Relative contributions of direct and indirect mechanisms mediating endothelin-induced contraction of guinea-pig trachea

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1 The present study was undertaken to determine the mechanism of action of endothelin-1 (ET-1)-induced contraction of the guinea-pig isolated trachea.

2 ET-1 (1 nM–0.3 µM) produces a concentration-dependent contraction of guinea-pig trachea with an EC₅₀ of approximately 25 nM. The combination of the peptidoleukotriene receptor antagonist, SK&F 104353 (10 µM) and the H₁-histamine receptor antagonist, mepyramine (10 µM), which abolishes antigen-induced contraction in guinea-pig trachea, was without effect on ET-1 concentration-response curves. Furthermore, the platelet-activating factor (PAF) receptor antagonist, WEB 2086, (1 or 10 µM) did not inhibit ET-induced contraction.

3 ET-1 (0.3 µM) did not stimulate histamine or immunoreactive peptidoleukotriene release from guinea-pig isolated trachea.

4 The release of various prostanoids from guinea-pig trachea was increased significantly by ET-1 (0.3 µM); the profile of release was prostaglandin D₂ (PGD₂) = PGE₂ = 6-keto PGF_{1α} (PGI₂ metabolite) > thromboxane B₂ = PGF_{2α} >> 9α, 11β PGF₂ (PGD₂ metabolite). ET-1-induced release of prostaglandins, which was about 30% of that elicited by antigen in sensitized tissues, was not affected by epithelium removal and was observed in tissues from which the smooth muscle had been removed. Previous studies in our laboratory indicated that indomethacin potentiated contraction produced by high concentrations of ET-1, whereas a thromboxane receptor antagonist was without appreciable effect on ET-1 concentration-response curves.

5 Pretreatment of tissues for 1 h with capsaicin (10 µM), which depletes different sensory neurones, produced a small, but significant, inhibitory effect on ET-1 concentration-response curves in the presence but not the absence of the epithelium. The combination of the NK₁ tachykinin receptor antagonist, CP-96,345 (0.1 µM), and the NK₂ tachykinin receptor antagonist, SR 48968 (0.1 µM), was without effect on ET-1 concentration-response curves but substantially antagonized capsaicin-induced contraction.

6 The present data suggest that in guinea-pig isolated trachea, ET-1 produces contraction predominantly via a direct mechanism: there is no significant contribution of the release of histamine, leukotrienes, PAF, or tachykinins (acting on NK₁ or NK₂ receptors). Although ET-1 evokes the release of an array of prostanoids from the trachea they do not appear to have a major influence on the contractile response.

Keywords: Endothelin-1; guinea-pig trachea; SK&F 104353; mepyramine; WEB 2086; capsaicin; thiorphan; tachykinin receptor antagonists; neutral endopeptidase; epithelium removal; prostanoid release

Introduction

In 1988, Yanagisawa and co-workers isolated, cloned and characterized pharmacologically a novel, potent 21-amino acid vasoconstrictor peptide, named endothelin (ET), that was released from porcine cultured endothelial cells (Yanagisawa *et al.*, 1988). Of the 3 different forms of ET that are now known to exist, ET-1 is the original form isolated from porcine, and subsequently also from human, endothelial cells. In addition to possessing potent vasoconstricting properties, ET-1 produces a plethora of activities in a variety of systems including the respiratory tract (Yanagisawa & Masaki, 1989). Specific binding sites for ET have been detected in airways, particularly the smooth muscle, of various species including man (Power *et al.*, 1989; Turner *et al.*, 1989; Kanse *et al.*, 1989; Hemsén *et al.*, 1990; Henry *et al.*, 1990). Furthermore, ET-1 is a potent contractile agonist of mammalian airways both *in vitro* and *in vivo* (Payne & Whittle, 1988; Uchida *et al.*, 1988; Lagente *et al.*, 1989; Macquin-Mavier *et al.*, 1989; Turner *et al.*, 1989; Maggi *et al.*, 1989; 1990; Hay, 1990; Hemsén *et al.*, 1990; Matsuse *et al.*, 1990).

