Mechanism of endothelin-induced contraction in guinea-pig trachea: comparison with rat aorta

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1 Endothelin (1 nM–0.3 μ M) produced a concentration-dependent contraction of guinea-pig epithelium-containing (intact) trachea (EC_{50} = 30.9 nM). Endothelin was a less potent agonist than leukotriene D₄ (LTD₄; EC_{50} = 0.77 nM), but was more potent than carbachol (EC_{50} = 0.15 μ M) or substance P (EC_{50} = 1.4 μ M). Endothelin was a more potent contractile agent in rat endothelium-denuded aorta (EC_{50} = 2.1 nM) than in guinea-pig trachea.

2 Endothelin-induced contraction in guinea-pig trachea was unaffected by mepyramine (10 μ M), atropine (1 μ M), SK&F 104353 (10 μ M), a leukotriene receptor antagonist, or SQ 29,548 (1 μ M), a thromboxane receptor antagonist. The contraction produced by 0.3 μ M endothelin was potentiated by cyclo-oxygenase inhibition with 5 μ M indomethacin.

3 Nicardipine (0.01 or 0.1 μ M) or incubation in calcium-free medium + 0.1 mM EGTA for 30 min had a relatively minor or no effect on endothelin concentration-response curves in guinea-pig intact trachea, but markedly inhibited responses produced by endothelin in endothelium-denuded aorta of the rat. Increasing the EGTA concentration in calcium-free medium to 1 mM abolished endothelin-induced contraction in guinea-pig trachea.

4 In guinea-pig trachea, ryanodine (10 μ M) produced a 2.1 fold shift to the right of endothelin concentration-response curves and reduced the maximum response elicited by 0.3 μ M endothelin.

5 Staurosporine (0.01 μ M and 0.1 μ M), a protein kinase C inhibitor, was without effect on endothelin- or carbachol-induced contraction in guinea-pig trachea, but markedly inhibited the response produced by endothelin in rat aorta.

6 Endothelin (3 nM–0.3 μ M) produced a concentration-dependent stimulation of phosphatidylinositol (PI) turnover in guinea-pig intact trachea, with an EC_{50} value of 45.9 nM.

7 Removal of the epithelium markedly potentiated endothelin-induced contraction in guinea-pig trachea, producing a 4.7 fold leftward shift in endothelin concentration-response curves and an increase in the contractile response elicited by 0.3 μ M endothelin.

8 These data indicate that endothelin is a potent agonist in guinea-pig trachea whose response is markedly enhanced by removal of the airway epithelium. Endothelin-induced contraction is not mediated to a marked extent by calcium influx via dihydropyridine-sensitive calcium channels and does not involve the release of histamine, acetylcholine, leukotrienes or thromboxane. Rather, endothelin appears to produce contraction of guinea-pig trachea via a direct action which involves stimulation of PI turnover and utilization of calcium from intracellular stores and, also, calcium influx via a pathway that is not sensitive to dihydropyridine calcium channel inhibitors. Endothelin-induced contraction of rat aorta was more sensitive to the effects of incubation in Ca²⁺-free medium, nicardipine or staurosporine, suggesting that differences exist in the relative mechanisms whereby endothelin produces contraction in different tissues.

Introduction

Endothelin is a recently discovered potent vasoconstrictor peptide isolated from porcine cultured endothelial cells (Yanagisawa *et al.*, 1988). This 21-amino acid peptide bears a close structural homology with a group of snake venom toxins, the sarafotoxins (Lee & Chiappinelli, 1988), and there is evidence that they may possess similar mechanisms of action (Kloog *et al.*, 1988; Van Renterghem *et al.*, 1988; Resink *et al.*, 1988; Ohlstein *et al.*, 1989). In a preliminary communication endothelin was shown to be an extremely potent bronchoconstrictor agent in guinea-pig trachea, possessing substantially greater potency than leukotriene D₄ (LTD₄) or neurokinin A (Uchida *et al.*, 1988).

Based on results of experiments in which the effects of calcium-free medium and a dihydropyridine calcium channel antagonist, nicardipine, were examined, it was hypothesized that endothelin produced contraction of vascular and airway smooth muscle by interacting with and activating voltage-dependent calcium channels (Yanagisawa *et al.*, 1988; Uchida *et al.*, 1988). Although this postulate is supported by a preliminary study in human vascular tissue (Hughes *et al.*, 1988), several recent publications provide strong evidence that endo-

thelin acts by a mechanism other than an interaction with dihydropyridine-sensitive calcium channels and stimulation of extracellular calcium influx (Van Renterghem *et al.*, 1988; Resink *et al.*, 1988; Hirata *et al.*, 1988; Miasiro *et al.*, 1988; Auguet *et al.*, 1988). These studies suggest that endothelin produces contraction of smooth muscle by interaction with a specific receptor and subsequent release of intracellular calcium as a consequence of stimulation of phosphatidylinositol (PI) turnover.

In the present study the mechanism(s) of the contractile effect of endothelin in guinea-pig trachea was investigated and a comparison was made with endothelin-induced contraction in rat aorta. A preliminary account of the results has been presented previously (Hay, 1989b).

Methods

Tissue preparation

Tracheae were removed from male Hartley guinea-pigs (Hazelton Research Animals, Denver, PA, U.S.A.; 450–650 g body weight) and placed in modified Krebs-Henseleit solution.

