Co-axial bioassay of a smooth muscle relaxant factor released from guinea-pig tracheal epithelium

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1 The ability of guinea-pig trachea to release an epithelium-derived relaxant factor (EpDRF) was assessed in a co-axial bioassay system.

2 Histamine (100 μM) and methacholine (25 μM) caused endothelium-dependent relaxation of rat isolated aorta, presumably via the release of endothelium-derived relaxant factor (EDRF). In contrast, endothelium-denuded rat aorta did not relax in response to these agents.

3 EDRF release was detected in response to methacholine in a co-axial bioassay system, consisting of intact rabbit aorta tube (EDRF donor) and endothelium-denuded rat aorta strip (assay preparation). These results indicated the transfer of EDRF from a donor to an assay preparation, thereby validating the co-axial bioassay method.

4 Substitution of endothelium-intact rabbit aorta tube by epithelium-intact guinea-pig tracheal tube tissue in co-axial assemblies, still allowed the assay preparation to relax in response to histamine or methacholine. Removal of the intact tracheal tube from the system, or removal of the epithelium from the donor tracheal tube in co-axial preparations, abolished such relaxant responses. These observations are consistent with histamine- or methacholine-induced release of an epithelium-derived relaxant factor (EpDRF) from the trachea.

5 In the co-axial assembly comprising intact guinea-pig trachea and endothelium-denuded rat aorta, histamine and methacholine produced concentration-dependent, EpDRF-induced aortic relaxation. Mean concentrations of histamine and methacholine producing 50% of the maximum relaxation (EC_{50}) were 39.8 μM and 2.7 μM respectively. Histamine-induced relaxation was inhibited in the presence of mepyramine (2 μM) and responses to methacholine were inhibited by atropine (0.1 μM).

6 Methylene blue (50 μM) had no effect on such relaxant responses, indicating that EpDRF does not activate guanylate cyclase. Furthermore, the cyclo-oxygenase inhibitor indomethacin (5 μM), the cyclo-oxygenase/lipoxygenase inhibitor BW 755C (150 μM) and the leukotriene receptor antagonist FPL 55712 (10 μM) each failed significantly to alter EpDRF-mediated relaxation of vascular smooth muscle suggesting that EpDRF is not a prostanoid. Platelet activating factor (Paf) failed to cause relaxation of endothelium-denuded rat aorta, indicating that this mediator was also not EpDRF.

7 EpDRF was also released from human bronchial segments.

8 This study provides direct evidence for the release of an EpDRF from non-diseased airway tissue and further suggests that healthy airway reactivity to spasmogens is modulated by the release of an endogenous protective, spasmolytic substance. The bronchial hyperreactivity of asthma may be partly caused by attenuated production of such an inhibitory signal.

Introduction

The cause of asthma and of the characteristic bronchial hyperreactivity to airway spasmogens is unknown (Boushey *et al.*, 1980; Barnes, 1986). Inhalation of spasmogens in normal subjects causes bronchoconstriction which quickly reaches a plateau. In asthmatics, not only does bronchoconstriction occur

at lower doses, but it continues in a dose-related manner as if some protective inhibitory mechanism is lacking (Woolcock *et al.*, 1984). Our group (Goldie *et al.*, 1986a) and others (Flavahan *et al.* 1985; Barnes *et al.*, 1985; Murlas, 1986; Hay *et al.*, 1986), have recently suggested that the airway epithelium in healthy lung produces a relaxant factor, perhaps similar to vascular endothelium-derived relaxant

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factor (EDRF) (Furchgott & Zawadzki, 1980) which modulates the responsiveness of the underlying smooth muscle to spasmogens. Such an airway epithelium-derived relaxant factor (EpDRF) generated in response to airway smooth muscle spasmogens as well as to osmotic stimuli (Munakata *et al.*, 1988) may limit bronchoconstriction in healthy lung. Conversely, in asthma when the airway epithelium is damaged (Laitinen *et al.*, 1985), the production of protective EpDRF would be attenuated or abolished.

Smooth muscle reactivity to spasmogens including histamine and methacholine, was significantly increased in epithelium-denuded isolated airway preparations from several animal species as well as from man (Raeburn *et al.*, 1986). However, studies describing smooth muscle reactivity changes following epithelium removal provide only indirect evidence for the existence of EpDRF. In contrast, Ilhan & Sahin (1986) and Guc *et al.* (1988) have demonstrated the release and transfer of a smooth muscle relaxant substance from airway epithelium. In the present study, we have directly measured the relaxant activity of a substance derived from guinea-pig trachea in a co-axial bioassay system utilizing rat thoracic aorta. These data further establish the existence of EpDRF, and support the concept that asthma-induced airway hyperreactivity may be at least partly due to the loss of such an inhibitory autacoid.