In guinea-pig isolated trachea it has been proposed that

ET-1 produces contraction predominantly directly via an interaction with a specific receptor, stimulation of phosphatidylinositol turnover and subsequent release of intracellular calcium (Hay, 1989). There is conflicting information on the relative contribution of indirect mechanisms to the contraction produced by ET-1 in guinea-pig trachea. For example, a role for released histamine and peptidoleukotrienes from mast cells has been proposed in preliminary reports (Ninomiya *et al.*, 1989; Nomura *et al.*, 1990), although another study found no evidence for a contribution from these mediators (Hay, 1989). In addition, there are discrepancies regarding the functional role of prostanoids in contractions of isolated airway smooth muscle elicited by ET-1 (Maggi *et al.*, 1989; Filep *et al.*, 1990; Hay, 1990; Henry *et al.*, 1990; Sarriá *et al.*, 1990). To attempt to clarify the role of prostanoids, and also histamine and peptidoleukotrienes in mediating ET-1-induced contraction in guinea-pig trachea, in the present study the ability of ET-1 to release these mediators was measured directly.

The epithelium has been shown to inhibit ET-1-induced contractions in guinea-pig isolated trachea (Hay, 1989) and bronchus (Maggi *et al.*, 1989). The effect was inhibited by phosphoramidon, an inhibitor of neutral endopeptidase (NEP) (Hudgin *et al.*, 1981) and was attributed to the

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epithelium acting as a significant site of metabolism for ET-1 (Hay, 1989). The ETs have been shown to be good substrates for NEP (Vijayaraghavan *et al.*, 1990; Fagny *et al.*, 1991). However, the possibility exists that the modulatory influence of the epithelium may reflect the release of epithelium-derived tachykinins, e.g. substance P, which are substrates for neutral endopeptidase (Skidgel *et al.*, 1984; Hooper & Turner, 1985; Hooper *et al.*, 1985) so that their effects would be potentiated by inhibitors of this enzyme, such as phosphoramidon (Fine *et al.*, 1989; Frossard *et al.*, 1989). To study this possibility the effect of capsaicin, which depletes sensory substance P-containing neurones (Buck & Burks, 1986; Maggi & Meli, 1988) on ET-1-induced contractions in guinea-pig trachea with and without epithelium was examined. In addition the influence of the potent and selective antagonists at NK₁ receptors (CP-96,345; Snider *et al.*, 1991) and NK₂ receptors (SR 48968; Advenier *et al.*, 1991) on contractions produced by ET-1 was investigated.

Accordingly, the purpose of the present study was to investigate further and clarify the mechanism(s) of action (direct versus indirect) of ET-1 in guinea-pig isolated trachea. A preliminary account of the results has been presented by Hay & Undem (1991).

Methods

Contraction studies

Tissue preparation Tracheae were removed from male Hartley guinea-pigs (Hazelton Research Animals, Denver, PA, U.S.A.; 450–750 g body weight). In some experiments the guinea-pigs were actively sensitized to ovalbumin with three intraperitoneal injections (10 mg kg⁻¹) on days 1, 3, and 5. The animals were killed beginning 21 days after the last injection. The tissues were placed in modified Krebs-Henseleit solution which was gassed with 95% O₂:5% CO₂ and maintained at 37°C. The composition of the Krebs solution was (mM): NaCl 113.0, KCl 4.8, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25.0 and glucose 5.5. Following careful removal of adherent fat and connective tissue, the trachea was cut open along its longitudinal axis, directly opposite the smooth muscle and strips consisting of two adjacent cartilage rings were prepared. The tissue preparations were then placed in 10 ml water-jacketed organ baths containing Krebs-Henseleit solution and connected via silk suture to Grass FT03C force-displacement transducers. Mechanical responses were recorded isometrically by multi-channel polygraphs. Tissues were equilibrated under 1.5 g resting load for at least 1 h, and washed every 15 min with fresh Krebs-Henseleit solution before the start of each experiment.

In some experiments, the epithelium was removed mechanically from alternate strips of trachea by gently rubbing the luminal surface with a cotton-tipped applicator. We have demonstrated previously that this procedure effectively removes the epithelium from guinea-pig trachea without producing obvious damage to the underlying mucosal and smooth muscle layers, and does not alter the basic mechanical properties of the tissue (Hay *et al.*, 1986; Fedan *et al.*, 1988).

Concentration-response curves Concentration-response curves for ET-1 were obtained by its cumulative addition to the organ bath in three fold increments according to the technique of Van Rossum (1963). In most experiments examining the effects of drugs, tissues were exposed to these agents for 30 min before addition of contractile agonists. In studies examining the effects of capsaicin, tissues were exposed to this drug for 1 h then washed thoroughly over a period of 45 min before construction of ET-1 concentration-response curves. In experiments examining the effects of the tachykinin receptor antagonists on ET-1- and capsaicin-induced contractions, tissues were incubated with CP-96,345 and SR 48968

for 30 min and 120 min, respectively, before starting the agonist concentration-response curves. All experiments were conducted in the presence of 5 µM indomethacin which was present throughout the study.