The composition of the Krebs-Henseleit solution, which was gassed with 95% O₂: 5% CO₂ and maintained at 37°C, was (mM): NaCl 113.0, KCl 4.8, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25.0 and glucose 5.5. The calcium-free physiological buffer used in some studies was of the same composition as Krebs-Henseleit solution except that CaCl₂ was omitted and 0.1 mM or 1 mM EGTA added. 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. Thoracic aortae were removed from male Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC, U.S.A.; 250–350 g body weight) and cleaned of connective tissue and adherent fat. The isolated arteries were placed round 21 g syringe needles and cut into strips of approximately 15 mm length and 2 mm width; endothelium was removed from aorta by rotation of the tissue several times round the syringe needle. The tissue preparations were then placed in 10 ml water-jacketed organ baths containing Krebs-Henseleit solution and connected via silk suture to Grass FTO3C force-displacement transducers. Mechanical responses were recorded isometrically by multi-channel polygraphs. Tissues were equilibrated, under 1.5 g and 1 g resting load for trachea and aorta, respectively, 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 (permitting paired analysis) by gently rubbing the luminal surface with a cotton-tipped applicator. It has been 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).

Concentration-response curves

Concentration-response curves for agonists were generally obtained by their cumulative addition to the organ bath in either 3 or 10 fold increments according to the technique of Van Rossum (1963). Each drug concentration was left in contact with the preparation until the response reached a plateau before addition of the subsequent agonist concentration. In experiments examining the effects of antagonists, tissues were exposed to these agents for 30 min before addition of contractile agonists. In some experiments the influence of extracellular calcium on agonist-induced contractions was examined by incubation of tissues with calcium-free Krebs-Henseleit solution + EGTA (0.1 or 1 mM) for 30 min before addition of agonist; during the incubation period in calcium-free Krebs-Henseleit solution tissues were washed several times with physiological buffer. Studies in which the influence of ryanodine on agonist-induced contractions was examined were conducted in calcium-free Krebs-Henseleit solution + 0.1 mM EGTA. Tissues were exposed to calcium-free Krebs-Henseleit solution + 0.1 mM EGTA containing ryanodine for 30 min before construction of agonist concentration-response curves; tissues were washed several times during the incubation period. Most experiments were conducted in the presence of 5 µM indomethacin, which was present throughout the study. LTD₄ concentration-response curves were performed in the presence of 3 mM L-cysteine, which inhibits the conversion of LTD₄ to LTE₄ (Snyder *et al.*, 1984). Studies in which the effects of KCl in guinea-pig trachea or rat aorta were examined were generally conducted in the presence of atropine (1 µM) or phentolamine (1 µM), respectively.

After the equilibration period, and before construction of concentration-response curves, tissues were exposed to either 10 µM carbachol for guinea-pig trachea or 60 mM KCl for rat aorta. 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.

Phosphatidylinositol turnover studies

PI turnover in guinea-pig trachea was measured by a modification of the method outlined by Berridge *et al.* (1983). Tracheae, from which the cartilage was removed, were incubated for 4 h with 10 µCi ml⁻¹ [³H]-myo-inositol (specific activity = 83.2 Ci mmol⁻¹) in 3 ml Krebs-Henseleit solution maintained at 37°C and gassed with 95% O₂: 5% CO₂. Following the loading period tissues were washed three times with 3 ml Krebs-Henseleit solution containing 10 mM Li⁺ and 1 mM myo-inositol; buffer was removed by aspiration. Tissues were placed in individual test-tubes containing 1 ml of Krebs-Henseleit solution + 10 mM Li⁺, which prevents the metabolism of inositol monophosphate (IP₁) to inositol (Allison & Blisner, 1976) and increases the accumulation and recovery of inositol phosphates (Berridge *et al.*, 1982). Following incubation with the appropriate concentration of endothelin for 60 min the reaction was stopped by addition of 3 ml of a mixture of chloroform: methanol: hydrochloric acid in a ratio of 2:1:0.1 (Best *et al.*, 1985). The test-tubes were vortexed vigorously and then centrifuged for 10 min at 150 g to separate the aqueous and chloroform phases. The upper aqueous phase was removed, diluted with 4 ml of distilled H₂O and then added to columns containing 1 ml of Dowex AG 1-X8 (100–200 mesh, formate form) anion exchange resin to permit separation and extraction of the inositol phosphates. The inositol phosphates were eluted by the following procedure: 5 ml of distilled H₂O was added to columns three times to elute free [³H]-myo-inositol, followed by three washes with 5 ml of 60 mM sodium formate: 5 mM sodium tetraborate which elutes glycerophosphoinositol. Total inositol phosphates were then eluted by three 5 ml additions of 1 M ammonium formate: 0.1 M formic acid. Radioactivity, measured by a TM Analytic counter (Elk Grove Village, IL) at an efficiency of 30%, was determined in a 5 ml aliquot of the combined eluate samples to which 2 ml distilled H₂O and 10 ml of Aquasol liquid scintillation cocktail (New England Nuclear, Boston, MA) had been added. Individual tissues were dried at room temperature before being weighed and data were expressed as d.p.m. per mg tissue weight. In addition, in some experiments radioactivity present in the chloroform phase was measured to determine [³H]-myo-inositol incorporation into phospholipids; 200 µl of the lower phase was dried overnight at room temperature and 10 ml of Aquasol was added before radioactivity was counted.

