsanguination. The coeliac artery was rapidly dissected out and rinsed with ice-cold modified Krebs solution. After removing superficial adipose and connective tissue, transverse strips (cut-open rings, about 1 × 10 mm) were prepared and

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suspended in 5 ml organ baths containing oxygenated (95% O_2, 5% CO_2) modified Krebs solution (pH 7.4, 37°C). Care was taken during this procedure to avoid contact with the luminal surface of the strips. The composition of the modified Krebs solution was as follows (mM): Na^+ 145.0, K^+ 5.95, Ca^{2+} 1.7, Mg^{2+} 1.2, Cl^- 128.15, HCO^-_3 25.0, $H_2PO^-_4$ 1.2, SO^{2-} 1.2, glucose 10.6 and disodium EDTA 0.025. Contraction force was monitored with a Statham isometric force tranducer connected to a d.c. amplifier and a pen recorder. The vascular strips were equilibrated for approximately 1 h during which the buffer was changed at 12 min intervals. Final resting force prior to experimentation was 1 g. Before the actual experiment all strips were contracted with noradrenaline (10^{-6} M) and then exposed to acetylcholine (10^{-6} M) as a functional test for endothelial integrity (Furchgott, 1984). Strips showing less than 40% relaxation to acetylcholine (10^{-6} M) were discarded from the study (about 5% of the total number).

Organ bath experiments

A typical experiment consisted of a control period during which the strips were contracted with noradrenaline (10^{-6} M). After tension had stabilized the preparations were relaxed by the addition of cumulative doses of one of the endothelium-dependent vasodilators, acetylcholine or substance P, and one of the endothelium-independent vasodilators, prostaglandin E_1 or glyceryl trinitrate. Noradrenaline HCl (Sigma, Munich, FRG) was dissolved and diluted in 0.001 N HCl containing ascorbic acid (1 mg ml^{-1}). Acetylcholine HCl (Sigma) and glyceryl trinitrate (10% triturated mixture in lactose, Mack, Illertissen, FRG) were dissolved in distilled water. Substance P (Sigma) was dissolved in phosphate buffer containing gelatine (1 mg ml^{-1}). Prostaglandin E_1 (Upjohn, Kalamazoo, MI, USA) was dissolved in 70% (v/v) ethanol. After appropriate dilution with modified Krebs solution the compounds were added to the organ bath in 50 µl. Exposures of the arterial strips to different relaxants were separated by 36 min. The first contraction-relaxation protocol was followed by a 20 min exposure of the strips to the polyphenolic antioxidant gossypol (3×10^{-5} M) (Hamasaki & Tai, 1985). Gossypol acetic acid (Sigma) was dissolved in dimethyl sulphoxide (DMSO) and added to the organ bath in 10 µl (the final bath concentration of DMSO was 0.2%). Then the tissues were washed 4 times with fresh modified Krebs solution to remove all gossypol from the organ bath. Tension was readjusted to 1 g before the second experimental cycle started which was identical to the first cycle described above. In other experiments the arterial strips were taken out of the organ bath after the first experimental cycle and their intimal surfaces were gently rubbed with the blunt side of a scalpel to remove the endothelial cell layer.

No significant changes were observed in contraction or relaxation responses over two experimental periods in control strips (exposed to 0.2% DMSO instead of gossypol). In sample preparations endothelial integrity was judged by lightmicroscopy after silver staining (method modified from Poole *et al.*, 1958) at the end of the experiment. Gossypol (or its solvent DMSO, 0.2%) did not lead to an appreciable loss of endothelial cells from the vascular strips whereas the rubbing procedure removed more than 95% of the endothelial cells.

Statistical analysis

Results are expressed as means ± s.e.mean. Differences in response to vasodilators before and after gossypol treatment or endothelial denudation were tested for statistical significance by one-way analysis of variance followed by the Fisher least-significant-difference test for comparison of different means (Snedecor & Cochran, 1967). A P value less than 0.05 was considered significant.

Results

Relaxation responses in the presence and absence of endothelium

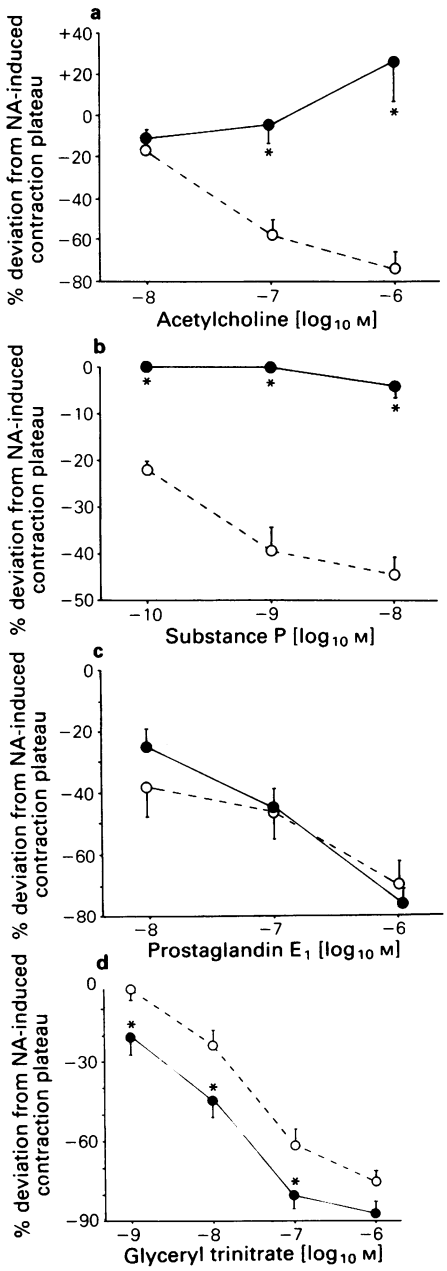
In preconstricted strips of rabbit coeliac artery with the endothelium intact all four vasodilators tested (acetylcholine, substance P, prostaglandin E_1, and glyceryl trinitrate) induced concentration-dependent relaxations (Figure 1). In rubbed preparations (endothelium removed) acetylcholine (10^{-8}–10^{-7} M) had no significant effect on the preparations, and acetylcholine (10^{-6} M) generally produced additional contractions (due to the direct contractile effect of the muscarinic agonist on vascular smooth muscle; cf. Furchgott, 1984). Substance P (10^{-10} M–10^{-8} M) had no effect at all in endothelium-denuded preparations. Concentration-response curves to prostaglandin E_1 (10^{-8} M–10^{-6} M) were essentially identical in rubbed and unrubbed strips, whereas responses to glyceryl trinitrate (10^{-9}–10^{-7} M) were significantly greater in the absence of endothelium. For example, glyceryl trinitrate (10^{-8} M) produced 29% ± 4% relaxation in unrubbed, but 44% ± 5% relaxation in rubbed strips (mean ± s.e.mean, $n = 8$); at 10^{-7} M the responses to glyceryl trinitrate were 65% ± 6% relaxation versus 82% ± 5% relaxation (mean ± s.e.mean, $n = 8$); the differences were significant at $P < 0.05$. This confirms previous observations obtained in other arterial tissues under induced tension (Shirasaki & Su, 1985; Shirasaki *et al.*, 1986; White *et al.*, 1986).