After the equilibration period, and before construction of concentration-response curves, tissues were exposed to 10 µM carbachol. Following plateau of this reference contraction, tissues were washed several times over 15–30 min until the tension returned to baseline level. The preparations were then left for at least 30 min before the start of the experiment.

Measurement of mediator release

Guinea-pig tracheas were isolated as outlined above, cut into 12 rings and incubated in 2 ml of Krebs-Henseleit solution, which was gassed with 95% O₂:5% CO₂ and maintained at 37°C. The physiological buffer was replaced at 15 min intervals for 90 min. Following this equilibration period, 2 ml of Krebs-Henseleit containing or lacking ET-1 (1 nM, 10 nM or 0.3 µM), was added for 15 min. After this time the supernatant was taken to assay histamine, prostanoic acid and immunoreactive leukotriene C₄ (i-LT) release. In addition, to determine the total tissue content of histamine, 2 ml of 0.4 N perchloric acid was added to the tissue, which was then placed in a boiling water bath for 15 min; the supernatant fluid was assayed to measure the total histamine content. The studies examining mediator release were conducted in the absence of indomethacin and generally employed epithelium-containing tissues. However, in some experiments the epithelium was removed from 6 rings (as described above) with the remaining 6 rings serving as paired controls. Similarly, the effect of ET-1 was compared to specific antigen challenge in a paired fashion by treating 6 rings with ovalbumin and 6 rings with ET-1. In one set of experiments the trachealis was dissected from the tissues with the aid of a dissecting microscope. The effect of ET-1 on mediator release from the non-trachealis portion of the trachea was then evaluated.

Histamine was assayed by the automated fluorometric technique described by Siraganian (1974). Prostaglandin release into the supernatant fluid was assayed by combined gas chromatography (negative ion chemical ionization) mass spectrophotometry (GC/MS) as described previously (Hubbard *et al.*, 1986). Pepidoleukotriene released from the trachea was assayed by the radioimmunoassay outlined previously (Undem *et al.*, 1987). The limit of sensitivity of this assay is approximately 10–25 pg, as defined by that amount required to inhibit [³H]-LTC₄ binding by 10%. The anti-pepidoleukotriene antibody is highly selective with little affinity (crossreactivity <1%) for a variety of heterologous eicosanoids. The antibody does not, however, distinguish markedly between leukotriene C₄ (LTC₄), LTD₄, and LTE₄. Standard curves with authentic LTC₄, LTD₄ and LTE₄ were parallel; the amounts of LTC₄, LTD₄ and LTE₄ required to inhibit [³H]-LTC₄ binding by 50% were found to be approximately 0.4, 0.5 and 0.6 pmol 0.1 ml⁻¹, respectively.

Analysis of data

Agonist-induced responses for each tissue were expressed as a percentage of the reference contraction. Geometric mean EC₅₀ values were calculated from linear regression analyses of data. Results for control- and treated-tissues were analysed for differences in both the EC₅₀s and also the maximum contractile response produced by ET-1. The prostanoic acids released from the trachea are expressed as pg g⁻¹ wet weight of tissue, whereas the release of i-LT and histamine are given as ng g⁻¹ and µg g⁻¹ or % total histamine content, respectively. All data are given as the mean ± s.e.mean. Statistical analysis was conducted by 2-tailed Student's *t* test for paired or unpaired samples. Where appropriate; a probability value less than 0.05 was regarded as significant.

Drugs

The following drugs were used: endothelin-1 (human, porcine) was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.), Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Calbiochem Corp. (La Jolla, CA, U.S.A.). SK&F 104353

(2(S)-hydroxy-3(R)-(2-carboxyethylthio)-3-[2-(8-phenyloctyl)phenyl]-propanoic acid) was synthesized at SmithKline Beecham Pharmaceuticals (King of Prussia, PA, U.S.A.). Carbachol, capsaicin, indomethacin, tetrodotoxin and mepyramine were obtained from Sigma Chemical Co. WEB 2086 {3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]-triazolo-[4,3-a][1,4]-diazepine-2-yl]-1-(4-morpholinyl)-1-propanone} was a gift from Boehringer Ingelheim (Richfield, CT, U.S.A.) and CP-96,345 {(2S,3S)-cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl)methyl]-1-azabicyclo[2.2.2]octan-3-amine} and SR 48968 {(S)-N-methyl-N[4-acetylamino-4 phenylpiperidino]-2-(3,4-dichlorophenyl) butyl} benzamide} were generously provided by ICI pharmaceuticals (Wilmington, DE, U.S.A.).