Analysis of data

Agonist-induced responses for each tissue were expressed as a percentage of the reference contraction or as absolute developed tension (in g). Geometric mean EC₅₀ values were calculated from linear regression analyses of probit-transformed data. All data are given as mean ± s.e.mean. Statistical analysis was conducted by ANOVA or 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 (human, porcine) was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.) or Calbiochem Corp. (La Jolla, CA, U.S.A.). Staurosporine was obtained from Eli Lilly Co. (Indianapolis, IN, 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.). Ryanodine and H-7 (1-(5-isquinolinesulphonyl)-2-methylpiperazine) were obtained from Calbiochem Corp. (La Jolla, CA, U.S.A.). Carbachol, atropine, EGTA, mepyramine and nifedipine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). SQ 29,548 ([1S-[1α,2β(5z),3β,4α]] - 7 - [3 - [[2 - [(phenylamino)carbonyl] -

hydrazino]-methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid) was a gift from Squibb Institute of Medical Research (Princeton, NJ, U.S.A.).

Results

Characteristics of endothelin-induced contraction

In intact isolated trachea of the guinea-pig endothelin produced a concentration-dependent contractile response. The threshold concentration of endothelin for eliciting contraction was 1 nM–10 nM. Endothelin did not appear to be a very efficacious agonist, with 0.3 μM endothelin eliciting a response of only 0.52 ± 0.11 g (n = 10), which represents only 57.3 ± 8.2% of the reference contraction to 10 μM carbachol. However, due to lack of availability, concentrations of endothelin greater than 0.3 μM could not be examined in this study. Therefore, although the data would suggest that contraction elicited by 0.3 μM endothelin is close to the maximum response produced by endothelin, a true comparison between the maximum contractile responses produced by carbachol and endothelin could not be ascertained. Indomethacin (5 μM) markedly potentiated contraction produced by higher concentrations of endothelin (0.03–0.3 μM), e.g., response to 0.3 μM endothelin was 1.24 ± 0.09 g (n = 32; P > 0.0005 compared to response in the absence of indomethacin), which corresponds to 77.7 ± 3.7% of the response to 10 μM carbachol. In view of the marked enhancement of endothelin-induced contraction by indomethacin, unless stated otherwise, the subsequent experiments with contractile agonists were performed in the presence of 5 μM indomethacin, to inhibit the release of prostanoids.

Contraction produced by endothelin in guinea-pig intact trachea was slow to develop with a time to 50% of peak response of 3.2 ± 0.2 min (n = 4) with 0.1 μM endothelin; contraction was initiated approximately 15 s after addition of endothelin. In addition, the response to endothelin was slow to washout: time to 50% relaxation was 28.1 ± 9.8 min for 0.1 μM endothelin; (n = 4). There was no difference in the concentration-response curves to endothelin (1 nM–0.1 μM) in normal and siliconized organ baths (data not shown).

In the presence of indomethacin, endothelin (1 nM–0.3 μM) produced a concentration-dependent contractile response with an EC₅₀ value = 30.9 nM (n = 32) (Figure 1; Table 1). A comparison was made between the concentration-response curves produced by endothelin, LTD₄, substance P and carbachol in intact guinea-pig trachea (Figure 1 and Table 1); quantities of endothelin and substance P sufficient to produce a maximum response were not available. LTD₄ was the most potent of the contractile agonists, followed by endothelin, carbachol and finally, substance P.

Atropine (1 μM), mepyramine (1 or 10 μM), SK&F 104353 (1 or 10 μM), a leukotriene receptor antagonist (Hay *et al.*, 1987) or SQ 29,548 (0.1 or 1 μM), a thromboxane receptor antagonist

Figure 1 Concentration-response curves to endothelin (●), carbachol (□), leukotriene D₄ (LTD₄) (○) and substance P (Δ) in guinea-pig intact trachea. Results are expressed as a percentage of the reference contraction to 10 μM carbachol and are the mean with vertical lines indicating s.e.mean; endothelin, n = 32; carbachol, n = 20; LTD₄, n = 4; substance P, n = 4. Experiments were conducted in the presence of 5 μM indomethacin.

(Ogletree *et al.*, 1985) were without effect on endothelin-induced contraction (Figure 2).

Removal of the epithelium increased the responsiveness of guinea-pig trachea to the contractile effects of endothelin, as reflected by a 4.7 fold decrease in the EC₅₀ and an increase in the contractile response produced by 0.3 μM endothelin (Figure 3); EC₅₀ (–log M): +epithelium = 7.50 ± 0.07 (n = 23); –epithelium = 7.84 ± 0.06 (n = 23, P < 0.0005 compared to +epithelium); contractile response with 0.3 μM endothelin (% of that to 10 μM carbachol): +epithelium = 87.0 ± 5.4 (n = 23); –epithelium = 108.5 ± 6.5 (n = 23; P < 0.005 compared to +epithelium).

Endothelin was a more potent agonist in endothelium-denuded aorta of the rat than in guinea-pig trachea, with an EC₅₀ value = 2.1 nM (–log M = 8.68 ± 0.17, n = 8; P < 0.0005 compared to guinea-pig intact or denuded trachea; Table 1).

Mechanism of endothelin-induced contraction

Extracellular calcium A series of experiments was conducted to determine the relative extracellular and intracellular calcium sources responsible for the endothelin-induced contraction in guinea-pig trachea and rat aorta.