Relaxation response before and after treatment with gossypol

The above findings raised the question as to whether the ability of endothelium to produce EDRF might be involved in the diminished response to glyceryl trinitrate. We therefore investigated the responses to all four vasodilators before and after treatment of coeliac arterial strips with the irreversible inhibitor of EDRF production, gossypol.

Exposure of the arterial preparations to gossypol (3×10^{-5} M for 20 min) abolished relaxations in response to acetylcholine and substance P (Figures 1a, b); relaxations in response to acetylcholine (10^{-6} M) were often converted into contractions (Figure 1a). This is due to the direct contractile effect of acetylcholine on vascular smooth muscle (cf. above). The endothelium-independent relaxations by prostaglandin E_1 remained unchanged after gossypol (Figure 1c). In contrast, the concentration-effect curve of glyceryl trinitrate was significantly shifted to the left after gossypol treatment (Figure 1d).

Treatment of the coeliac artery strips with gossypol had no effect on noradrenaline (10^{-6} M)-induced tension (1.02 ± 0.15 g before gossypol versus 1.07 ± 0.21 g after gossypol, $n = 26$).



Discussion

The present results demonstrate that gossypol blocked selectively and irreversibly EDRF-mediated relaxations in rabbit coeliac artery. Relaxations in response to the endothelium-independent agent prostaglandin E_1 were not affected by this inhibitor. Previous bioassay experiments have shown that gossypol inhibits the formation and/or release of EDRF and does not interact with the factor after release or with its effect on the smooth muscle cells (Busse & Förstermann, 1986; Förstermann *et al.*, 1986a). The finding that relaxations by glyceryl trinitrate were increased after treatment of the arterial strips with gossypol suggests that the basal formation of EDRF could somehow interfere (or compete) with the response of the artery to glyceryl trinitrate. Basal (unstimulated)

Figure 1 Effect of four different vasodilators on endothelium-intact strips of rabbit coeliac artery. The arterial strips were preconstricted with noradrenaline (NA, 10^{-6} M). The effects of different concentrations of the vasodilators were tested before (Control, O- - -O) and after gossypol (3×10^{-5} M, ●—●). Relaxation is expressed as percentage of the response to NA. All values are given as means with s.e.mean shown by vertical lines. Asterisks indicate significant differences ($P < 0.05$) versus control. (a) Effect of the endothelium-dependent relaxant acetylcholine (10^{-8}–10^{-6} M, $n = 13$); (b) effect of the endothelium-dependent relaxant substance P (10^{-10}–10^{-8} M, $n = 4$–8); (c) effect of prostaglandin E_1 (10^{-8}–10^{-6} M, $n = 13$, not mediated by endothelial cells); (d) effect of glyceryl trinitrate (10^{-9}–10^{-6} M, $n = 12$–16; not mediated by endothelial cells).

EDRF production has been demonstrated by bioassay (Griffith *et al.*, 1984) and it is also suggested by the finding of higher concentrations of cyclic GMP in (unstimulated) endothelium-intact arteries compared with endothelium-denuded preparations (Rapoport & Murad, 1983). Abolition of the EDRF-production may also explain the increased response to nitrovasodilators observed in other arteries after endothelial denudation (Shirasaki & Su, 1985; Shirasaki *et al.*, 1986; White *et al.*, 1986). This finding was confirmed here for glyceryl trinitrate in rabbit coeliac artery.

In view of the assumed common mechanism of action of EDRF and nitrovasodilators (Murad, 1986; Förstermann *et al.*, 1985b), the interference (or competition) could occur at the level of the soluble guanylate cyclase. Agents leading to increases in cyclic GMP by mechanisms other than activation of soluble guanylate cyclase (like atrial natriuretic peptide or 8-bromo-cyclic GMP) are apparently not influenced by the presence of endothelium (Shirasaki *et al.*, 1986). Also the relaxant action of prostaglandin E_1 remained unimpaired after inhibition of EDRF production with gossypol (this study). Effects of prostaglandin E_1 are unlikely to involve activation of a guanylate cyclase; they are rather associated with increases in cyclic adenosine monophosphate (Vegesna & Diamond, 1986).

The mechanism by which nitrovasodilators and EDRF possibly interact at the smooth muscle cell requires further investigation.

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