Methods

Tissue preparation for organ bath experiments

Male Wistar rats (220–250 g) were killed by a blow to the head and the thoracic aorta removed. After removal of adhering fat and connective tissue, a segment of the vessel was cut open along the longitudinal axis. For some experiments, care was taken to preserve the integrity of the endothelium during the production of cross-cut vessel strip preparations (7–8 mm in length). For other experiments, the endothelium was removed by gentle rubbing with a cotton wool probe. All preparations were suspended under 500 mg tension in Krebs solution maintained at 37°C and gassed with 5% CO₂ in O₂ and equilibrated for 1 h in the presence of indomethacin (5 µM), which was present thereafter in all experiments.

Aortae were then precontracted with the α -adrenoceptor agonist phenylephrine (Phe). Initially, all preparations were challenged with 0.2 µM Phe. After washout and equilibration for 45 min, preparations were re-challenged with 0.05 µM Phe. This second challenge was repeated to confirm the production of a stable, submaximal increase in tone.

Preparations were then exposed to histamine (100 µM) or methacholine (25 µM). These tests were designed to confirm the ability of histamine and methacholine to induce the release of EDRF from endothelium-intact rat aorta as assessed by relaxation responses and to verify the successful deliberate removal of the endothelium by the absence of such effects in appropriate preparations. In some experiments, endothelium-intact and endothelium-denuded vessel strips were challenged with the β -adrenoceptor agonist (–)-isoprenaline (13 nM–1 µM) or the inflammatory mediator platelet activating factor (Paf, 1–100 nM).

Co-axial bioassay assemblies

Guinea-pig trachea Male guinea-pigs (SR/C Tricolour) weighing 450–500 g were killed by cervical dislocation and the tracheae removed, dissected free of surrounding tissue and cut into tubular segments approximately 7 mm in length. The epithelium of some tube segments was deliberately removed by gentle rubbing of the luminal surface with a cotton wool-coated probe as previously described for tracheal ring preparations (Goldie *et al.*, 1986a). Complete removal of the airway epithelium from all such preparations was verified by subsequent histological examination.

Following initial pharmacological verification of the presence or absence of endothelium, aorta preparations were set-up in co-axial assemblies under 500 mg resting tension, within guinea-pig tracheal tube segments. In such assemblies, the tracheal tube freely surrounds the vessel preparation without any direct contact between the tissues. After a 45 min equilibration period, the vessel within the assembly was precontracted with Phe (0.05 µM) and re-challenged with either histamine (5–200 µM) or methacholine (2.5–200 µM) in the presence or absence of the histamine receptor antagonists mepyramine (H₁-selective; 2 µM) or cimetidine (H₂-selective; 25 µM), the muscarinic cholinergic antagonist atropine (0.1 µM), the cyclo-oxygenase/lipoxygenase inhibitor BW 755C (150 µM), the leukotriene receptor antagonist FPL 55712 (10 µM), or the guanylate cyclase inhibitor methylene blue (50 µM). Indomethacin (5 µM) was also present throughout these experiments.

Human bronchus Bronchial tubes (2–3 mm, i.d.) were dissected as previously described (Goldie *et al.*, 1986b), from samples of non-diseased human lung obtained 2–4 h post-mortem from the victims of motor vehicle accidents. Tube segments (approximately 7 mm) were mounted co-axially with endothelium-denuded rat aorta and tested pharmacologically as described for guinea-pig trachea.

Figure 1 Responses to histamine (H; 100 μ M) or methacholine (M; 25 μ M) in (a) an endothelium-intact rat aorta preparation, (b) an endothelium-denuded rat aorta preparation, (c) an endothelium-denuded rat aorta preparation mounted co-axially within the lumen of an endothelium-intact rabbit aortic tube and (d) an endothelium-denuded rat aorta preparation mounted co-axially within the lumen of an endothelium-denuded rabbit aortic tube. All rat aortic preparations were precontracted with phenylephrine (Phe, 0.05 μ M; ▲). Indomethacin (5 μ M) was present throughout all the experiments.

Rabbit aorta The smooth muscle relaxant activity of EDRF released from rabbit aorta was also measured on endothelium-denuded rat aorta in co-axial bioassay. Endothelium-denuded aorta preparations were suspended under 500 mg tension within the lumen of tube segments (approximately 7 mm in length) of intact and endothelium-denuded rabbit aorta. Aortae were precontracted with Phe (0.05 μ M) and the assemblies then challenged with methacholine (25 μ M). These tests were conducted with a

view to validating the co-axial bioassay method with respect to the transfer of an endogenously produced and secreted relaxant substance.