In guinea-pig trachea, incubation for 30 min in calcium-free Krebs + 0.1 mM EGTA was without effect on endothelin concentration-response curves (Figure 4a). Calcium-deprivation for 30 min had a significant inhibitory effect against contractions induced by carbachol (Figure 4c) or KC1 (Figure 4e). Increasing the concentration of EGTA in calcium-free medium to 1 mM abolished contractions produced by endothelin (Figure 4b) or KC1 (Figure 4f). Further inhibition

Table 1 Comparison of potencies and efficacies of endothelin, substance P, leukotriene D₄ (LTD₄) and carbachol in guinea-pig intact isolated trachea and contraction to endothelin in rat endothelium-denuded aorta

Agonist	n	EC ₅₀ (−log M)	Maximum contraction	
			% reference contraction	g tension
Guinea-pig trachea				
Endothelin	32	7.51 ± 0.06	77.7 ± 3.7	1.24 ± 0.09
LTD ₄	4	9.12 ± 0.19**	97.9 ± 10.7*	1.39 ± 0.42
Carbachol	20	6.84 ± 0.15**	122.6 ± 3.4**	2.81 ± 0.21**
Substance P	4	5.85 ± 0.20**	97.5 ± 21.2	1.63 ± 0.36
Rat aorta				
Endothelin	8	8.86 ± 0.17**	197.4 ± 14.7	0.81 ± 0.15*

Results are expressed as EC₅₀ values (–log M) and contractile response (% reference contraction or g tension) and are the means ± s.e.mean. Studies were performed in the presence of 5 μM indomethacin.

Significantly different from endothelin in guinea-pig trachea, *P < 0.05, **P < 0.0005.

* Reference contraction was 10 μM carbachol for guinea-pig trachea and 60 mM KC1 for rat aorta.

Figure 2 Effects of (a) atropine ($1\mu\text{M}$); (b) mepyramine ($10\mu\text{M}$); (c) SK&F 104353 ($10\mu\text{M}$) and (d) SQ 29,548 ($1\mu\text{M}$) on endothelin concentration-response curves in guinea-pig intact trachea. Results are expressed as a percentage of the reference contraction to $10\mu\text{M}$ carbachol and are the mean, with vertical lines indicating s.e.mean (●—●) Control tissues; (○---○) treated tissues; (a) $n = 6$; (b) $n = 7$; (c) $n = 4$; (d) $n = 4$. Experiments in which the effects of atropine, mepyramine and SK&F 104353 were examined, but not those with SQ 29,548, were conducted in the presence of $5\mu\text{M}$ indomethacin.

of carbachol concentration-response curves was also produced by incubation for 30 min in calcium-free medium + 1 mM EGTA, although an appreciable contractile response persisted (Figure 4d). Addition of 2.5 mM CaCl_2 at the end of the experiment returned endothelin-induced contraction to control value (Figure 4b) and partially restored responses produced

Figure 3 Effects of removal of the epithelium on endothelin concentration-response curves in guinea-pig isolated trachea. Results are expressed as a percentage of the reference contraction to $10\mu\text{M}$ carbachol and are the mean of 23 tissues; vertical lines show s.e.mean. (●—●) + Epithelium; (○---○) -epithelium. Experiments were conducted in the presence of $5\mu\text{M}$ indomethacin.

by carbachol (Figure 4d) or KCl (Figure 4f); addition of 2.5 mM CaCl_2 did not appreciably affect the level of tone in control tissues.

Nicardipine ($0.01\mu\text{M}$) had no effect on endothelin concentration-response curves ($n = 3$; data not shown). Increasing the concentration of nicardipine to $0.1\mu\text{M}$ produced significant inhibition of contractions produced by 0.03 and $0.1\mu\text{M}$ endothelin but had no effect on responses produced by lower endothelin concentrations (Figure 5a). Nicardipine ($0.1\mu\text{M}$) inhibited contractions produced by low concentrations of carbachol (0.01 – $0.1\mu\text{M}$) but had no effect on responses produced by higher carbachol concentrations (Figure 5b). KCl-induced contractions were inhibited to a greater extent by nicardipine ($0.1\mu\text{M}$) than those produced by endothelin and, especially, carbachol (Figure 5c).

In contrast to guinea-pig trachea, incubation of rat aorta in calcium-free medium + 0.1 mM EGTA for 30 min produced substantial inhibition of endothelin-induced contraction (Figure 6a). At the end of the experiment, addition of 2.5 mM CaCl_2 to tissues exposed to $0.1\mu\text{M}$ endothelin increased the contractile response to the same level as that observed in control tissues; addition of 2.5 mM CaCl_2 had no effect in control tissues. Nicardipine ($0.01\mu\text{M}$) produced appreciable inhibition of endothelin-induced contraction in rat aorta (Figure 6b). Increasing the concentration of nicardipine to $0.1\mu\text{M}$ did not elicit significant further inhibition of contraction produced by endothelin (Figure 6c). Incubation for 30 min in calcium-free Krebs + 0.1 mM EGTA produced marked inhibition and $0.1\mu\text{M}$ nicardipine essentially abolished contraction produced by KCl in rat aorta (data not shown).