Figure 1 Effects of (a) the combination of the leukotriene receptor antagonist, SK&F 104353 (10 μM) and the H_1 -histamine receptor antagonist, mepyramine (10 μM), and (b) the PAF receptor antagonist, WEB 2086 (1 or 10 μM), on endothelin-1 (ET-1) concentration-response curves in epithelium-containing guinea-pig trachea. Results are expressed as a percentage of the response to 10 μM carbachol and are the mean $\pm$ s.e. mean of 7 experiments. (a): (●) control; (○) SK&F 104353 and mepyramine; (b): (●) control; (□) 1 μM WEB 2086; (Δ) 10 μM WEB 2086. Studies were conducted in the presence of 5 μM indomethacin.

Results

Endothelin-1 (ET-1) (1 nM–0.3 μM) produced a concentration-dependent contraction of epithelium-containing (intact) guinea-pig isolated trachea with an EC_{50} of 23.3 nM. The maximum contractile response elicited by ET-1 (0.3 μM) was $78.5 \pm 5.4\%$ ($n = 15$) of that induced by 10 μM carbachol.

Effects of receptor antagonists and tetrodotoxin (TTX)

The combination of the leukotriene receptor antagonist, SK&F 104353 (10 μM), and the H_1 -histamine receptor antagonist, mepyramine (10 μM), was without effect on ET-1 concentration-response curves (Figure 1a). In addition, the PAF antagonist, WEB 2086 (1 or 10 μM), did not significantly affect contractions induced by ET-1 (Figure 1b). In confirmation of previous findings in guinea-pig epithelium-intact trachea (Maggi *et al.*, 1989), TTX (1 μM) did not inhibit ET-1-induced contractions (data not shown).

Release of mediators

The release of histamine from the guinea-pig trachea was measured, as outlined in Methods, under basal conditions and following addition of 0.3 μM ET-1 for 15 min. This concentration of ET-1, which produces the maximum or close to the maximum contractile response, did not stimulate the release of histamine above basal levels. Thus, the spontaneous release and that elicited in the presence of ET-1 were both < 2 ng, which represents $< 0.5\%$ of the total histamine content of $5.1 \pm 0.3 \mu\text{g g}^{-1}$ tissue ($n = 11$, Table 1). Furthermore, in trachea obtained from guinea-pigs actively sensitized with ovalbumin, ET-1 (0.3 μM) did not stimulate histamine release (Table 1). In contrast, ovalbumin (0.01 mg ml $^{-1}$)

Table 1 Endothelin-1 (ET-1)-induced mediator release from the guinea-pig trachea*

Mediator	Spontaneous release (pg g $^{-1}$)	ET-1-induced release (net) (pg g $^{-1}$)	n
Histamine	Not detectable ^b ($< 0.5\%$ total)	Not detectable ($< 0.5\%$ total)	11
i-LT	Not detectable ^b	Not detectable	4
PGD ₂	496 ± 210	$4120 \pm 573^*$	13
9 α , 11 β -PGF ₂	7 ± 6	$36 \pm 12^*$	13
PGE ₂	565 ± 100	$3276 \pm 381^*$	13
PGF _{2\alpha}	166 ± 35	$879 \pm 113^*$	13
TxB ₂	241 ± 74	$1036 \pm 168^*$	13
6-keto PGF _{1\alpha}	397 ± 69	$2632 \pm 318^*$	13

*Tracheae were isolated from naive ($n = 6$) or activity sensitized ($n = 7$) guinea-pigs (see Methods). The basal release of mediators and that evoked by the addition of ET-1 (0.3 μM) was quantified. There was no appreciable differences in the release of mediators from naive and actively sensitized trachea and, therefore, the values were pooled. These data are derived from the results illustrated in Figures 2a and 2b.

^bThe amount of histamine and immunoreactive leukotriene C₄ (i-LT) released from the trachea was below our limits of detection. The histamine assay is sensitive to about 1 ng per sample (5–10 ng g $^{-1}$ in our experiments); the limit of detection for i-LT was about 5 ng g $^{-1}$.

*Statistically significantly different from spontaneous release; $P < 0.05$.