Analysis of results

Unless otherwise indicated, numerical data are presented as mean $\pm$ standard error of the mean ($\pm$ s.e.mean). The potencies of agonists causing concentration-dependent responses of rat aorta

Figure 2 Responses to histamine (H, 100 μ M) and methacholine (M, 25 μ M) in endothelium-denuded rat aorta (a) mounted co-axially within the epithelium-intact guinea-pig trachea or (b) mounted co-axially within epithelium-denuded guinea-pig trachea. All preparations were pre-contracted with phenylephrine (Phe, 0.05 μ M; $\blacktriangle$). Indomethacin (5 μ M) was present throughout all the experiments.

preparations are expressed as pD_2 values or as molar EC_{50} values, i.e. concentrations producing 50% of the maximum response, derived from mean estimates of pD_2 where $pD_2 = -\log_{10}(EC_{50})$. The effects of competitive antagonists on the potency of agonists were assessed as mean shifts (with 95% confidence limits) derived from paired data i.e. from pD_2 values for the agonists obtained in the presence and absence of the appropriate antagonist.

Drugs and solutions

Drugs used were: cimetidine, histamine diphosphate, indomethacin, isoprenaline hydrochloride, methacholine chloride, mepyramine maleate, phenylephrine hydrochloride, L- α -phosphatidylcholine β -acetyl- γ -O-alkyl (Paf) (each from Sigma); atropine sulphate (D.H.A. Laboratories); 3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2 pyrazoline (BW 755C, Wellcome); 7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid (FPL 55712, Fisons); methylene blue (Gurr). Indomethacin was prepared in 5 mg ml⁻¹ sodium carbonate solution. Isoprenaline was prepared in 0.9% w/v NaCl solution (saline) containing ascorbic acid 20 μ g ml⁻¹. Stock solutions of Paf were prepared in 0.25% bovine serum albumin (BSA; Sigma) in polypropylene containers.

Dilutions of Paf stock solution were also prepared using 0.25% BSA. The Krebs solution used throughout the study had the following composition (mM): NaCl 117.6, KCl 5.4, NaHCO₃ 25, KH₂PO₄ 1.03, MgSO₄ 0.57, D-glucose 11.1 and CaCl₂ 2.5.

Results

Endothelium-dependent rat thoracic aorta preparations were precontracted with phenylephrine (Phe; 0.05 μ M). This concentration of Phe produced stable increases in tone averaging 233 ± 8 mg of force ($n = 42$ preparations from 7 animals). This was equivalent to $98 \pm 1\%$ ($n = 6$) of the maximum response to this agonist. In endothelium-intact aortic strips, histamine (100 μ M) and methacholine (25 μ M) reduced Phe-induced tone by $78 \pm 5\%$ ($n = 15$ preparations from 6 animals) and $77 \pm 2\%$ ($n = 14$ preparations from 4 animals), respectively, presumably as a result of the release of EDRF (Figure 1a). In contrast, endothelium-denuded rat aorta failed to respond to these agents, thereby verifying the absence of endothelial cells and of EDRF (Figure 1b). Nevertheless, the β -adrenoceptor agonist (–)-isoprenaline (13 nM–1 μ M) caused complete concentration-dependent relaxation of the

Figure 3 Concentration-dependent relaxation of endothelium-denuded rat aorta caused by (a) histamine (H; 5–200 μM) or (b) methacholine (M; 2.5–50 μM) induced release of epithelium-derived relaxing factor (EpDRF) from co-axially mounted guinea-pig trachea. Histamine-induced relaxation of rat aorta caused by EpDRF released from guinea-pig trachea in the (c) absence or (d) presence of the guanylate cyclase inhibitor methylene blue (50 μM). All preparations were pre-contracted with phenylephrine ($\blacktriangle$; 0.05 μM). Indomethacin (5 μM) was present throughout all the experiments.

endothelium-denuded tissue. The mean pD_2 value for isoprenaline-induced relaxation was 7.77 ± 0.05 ($n = 8$). Paf (1 nM–100 nM) was virtually inactive in endothelium-denuded rat aorta.