Intracellular calcium The effects of ryanodine ($10\mu\text{M}$) on responses elicited by endothelin, carbachol and KCl in intact guinea-pig trachea are summarized in Figure 7. These experiments were conducted in the presence of calcium-free medium + 0.1 mM EGTA. Ryanodine ($10\mu\text{M}$) produced significant inhibition of endothelin-induced contraction (Figure 7a). Ryanodine produced a small but significant 2 fold shift to the right of carbachol concentration-response curves, without affecting the maximum contractile response (Figure 7b), but had no effect against KCl-induced responses (Figure 7c).

Protein kinase C Staurosporine, a purported protein kinase C inhibitor, at concentrations of $0.01\mu\text{M}$ (data not shown) or $0.1\mu\text{M}$ (Figure 8a) did not inhibit endothelin-induced contraction. Responses to KCl were inhibited to a small extent by $0.1\mu\text{M}$ staurosporine whereas those produced by carbachol were unaffected (data not shown). Another purported protein kinase C inhibitor, H-7, at concentrations of $3\mu\text{M}$ or $10\mu\text{M}$ was without effect on endothelin-induced contraction. A

Figure 4 Effects of 30 min incubation in calcium-free Krebs + 0.1 mM EGTA (a, c, e) or + 1 mM EGTA (b, d, f) on concentration-response curves produced by endothelin (a, b), carbachol (c, d) or KCl (e, f) in guinea-pig intact trachea. Results are expressed as a percentage of the reference contraction to 10 μ M carbachol and are the mean with vertical lines indicating s.e.mean. (●—●) Control tissues; (○---○) treated tissues; (a) $n = 4$; (b) $n = 4$; (c) $n = 8$; (d) $n = 4$; (e) $n = 7$; (f) $n = 4$. Studies examining KCl-induced contractions were conducted in the presence of 1 μ M atropine. Re-addition of 2.5 mM CaCl₂ to tissues is indicated by + CaCl₂. Experiments were conducted in the presence of 5 μ M indomethacin.

higher concentration of H-7, 30 μ M, produced substantial inhibition of endothelin concentration-response curves (data not shown).

In rat aorta, 0.01 μ M staurosporine produced marked inhibition (Figure 8b) and 0.1 μ M staurosporine abolished endothelin-induced contractions (Figure 8c). Staurosporine (0.1 μ M) also abolished the contraction produced by KCl (data not shown).

Phosphatidylinositol turnover in guinea-pig trachea In the presence of 5 μ M indomethacin, endothelin (3 nM–0.3 μ M) produced a concentration-dependent stimulation of PI turnover, measured as total water-soluble inositol phosphates, in epithelium-containing guinea-pig trachea from which the cartilage had been removed (Figure 9). The calculated EC₅₀ ($-\log$ M) was 7.34 ± 0.20 , ($n = 8$), which is in close agreement with the EC₅₀ value for producing contraction (7.51 ± 0.06 ; Table 1). Endothelin (0.3 μ M) did not stimulate [³H]-myo-inositol incorporation into glycerophosphoinositol or the phospholipid (chloroform) phase (data not shown).

Discussion

In a preliminary communication by Uchida and co-workers (1988) endothelin was observed to be a potent contractile

agonist in guinea-pig isolated trachea, with a potency of more than 2 log units greater than neurokinin A or LTD₄. In the present study it was confirmed that endothelin is a potent constrictor of guinea-pig isolated trachea. However, endothelin was approximately 60 fold less potent in this series of experiments (EC₅₀ = 30.9 nM) than was previously determined (EC₅₀ = 0.53 nM) (Uchida *et al.*, 1988). The reason(s) for this discrepancy is unknown but may be related to different sources of endothelin, differences in the integrity of the airway epithelium, in addition to different strains of guinea-pigs used. It should be noted that the potency of endothelin in this study is similar to that presented in a recent publication also using guinea-pig trachea (Maggi *et al.*, 1989). In the present set of experiments endothelin was a more potent agonist in rat aorta than guinea-pig trachea.

Endothelin-induced contraction appears to involve a direct action on smooth muscle. Thus, the response was unaffected by mepyramine, atropine, SK&F 104353, a leukotriene receptor antagonist (Hay *et al.*, 1987) or SQ 29,548, a thromboxane receptor antagonist (Ogletree *et al.*, 1985), indicating that it does not involve the release of histamine, acetylcholine, leukotrienes or thromboxane. Indomethacin potentiated contractions produced by high concentrations of endothelin (0.03–0.3 μ M), suggesting that, as is the case with several other bronchoconstrictor agonists (Orehek *et al.*, 1975; Saad &

Figure 5 Effects of nicardipine ($0.1 \mu\text{M}$) on concentration-response curves produced by (a) endothelin, (b) carbachol and (c) KCl in guinea-pig intact trachea. Results are expressed as a percentage of the reference contraction to $10 \mu\text{M}$ carbachol and are the mean with vertical lines indicating s.e.mean. (●—●) Control tissues; (○---○) treated tissues; (a) $n = 7$; (b) $n = 4$; (c) $n = 4$. Studies examining KCl-induced contractions were conducted in the presence of $1 \mu\text{M}$ atropine. Experiments were conducted in the presence of $5 \mu\text{M}$ indomethacin.