Figure 2 (a) Effects of endothelin-1 (ET-1, 10 nM or 0.3 μM) on prostanoid release from epithelium-containing guinea-pig trachea, and (b) comparison of the effects of ET-1 (0.3 μM) or ovalbumin (0.01 mg ml⁻¹) on prostanoid release from paired, epithelium-containing trachea removed from guinea-pigs actively sensitized with ovalbumin. Data are given as pg g⁻¹ tissue wet weight and are the mean ± s.e.mean. (a) Solid columns: 10 nM ET-1, *n* = 3; hatched columns: 0.3 μM ET-1: *n* = 6. (b) Solid columns: ovalbumin, *n* = 7; hatched columns ET-1, *n* = 7. Prostanoid levels were measured by gas chromatography-mass spectrophotometry. Note the different scales for the y-axes of (a) and (b).

induced the release of $12.9 \pm 3.2\%$ (*n* = 7) of the total histamine stores. ET-1 (0.3 μM) also failed to elicit i-LT release from the trachea. However, it should be noted that we could not detect i-LT release much below 5 ng g⁻¹ of trachea. Nevertheless, ovalbumin (0.01 mg ml⁻¹) evoked the release of detectable amounts of i-LT, averaging 12.6 ± 2.6 ng g⁻¹ (*n* = 6).

In epithelium-intact guinea-pig trachea, ET-1 (0.3 μM) stimulated the release of a variety of prostanoids, measured using GC/MS techniques (Figure 2a, Table 1). The profile of release was: PGD₂ = PGE₂ = 6 keto PGF_{1α} (PGI₂ metabolite) > PGF_{2α} = TxB₂ >> 9α, 11β PGF₂ (PGD₂ metabolite). The threshold concentration of ET-1 to elicit prostanoid release appeared to be about 10 nM, as 1 nM ET-1 was without effect in two preparations (data not shown), whereas 10 nM elicited a small but detectable release of most of the prostanoids; the magnitude of the release was less than 10% of that produced by 0.3 μM ET-1 (Figure 2a).

As indicated in Figure 2b a comparison was made of the ability of ovalbumin (0.01 mg ml⁻¹, a maximally effective concentration) and ET-1 (0.3 μM) to stimulate prostanoid release from trachea taken from guinea-pigs that were actively sensitized with ovalbumin. Two main observations were apparent from this study. First, the total prostanoid

Figure 3 (a) Effect of epithelium removal on prostanoid released from paired guinea-pig tracheae induced by 0.3 μM endothelin-1 (ET-1) and (b) prostanoid release elicited by 0.3 μM ET-1 from epithelium-containing guinea-pig trachea with or without the smooth muscle (i.e. predominantly cartilage). Data are given as pg g⁻¹ tissue wet weight and are the mean ± s.e.mean. (a) Solid columns: + epithelium, *n* = 4; hatched columns - epithelium, *n* = 4. (b) Solid columns: preparations without smooth muscle, *n* = 4; hatched columns: preparations containing smooth muscle, *n* = 4-6. Prostanoid levels were measured by gas chromatography-mass spectrophotometry.

release induced by ovalbumin (37,588 pg g⁻¹ tissue net weight) was about three fold higher than that elicited by ET-1 (11,635 pg g⁻¹ tissue net weight). Secondly, the profile of prostanoid release produced by ovalbumin (PGD₂ >> PGE₂ = TxB₂ > 6 keto PGF_{1α} > PGF_{2α} > 9α, 11β PGF₂) was different from that observed with ET-1 (see above).

The influence of the epithelium on ET-1-induced prostanoid release was analysed. In the four tissues studied, epithelium removal did not inhibit the prostanoid release induced by ET-1 (0.3 μM) (Figure 3a); furthermore, the relative profile of release of the individual prostanoids was not significantly altered. The total amount of prostanoid release in epithelium-denuded tissues (22,952 pg g⁻¹ tissue net weight) was more than two fold higher than that observed in epithelium-containing preparations (10,302 pg g⁻¹ tissue net weight); due to the intraexperimental variability these differences were not statistically significant, *P* > 0.3.

The influence of the smooth muscle (trachealis) on ET-1-induced prostanoid release was studied. In preparations from which the smooth muscle had been carefully removed, ET-1 (0.3 μM) elicited prostanoid release which was very similar, both in terms of the absolute and relative amounts of the individual prostanoids, to that observed in preparations containing smooth muscle (Figure 3b).

Figure 4 Effect of capsaisin ($10\ \mu\text{M}$; 1 h exposure) on endothelin-1 (ET-1) concentration-response curves in guinea-pig trachea in the presence (a) and the absence (b) of the epithelium. Results are expressed as a percentage of response to $10\ \mu\text{M}$ carbachol and are the mean $\pm$ s.e. mean of 15 experiments; (●) control; (○) plus capsaisin. Studies were conducted in the presence of $5\ \mu\text{M}$ indomethacin. * $P < 0.05$.