When endothelium-denuded rat aortic preparations were set-up co-axially within the lumen of endothelium-intact tubular segments of rabbit aorta, methacholine again caused relaxation equivalent to $59 \pm 5\%$ ($n = 16$ preparations from 5 animals) of Phe-induced tone (Figure 1c). Thus, these co-axial assemblies could function as bioassay systems, with the intact vessel tubular segment acting as a donor of EDRF and the endothelium-denuded preparation responding with relaxation. Removal of the endothelium from tubular rabbit aorta donor preparations, or exposure to the guanylate cyclase antagonist methylene blue (50 μM) abolished the relaxant effect

of methacholine. This demonstrated the endothelium dependency of this response in this co-axial system (Figure 1d).

As seen in Figure 2a, when a segment of epithelium-intact guinea-pig trachea was substituted for the endothelium-intact rabbit aortic tube, endothelium-denuded rat aorta again relaxed in response to both histamine and methacholine in the presence of the cyclo-oxygenase inhibitor indomethacin (5 μM). On average, these preparations lost $78 \pm 5\%$ ($n = 36$ preparations from 9 animals) and $75 \pm 3\%$ ($n = 31$ preparations from 10 animals) of their phenylephrine-induced tone in response to histamine (100 μM) and methacholine (25 μM) respectively. When the surrounding tracheal ring segments were removed, the relaxant effect of these agents was abolished. Similarly, if the luminal surface of intact tracheal rings was subsequently rubbed with a cotton wool swab to remove the epithelium and remounted in co-axial assemblies, both histamine and methacholine again failed to cause relaxation of rat aortae (Figure 2b).

Ring samples of guinea-pig and human airway tubular preparations in which the epithelium had not been deliberately denuded, were examined histologically prior to and following their use in co-axial bioassay assemblies. Despite the handling of these airway tubes during assembly and dismantling of co-axial preparations, the epithelium remained virtually intact around the luminal circumference. Histological examination revealed that the endothelium of rabbit aorta preparations was also apparently intact prior to testing in the organ bath. Furthermore, the endothelium was similar in appearance before and after such testing.

It can be seen that both histamine (Figure 3a) and methacholine (Figure 3b) caused concentration-dependent release of EpDRF from guinea-pig trachea as indicated by the concentration-dependent relaxations produced in response to these agonists. The mean pD_2 for histamine was 4.40 ± 0.08 ($n = 13$ preparations) and for methacholine was 5.57 ± 0.08 ($n = 14$ preparations). The histamine H_1 -receptor antagonist mepyramine (2 μM) caused a mean reduction in the relaxant potency of histamine of 13 fold (95% confidence limits: 7–23; $n = 6$ preparations), while the histamine H_2 -receptor antagonist cimetidine (25 μM) was without effect. Atropine (0.1 μM) reduced the relaxant potency of methacholine by 52 fold (95% confidence limits: 27–102; $n = 7$ preparations).

At 300 μM the cyclo-oxygenase/lipoxygenase inhibitor BW 755C has been shown to cause 80% inhibition of endothelium-dependent, acetylcholine-induced relaxation of rabbit aorta pre-contracted with noradrenaline. However, vessel tone was depressed by 40% (Forstermann *et al.*, 1988). In the

present study, BW 755C ($150\text{ }\mu\text{M}$) significantly reduced phenylephrine-induced tone, but histamine and methacholine-induced relaxation was not inhibited. Similarly, the leukotriene receptor antagonist FPL 55712 ($10\text{ }\mu\text{M}$), which has been shown to be active between 2 and $20\text{ }\mu\text{M}$ while not altering drug-induced vascular tone (Jancar *et al.*, 1987), also failed to affect such relaxations. Thus EpDRF was not a prostanoid substance. Relaxant responses to histamine and methacholine were also unaltered in the presence of the guanylate cyclase inhibitor methylene blue ($50\text{ }\mu\text{M}$) (Figure 3c,d), which is known to selectively inhibit EDRF-induced responses (Martin *et al.*, 1985).

Figure 4 shows the rat aorta again relaxed in response to both histamine and methacholine in the presence of indomethacin when surrounded by epithelium-intact, human bronchial ring preparations in co-axial assemblies. Removal of the bronchial epithelium caused abolition of the vascular relaxant response. Thus, an EpDRF was also released from human airway tissue *in vitro*.