Burka, 1983), responses produced by endothelin in guinea-pig trachea are normally attenuated by a bronchodilator prostanoid(s), presumably prostaglandin E_2 (PGE_2). This finding is in contrast to the effects of indomethacin against bronchoconstriction produced by administration of endothelin *in vivo*. Thus, indomethacin abolished bronchospasm induced by intravenous administration of endothelin (Payne & Whittle, 1988) and significantly inhibited bronchoconstriction produced by aerosol administration of endothelin in guinea-pigs (Lagente *et al.*, 1989). This suggests that administration of endothelin *in vivo*, especially via the intravenous route, produced bronchospasm in guinea-pigs indirectly via the release of constrictor cyclo-oxygenase products of arachidonic acid. One likely candidate is thromboxane, a potent bronchoconstrictor in the guinea-pig (Svensson *et al.*, 1977), as it has been shown that endothelin releases eicosanoids, including thromboxane, from guinea-pig isolated lung (De Nucci *et al.*, 1988). However, this mechanism is not involved in endothelin-induced contraction in guinea-pig isolated trachea, as reflected by the lack of inhibitory effect of indomethacin, or a specific thromboxane receptor antagonist SQ 29,548 (Ogletree *et al.*, 1985), in concentrations that produced sub-

Figure 6 Effects of (a) 30 min incubation in calcium-free Krebs + 0.1 mM EGTA, (b) $0.01 \mu\text{M}$ nicardipine or (c) $0.1 \mu\text{M}$ nicardipine on endothelin concentration-response curves in rat endothelium-denuded aorta. Results are expressed as a percentage of the reference contraction to 60 mM KCl and are the mean with vertical lines indicating s.e.mean. (●—●) Control tissues; (○---○) treated tissues; (a) $n = 8$; (b) $n = 4$; (c) $n = 4$. Re-addition of 2.5 mM CaCl_2 to tissues is indicated by $+\text{CaCl}_2$. Experiments were conducted in the presence of $5 \mu\text{M}$ indomethacin.

stantial inhibition of contractions elicited by the thromboxane mimetic, U-44069 (data not shown).

Although the data indicate that endothelin produces contraction of guinea-pig isolated trachea by a direct action, the response is influenced by the epithelium. Removal of the epithelium increased the responsiveness of the tracheal smooth muscle to the contractile effects of endothelin, similar to the observations obtained for other bronchoconstrictor and bronchorelaxant agents in airways from several species including man (Fedan *et al.*, 1988). The mechanism(s) responsible for this modulatory influence of the epithelium remains controversial, although postulates for which there is relatively convincing evidence include: (i) the epithelium is a source of prostanoid and non-prostanoid factors (Flavahan *et al.*, 1985; Hay *et al.*, 1986; Tschirhart *et al.*, 1987; Vanhoutte, 1988; Braunstein *et al.*, 1988) and (ii) the epithelium is a site of metabolism for some agents including catecholamines (Farmer *et al.*, 1986) and neurokinins (Devillier *et al.*, 1988; Frossard *et al.*, 1989). There is preliminary evidence to suggest that the epithelium inhibits contraction produced by endothelin in guinea-pig isolated trachea by acting as a locus for its meta-

Figure 7 Effects of 10 μM ryanodine on concentration-response curves produced by (a) endothelin, (b) carbachol and (c) KCl in guinea-pig intact trachea. Results are expressed as a percentage of the reference contraction to 10 μM carbachol and are the mean with vertical lines indicating s.e.mean. (●—●) Control tissues; (○---○) treated tissues; (a) $n = 8$; (b) $n = 4$; (c) $n = 4$. Studies examining KCl-induced contractions were conducted in the presence of 1 μM atropine. These studies were performed in calcium-free Krebs + 0.1 mM EGTA (30 min pretreatment). Experiments were conducted in the presence of 5 μM indomethacin.

bolism (Hay *et al.*, 1989a). It has recently been shown that the epithelium also attenuates endothelin-induced contraction in the guinea-pig main bronchus (Maggi *et al.*, 1989).

In the first publication on endothelin, contraction of porcine coronary artery was shown to be abolished by incubation in calcium-free medium + 1 mM EGTA and substantially inhibited by nicardipine, a dihydropyridine calcium channel inhibitor (Yanagisawa *et al.*, 1988). Based on these findings it was proposed that endothelin elicited contraction by activation of the dihydropyridine-sensitive calcium channels, i.e., by acting as an endogenous calcium channel 'agonist' or activator (Yanagisawa *et al.*, 1988). A similar mechanism was postulated to account for the endothelin-induced contraction of guinea-pig isolated trachea, a response which was partially inhibited by nicardipine (Uchida *et al.*, 1988). However,

Figure 8 Effects of staurosporine (0.01 μM or 0.1 μM) on concentration-response curves produced by endothelin in (a) guinea-pig intact trachea or (b, c) rat endothelium-denuded aorta. Results are expressed as a percentage of the reference contraction to (a) 10 μM carbachol or (b, c) 60 mM KCl and are the mean with vertical lines indicating s.e.mean. (●—●) Control tissues; (○---○) treated tissues; (a) $n = 9$; (b) $n = 4$; (c) $n = 4$. Tissues were exposed to 0.1 μM staurosporine (a and c) or 0.01 μM staurosporine (b) for 30 min before construction of agonist concentration-response curves. Experiments were conducted in the presence of 5 μM indomethacin.