Effects of the epithelium, capsaisin and tachykinin receptor antagonists on ET-1-induced contractions

We tested the hypothesis that ET-1 releases tachykinins from capsaisin-sensitive nerve fibres; these experiments were conducted in the presence of $5\ \mu\text{M}$ indomethacin. Addition of capsaisin ($10\ \mu\text{M}$) resulted in a significant elevation in tone which returned to baseline after approximately 45–60 min. Pretreatment for 1 h with capsaisin ($10\ \mu\text{M}$), followed by washout over 30–45 min, abolishes the response to subsequent exposure to capsaisin, as well as the response to stimulation of tachykinin-containing nerves in the trachea (Ellis & Undem, 1990). Treatment of the tissues with capsaisin in this fashion had a slight, but significant inhibitory effect on ET-1 concentration-response curves in epithelium-containing tissues with an approximately 20% reduction in the maximum response to $0.3\ \mu\text{M}$ ET-1. In contrast, capsaisin had no effect on the ET-1-induced contractions in epithelium-denuded preparations (Figure 4). Capsaisin ($10\ \mu\text{M}$, 1 h pretreatment) had no effect on carbachol concentration-response curves in either the presence or absence of the epithelium (data not shown).

It was observed that the combination of the NK_1 tachykinin receptor antagonist, CP 96,345 ($0.1\ \mu\text{M}$) and the NK_2 tachykinin receptor antagonist, SR48986 ($0.1\ \mu\text{M}$) was without effect on ET-1 concentration-response curves in epithelium-containing guinea-pig trachea (Figure 5a). In con-

Figure 5 Effect of the combination of the NK_1 tachykinin receptor antagonist CP 96,345 ($0.1\ \mu\text{M}$) and the NK_2 tachykinin receptor antagonist SR 48968 ($0.1\ \mu\text{M}$) on (a) endothelin-1 (ET-1) and (b) capsaisin concentration-response curves in epithelium-containing guinea-pig trachea. Results are expressed as a percentage of the response to $10\ \mu\text{M}$ carbachol and are the mean $\pm$ s.e. mean of 6 experiments; (●) = control; (○) plus CP 96,345 and SR 48968. Studies were conducted in the presence of $5\ \mu\text{M}$ indomethacin.

trast, the combination of these antagonists virtually abolished the contractile response to capsaisin (Figure 5b).

Discussion

In a previous paper it was concluded that in guinea-pig trachea, ET-1 produces contraction predominantly via a direct mechanism (Hay, 1990). Thus, atropine, mepyramine, SK&F 104353 (the peptidoleukotriene receptor antagonist) and SQ 29,548 (the thromboxane receptor antagonist) were without significant effect on ET-1-induced contraction (Hay, 1990). The data from the present study provide further evidence in support of the above postulate in that the combination of mepyramine ($10\ \mu\text{M}$) and SK&F 104353 ($10\ \mu\text{M}$) did not inhibit the contraction induced by ET-1. This experiment was performed as it had been noted previously that, either agent alone produced only partial inhibition of the antigen-induced, mast-cell dependent contraction in guinea-pig isolated trachea, whereas the combination of the two antagonists actually abolished the response (Hay *et al.*, 1987). The lack of effect of the combination of mepyramine and SK&F 104353 against contraction to ET-1 suggests that the release of histamine and peptidoleukotrienes from mast cells are not involved in this response. This is supported by the lack of ability of ET-1 to stimulate the release of histamine or peptidoleukotrienes from trachea. The levels of histamine

or i-LTC₄ released basally or in the presence of a concentration of ET-1 which produces the maximum or close to the maximum contraction, 0.3 μ M, contrasted with the substantial release from trachea isolated from actively sensitized guinea-pigs induced by a maximally effective concentration of antigen. These data, considered along with our previous findings that the thromboxane receptor antagonist, SQ 29,548 had no effect, and indomethacin slightly enhanced ET-induced contractions of the isolated trachea (Hay, 1990) support the hypothesis that ET contracts the trachea primarily via a direct effect on the trachealis. Additional support of this can be found from experiments in which intra-arterial injection of ET in guinea-pig perfused lung did not stimulate the release of histamine into the lung effluent (Touvy *et al.*, 1990). Furthermore, mepyramine did not influence ET-induced bronchoconstriction in guinea-pigs *in vivo* (Lagente *et al.*, 1989; Nambu *et al.*, 1990). In addition, in the present study, WEB 2086, the potent, selective PAF receptor antagonist (Casals-Stenzel, 1987a,b) was without effect on the response produced by ET-1.