Discussion

This study has provided direct evidence for the production and transfer of EpDRF from intact guinea-pig trachea and human bronchi in response to airway spasmogens, in a co-axial bioassay system. The validity of this approach was demonstrated by the successful transfer of EDRF produced in response to methacholine, from intact rabbit aortic tube donor to cause relaxation of endothelium-denuded rat aorta. In contrast, methacholine did not relax isolated endothelium-denuded rat aorta prep-

arations in the absence of rabbit aortic tube tissue. The failure of histamine ($100\text{ }\mu\text{M}$) or methacholine ($25\text{ }\mu\text{M}$) to cause relaxation following removal of the endothelium suggests that these agonists also did not cause non-specific spasmolytic effects in these preparations. Previous studies have also demonstrated specific endothelium-dependent relaxation of rat aorta in response to histamine at concentrations up to 1 mM and to acetylcholine up to $100\text{ }\mu\text{M}$ (Van de Voorde & Leusen, 1983; Rapoport & Murad, 1983).

Isoprenaline caused complete relaxation of isolated endothelium-denuded rat aorta preparations, indicating that removal of the endothelium had not caused significant damage to the underlying smooth muscle. Most importantly, histamine and methacholine caused concentration-dependent relaxation of endothelium-denuded rat aorta when these preparations were placed in co-axial assemblies with intact guinea-pig tracheal or intact human bronchial tubes. Removal of the epithelium from airway tubes caused complete abolition of the relaxant response. Clearly, relaxation observed in co-axial assemblies resulted from the release of a substance derived from the tracheal epithelium i.e. epithelium-derived relaxant factor (EpDRF).

EpDRF-induced relaxation of assay preparations in response to histamine was inhibited by mepyramine ($2\text{ }\mu\text{M}$; H_1 -selective) but not by cimetidine ($25\text{ }\mu\text{M}$; H_2 -selective). Similar concentrations of these agents have previously been shown to cause histamine receptor subtype-selective antagonism in guinea-pig trachea (Duncan *et al.*, 1980). Similarly, the muscarinic cholinergic antagonist atropine ($0.1\text{ }\mu\text{M}$) inhibited the relaxant effect of methacholine. Thus, the generation of EpDRF was probably initiated following the activation of epithelial histamine H_1 -receptors or muscarinic cholinergic receptors, as is apparently the case for EDRF (Furchgott & Zawadzki, 1980).

While the identity of vascular EDRF remains the subject of debate (Palmer *et al.*, 1987; Shikano *et al.*, 1987), some of its properties are established. EDRF behaves like a nitro-dilator, in that relaxation of vascular smooth muscle results from increased levels of intracellular cyclic-GMP. Thus, responses to EDRF were selectively inhibited by the guanylate cyclase inhibitor methylene blue ($50\text{ }\mu\text{M}$) (Martin *et al.*, 1985). In contrast, methylene blue ($50\text{ }\mu\text{M}$) failed to alter relaxation responses of rat aorta produced by histamine-induced release of EpDRF from guinea-pig trachea. Thus, EpDRF was not a guanylate cyclase stimulant, and therefore was neither EDRF nor nitric oxide. In addition, EpDRF does not appear to be a cyclo-oxygenase or lipoxygenase product of arachidonic acid metabolism, since indomethacin, BW 755C and FPL 55712 all failed to alter significantly the relaxation induced by this

Figure 4 Histamine (H; $100\text{ }\mu\text{M}$) and methacholine (M; $25\text{ }\mu\text{M}$) induced relaxation of phenylephrine-contracted (Phe, $0.05\text{ }\mu\text{M}$; $\blacktriangle$), endothelium-denuded rat aorta mounted co-axially within the lumen of segments of epithelium-intact human bronchial tube preparations. Indomethacin ($5\text{ }\mu\text{M}$) was present throughout all the experiments.

autacoid. The failure of Paf to induce relaxation of rat aorta preparations suggests that EpDRF cannot be Paf.

This study has demonstrated that guinea-pig tracheal epithelium produced a non-prostanoid inhibitory factor capable of attenuating smooth muscle responsiveness to spasmogens. The endothelium-denuded rat thoracic aorta strip provides a sensitive assay preparation for this spasmolytic substance. The loss of such an inhibitory mediator in airway preparations deliberately denuded of epithelium most probably explains their increased sensitivity to spasmogens including histamine, methacholine, acetylcholine, 5-hydroxytryptamine and prostaglandin $F_{2\alpha}$ (Flavahan *et al.*, 1985; Barnes *et al.*, 1985;

Goldie *et al.*, 1986a; Aizawa *et al.*, 1988; Raeburn *et al.*, 1988). This relaxant autacoid may help to protect the healthy respiratory tree against inhaled spasmogens as well as mediators released within the lung. Reduced EpDRF activity due to epithelial damage such as is commonly observed in asthma (Hogg & Eggleston, 1984; Laitinen *et al.*, 1985), may partially explain the bronchial hyperreactivity associated with this disease.

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