the majority of studies published subsequent to the original study provide evidence that endothelin acts largely via a mechanism other than activation of dihydropyridine-sensitive channels (see below). In the present study 30 min incubation in calcium-free medium + 0.1 mM EGTA had no effect on endothelin-induced contraction of guinea-pig trachea. In addition nicardipine, at a concentration of 0.01 μM, also had no effect on responses produced by endothelin, whereas increasing the concentration of nicardipine to 0.1 μM had a significant inhibitory effect. A high concentration of nifedipine (1 μM) was shown to possess only a small inhibitory effect against response produced by endothelin in guinea-pig isolated trachea (Maggi *et al.*, 1989). These data would suggest that only a small component of the endothelin-induced contraction is mediated by calcium influx via dihydropyridine-sensitive

Figure 9 Endothelin-induced stimulation of phosphatidylinositol turnover in epithelium-containing guinea-pig trachea from which the cartilage had been removed. Results are expressed as d.p.m. mg⁻¹ tissue dry weight and are the mean with bars showing s.e. mean of 7–8 tissues. Tissues were exposed to endothelin for 60 min and total inositol phosphates were then extracted as outlined in the Methods section. The calculated EC₅₀ (–log M) was 7.51 ± 0.06. Experiments were conducted in the presence of 5 μM indomethacin.

calcium channels. In fact, the inhibitory effect of these high concentrations of the dihydropyridines may be mediated via a non-specific mechanism other than their recognized action of inhibition of voltage-dependent calcium channels; for example, phosphodiesterase inhibition (Norman *et al.*, 1983).

Increasing the concentration of EGTA in the calcium-free medium to 1 mM abolished endothelin-induced contraction. One postulate is that the contraction of guinea-pig trachea induced by endothelin is mediated by utilization of a high affinity, superficially-bound (i.e., membrane) source of calcium that is removed by high concentrations of EGTA. However, one has to consider that incubation in calcium-free medium + 1 mM EGTA for 30 min may produce attenuation of endothelin-induced contraction by depleting the intracellular calcium stores responsible for the response, rather than by chelation of extracellular or superficially bound calcium (Wheeler & Weiss, 1979; Guan *et al.*, 1988). For example, responses induced by carbachol were further inhibited following incubation for 30 min in calcium-free medium + 1 mM EGTA, suggesting significant depletion of intracellular calcium stores. The differences in the effects of calcium-free medium + 1 mM EGTA on carbachol- and endothelin-induced contraction suggests differences in the mechanism(s) and relative contributions of extracellular and intracellular calcium sources responsible for these responses. Furthermore, the larger inhibitory influence of ryanodine on endothelin-induced contraction compared to those produced by carbachol may indicate differences in the relative efficacies of endothelin and carbachol for stimulating the release of intracellular calcium.

In rabbit aorta, although the endothelin-induced contraction is resistant to calcium channel inhibitors, it is markedly attenuated (approximately 70–80% reduction in the maximum response) by incubation in calcium-free medium + EGTA (Ohlstein *et al.*, 1989; Sugiura *et al.*, 1989) suggesting that it involves utilization of a superficial or membrane-associated pool of calcium that is accessible to EGTA. In contrast, in the present study endothelin-induced contraction in rat aorta was sensitive to inhibition either by nicardipine or by incubation in calcium-free medium + EGTA. This suggests that both tissue and species differences exist in the relative calcium sources responsible for endothelin-induced contraction.

It is clear that the endothelin-induced contraction of guinea-pig isolated trachea is partially resistant to the effects of the dihydropyridine calcium channel inhibitors and also calcium-deprivation with 0.1 mM EGTA, suggesting that the response is mediated to a significant extent by mobilization of calcium from intracellular stores. This proposal is supported by the data from these studies using ryanodine. Ryanodine is

a naturally occurring alkaloid that has been shown to affect calcium mobilization specifically from the sarcoplasmic reticulum in a variety of tissues including vascular and airway smooth muscles (Ito *et al.*, 1986; Hwang & Van Breemen, 1987; Gerthoffer *et al.*, 1988). In the present study, ryanodine produced more inhibition of the endothelin-induced contraction than of responses produced by carbachol, and did not inhibit KCl-induced responses. These findings are similar to those obtained by Gerthoffer and co-workers (Gerthoffer *et al.*, 1988) who observed that in canine trachealis ryanodine inhibited carbachol-induced contraction—which appears to involve predominantly mobilization of intracellular calcium release—but not KCl- or 5-hydroxytryptamine (5-HT)-induced contractions which are more reliant on the influx of extracellular calcium (Gerthoffer, 1986; Gerthoffer *et al.*, 1988).

Further support for endothelin inducing contraction of guinea-pig trachea partly by release of calcium from intracellular stores is the finding that endothelin stimulates PI turnover which, in a variety of tissues including smooth muscle, has been shown to stimulate release of calcium from the sarcoplasmic reticulum (Streb *et al.*, 1983; Hashimoto *et al.*, 1985; Somolyo *et al.*, 1987). In airway smooth muscle, agonists whose contractile responses are relatively resistant to calcium deprivation, suggesting they involve the mobilization of intracellular calcium, stimulate PI turnover and formation of IP₃ (Baron *et al.*, 1984; Hashimoto *et al.*, 1985) Takuwa *et al.*, 1986; Duncan *et al.*, 1987). In the present study the EC₅₀ for endothelin-induced stimulation of PI turnover in guinea-pig intact trachea was comparable to the EC₅₀ for producing contraction (45.9 nM vs 30.9 nM, respectively), suggesting that the two parameters may be linked. Endothelin stimulates PI metabolism and increases the levels of the inositol phosphates in cultured vascular smooth muscle cells from a variety of species (Resink *et al.*, 1988; Van Renterghem *et al.*, 1988; Araki *et al.*, 1989; Marsden *et al.*, 1989; Sugiura *et al.*, 1989). In addition, endothelin elevated the intracellular free concentration of Ca²⁺, measured fluorometrically (Kai *et al.*, 1989; Marsden *et al.*, 1989) and stimulated ⁴⁵Ca efflux (Miasiro *et al.*, 1988; Marsden *et al.*, 1989).