In contrast to the above data, there are some reports that ET-1 produces contraction of guinea-pig trachea, in part, via an indirect mechanism involving the release of secondary mediators. For example, a PAF antagonist, BN 52021, attenuated ET-1-induced contraction of guinea-pig trachea and also bronchi (Battistini *et al.*, 1990); although no data were shown, these authors indicated that similar results were obtained with WEB 2086, the PAF antagonist used in the present study. Accordingly, it was proposed that PAF may mediate, in part, ET-induced contraction in guinea-pig airways. It is difficult to envisage a prominent role for PAF in mediating ET-1-induced contraction of guinea-pig isolated trachea in view of the unresponsiveness of this preparation to the contractile effects of PAF that is generally observed (Vargaftig *et al.*, 1980; Prancan *et al.*, 1982; Cerrina *et al.*, 1983). Furthermore, autoradiography or binding studies revealed few if any specific binding sites for PAF in guinea-pig trachea (Hwang *et al.*, 1983; Goldie *et al.*, 1990). Filep and co-workers concluded, following studies examining the effects of various receptor antagonists, that ET-1-induced contraction and thromboxane release in guinea-pig trachea were, in part, mediated by the release of peptidoleukotrienes and PAF (Filep *et al.*, 1991b). It has been reported that a histamine receptor antagonist alone inhibited ET-induced contraction in this tissue (Ninomiya *et al.*, 1989; Nomura *et al.*, 1990). Furthermore, based on the effects of thromboxane receptor antagonists, there is some discrepancy as to the role of thromboxane in the response produced by ET-1 in guinea-pig isolated trachea (Hay, 1990; Filep *et al.*, 1990). A recent study indicated that ET-1 stimulated the release of histamine from guinea-pig pulmonary, but not peritoneal mast cells (Uchida *et al.*, 1992). The reason(s) for the differences between some of the findings from the above studies and the data of the present study is unknown.

ET was an efficacious stimulator of prostanoid production in the trachea. Interestingly, PGD₂, along with PGE₂, was the major prostanoid produced. PGD₂ is synthesized by mast cells (Lewis *et al.*, 1982), and is released from the guinea-pig trachea by antigenic stimulation (Undem *et al.*, 1988). If ET-1 is stimulating the mast cell to synthesize PGD₂ it would appear to be doing so in the absence of degranulation and histamine release. Alternatively, cells other than mast cells may be synthesizing the PGD₂ in the guinea-pig trachea. The cell types stimulated by ET-1 to synthesize the prostanoids cannot be discerned from our results. Nevertheless, the findings with epithelium-denuded trachea, and trachealis-free trachea indicate that neither the cells associated with the epithelium or the smooth muscle are obligatory for prostanoid production. ET-1 potently stimulates 6-keto PGF_{1 α} from cultured bovine aortic endothelial cells with an EC₅₀ of 3 nM (Filep *et al.*, 1991a). In contrast, 10 nM ET-1 was reported to be without effect on the release of PGF_{2 α} , and also histamine, from guinea-pig trachealis smooth muscle

(Nagai *et al.*, 1991), although Filep and co-workers observed that ET-1 stimulated thromboxane release from guinea-pig trachea (Filep *et al.*, 1990; 1991b). Furthermore, ET-1 (0.1 μ M–10 μ M) stimulated the release of the cyclo-oxygenase products PGD₂, PGE₂ and TxB₂ and also 15-HETE, but not 6-keto PGF_{1 α} or the peptidoleukotrienes, from human cultured nasal mucosa (Wu *et al.*, 1992). It is noteworthy that the greatest effect was on the release of PGD₂ and it was also observed that the ET-1-induced release of eicosanoids was not affected by a PAF receptor antagonist (Wu *et al.*, 1992).