Although some discrepancies exist, the majority of evidence to date is at odds with the original postulate that endothelin produces contraction of smooth muscle by activating dihydropyridine-sensitive calcium channels (Yanagisawa *et al.*, 1988). Thus, the response appears to involve mobilization of intracellular calcium, probably as a consequence of stimulation of PI turnover. A mechanism of action other than an interaction with dihydropyridine calcium channels is supported by data from binding studies, which indicate that, in rat cardiac membrane fragments, endothelin did not affect the affinity or the number of dihydropyridine binding sites and did not displace ligands for dihydropyridines and two other classes of calcium channel inhibitors (Gu *et al.*, 1989). In skeletal muscle T-tubule membrane endothelin did not affect binding of four different classes of calcium channel inhibitors (Van Renterghem *et al.*, 1988). Furthermore, specific high affinity binding of [¹²⁵I]-endothelin in rat aortic vascular smooth muscle cells was unaffected by nicardipine, verapamil or diltiazem (Hirata *et al.*, 1988).

However, there appears to be a component of the response to endothelin that does involve stimulation of extracellular calcium influx via dihydropyridine-sensitive membrane channels. The relative contribution of this mechanism to the endothelin-induced response is likely to depend on the tissue. For example, in the present study, endothelin-induced contraction of rat aorta was more susceptible to the effects of removal of extracellular calcium and nicardipine than was the response in the guinea-pig isolated trachea. In porcine coronary artery, endothelin-induced contraction was markedly attenuated by nicardipine (Yanagisawa *et al.*, 1988), whereas in rabbit aorta, a dihydropyridine calcium channel inhibitor had only a slight (Marsden *et al.*, 1989) or no effect (Ohlstein *et al.*, 1989) on the response. In agreement with the present study, Van Renterghem and co-workers (Van Renterghem *et al.*

al., 1988) observed that endothelin-induced contraction of rat aorta was inhibited markedly by a dihydropyridine calcium channel inhibitor, whereas in another study no effect was observed (Auguet *et al.*, 1988).

The role of diacylglycerol (DAG) and protein kinase C activation in endothelin-induced contraction of smooth muscle remains to be clarified. In the present study, staurosporine (0.01 and 0.1 μM) and H-7 (3 and 10 μM), two purported protein kinase C inhibitors (Hidaka *et al.*, 1984; Inagaki *et al.*, 1985; Tamaoki *et al.*, 1986) had no effect against endothelin-induced contraction of guinea-pig trachea. A higher concentration of H-7, 30 μM , markedly attenuated endothelin-induced contraction. However, the inhibitory effect of this high concentration of H-7 may be due to an action other than inhibition of protein kinase C. Thus, the data from the present study would suggest that protein kinase C is not intimately involved in endothelin-induced contraction of guinea-pig isolated trachea. In contrast, staurosporine produced marked inhibition of response to endothelin in rat aorta suggesting an involvement of activation of protein kinase C in this tissue. This highlights further the apparent differences in the fundamental mechanisms responsible for endothelin-induced contraction in rat aorta and guinea-pig trachea. However, the ability of staurosporine (0.1 μM) to abolish KCl-induced contraction suggests a non-specific mechanism of action of staurosporine, a feature of many purported selective protein kinase C inhibitors (Rüegg & Burgess, 1989). Furthermore, in rabbit aorta staurosporine (Ohlstein *et al.*, 1989) and H-7 (Sugiura *et al.*, 1989) inhibited endothelin-induced contraction.

In addition, endothelin stimulated DAG formation and protein kinase C phosphorylation in cultured vascular smooth muscle cells (Griendling *et al.*, 1989; Resink *et al.*, 1989), and it was suggested that this pathway may be involved in mediating endothelin-induced vasoconstriction.

In summary, endothelin is a potent constrictor of guinea-pig isolated trachea, and appears to act predominantly, if not exclusively, by a direct action on the smooth muscle cell. Endothelin-induced contraction is inhibited markedly by the epithelium. A minor component of the response is sensitive to inhibition by a dihydropyridine-sensitive calcium channel inhibitor. However, the endothelin-induced contraction appears to involve, predominantly, a mechanism other than activation of dihydropyridine-sensitive calcium channels, namely stimulation of PI turnover and subsequent release of calcium from intracellular stores, in addition to calcium influx via a pathway that is not blocked by dihydropyridine calcium channel inhibitors but is sensitive to calcium-free medium containing a high concentration of EGTA (1 mM). Endothelin-induced contraction of rat aorta was more sensitive to the effects of incubation in Ca^{2+} -free medium, nicardipine or staurosporine, suggesting that differences exist in the relative mechanisms whereby endothelin produces contraction in different tissues.

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