Notwithstanding the relatively minor effects of the cyclo-oxygenase inhibitor, indomethacin, on ET-induced contractions of the isolated trachea (Maggi *et al.*, 1989; Hay, 1990; Henry *et al.*, 1990; Sarriá *et al.*, 1991b), cyclo-oxygenase products may contribute to the effects of ET on the airways *in vivo*. Indeed, from the limited *in vivo* studies conducted to date, there seems to be a consensus that ET-1 produces bronchoconstriction in guinea-pigs, in part, indirectly by the release of secondary mediators, of which the predominant one appears to be thromboxane. For example, the bronchoconstriction elicited by intravenous administration of ET was essentially abolished by pretreatment with a cyclo-oxygenase inhibitor (Payne & Whittle, 1988; Macquin-Mavier *et al.*, 1989) or a thromboxane A₂ synthase inhibitor (Nambu *et al.*, 1990). In addition, indomethacin, or the PAF antagonist, BN 52021, produced approximately 50–60% inhibition of aerosolized ET-induced bronchoconstriction in guinea-pigs (Lagente *et al.*, 1989). Furthermore, it is possible that released prostanoids contribute significantly to effects of the ETs in airways other than bronchoconstriction.

Previous studies from our laboratory indicated that epithelium removal potentiates the response to ET-1 in guinea-pig trachea (Hay, 1989). Phosphoramidon, the NEP inhibitor (Hudgin *et al.*, 1981), inhibited the potentiating effect of epithelium removal on endothelin-induced contraction, suggesting that the phenomenon was due to the removal of an epithelium-derived phosphoramidon-sensitive peptidase, presumably NEP, that normally metabolizes endothelin (Hay, 1989). The members of the ET family have been shown to be good substrates for NEP (Vijayaraghavan *et al.*, 1990; Fagny *et al.*, 1991). Other researchers have recently provided further data in support of a modulatory role for epithelium-derived NEP on ET-1-induced contraction in guinea-pig isolated trachea (Noguchi *et al.*, 1991; Di Maria *et al.*, 1992; Yamaguchi *et al.*, 1992). For example, recombinant human NEP decreased ET-1-induced contraction (Di Maria *et al.*, 1992). Alternatively, however, the effects could be, in part, due to ET-1 releasing from the epithelium a peptide, e.g. a tachykinin such as substance P, that is metabolized by NEP. Substance P-containing nerves have been localized in the airway epithelium of several species including guinea-pigs (Lundberg *et al.*, 1984). To examine this other postulate the effects of capsaicin, which depletes afferent sensory neurones (Buck & Burks, 1986; Maggi & Meli, 1988), on ET-1-induced responses was examined. Capsaicin pretreatment produced a statistically significant inhibition of ET-1-induced contraction in guinea-pig trachea with an intact epithelium but not in preparations in which the epithelium had been removed. This suggests that in intact preparations a component of the response to ET-1 is due to the release of a substance from capsaicin-sensitive nerves. However, the contribution is a relatively minor one as capsaicin produced only approximately a 20% reduction in the maximum contraction produced by ET-1. The lack of effect of the combination of the potent and selective NK₁ and NK₂ receptor antagonists, CP-96,345 (Snider *et al.*, 1991) and SR 48968 (Advenier *et al.*, 1991), respectively, on ET-1-induced contraction in epithelium-containing guinea-pig trachea, at concentrations which essentially abolished contraction elicited by capsaicin, suggests that the inhibitory effect of capsaicin in these tissues is not due to the depletion of a substance (e.g. substance P or neurokinin A) which activates NK₁ or NK₂ tachykinin receptors. It is more likely

that the effect of capsaicin involved the release of another substance acting through another receptor system. Alternatively, the effect of capsaicin may not be related to its recognized ability to deplete afferent sensory neurones (Buck & Burks, 1986; Maggi & Meli, 1988); it is not due to a non-specific action on smooth muscle responsiveness as the inhibitory effect on responses to ET-1 was observed only in epithelium-containing tissues and capsaicin was without effect on carbachol-induced contraction in either the presence or absence of the epithelium. It is of interest that Yamaguchi and co-workers reported recently that phosphoramidon potentiated the response to ET-1 in guinea-pig trachea even after treatment of tissues with capsaicin (Yamaguchi *et al.*, 1992).

In summary, the present results provide further evidence that in guinea-pig isolated trachea, ET-1 produces contraction predominantly via a direct mechanism; there is no significant contribution of released histamine, leukotrienes, or

PAF. There is circumstantial evidence of a contribution, albeit minor, of an epithelium-derived substance(s) that is released from capsaicin-sensitive sensory neurones. However, this does not appear to involve tachykinins acting on NK₁ or NK₂ receptors. ET-1 stimulates the release of various prostanoids, most notably PGD₂, PGE₂ and prostacyclin, but only at relatively high concentrations (≥ 10 nM). Although these prostanoids do not appear to play a major role in ET-induced contraction of the isolated trachea, they may contribute to the overall pulmonary response to ET-1 exposure in the guinea-pig.

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