Endothelin-1-induced [³H]-inositol phosphate accumulation in rat trachea

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1   The effects of endothelin-1 (ET-1) and of the muscarinic cholinoceptor agonist, carbachol, on [³H]-inositol phosphate ([³H]-InsP) accumulation and smooth muscle contraction were determined in rat isolated tracheal tissue.

2   ET-1 (1 μM) and carbachol (10 μM) induced significant accumulation of [³H]-InsPs in *myo*-[2-³H]-inositol-loaded rat tracheal segments. Several components of the tracheal wall including the airway smooth muscle band, the cartilaginous region and the intercartilaginous region generated significant levels of [³H]-InsPs in response to ET-1 and carbachol. Following stimulation with ET-1, a greater proportion of tracheal [³H]-InsPs were generated in the intercartilaginous region (49%) than in either the airway smooth muscle band (25%) or cartilaginous region (26%). However, when the respective weights of these regions is taken into account, ET-1-induced accumulation of [³H]-InsPs was greatest in the airway smooth muscle band. The tracheal epithelium did not appear to generate [³H]-InsPs in response to ET-1 or modulate either basal or ET-1-induced accumulation of [³H]-InsPs in rat tracheal segments.

3   In the rat tracheal smooth muscle band, ET-1 caused a time- and concentration-dependent accumulation of [³H]-InsPs. Concentrations of ET-1 as low as 10 nM produced significant accumulation of [³H]-InsPs (1.23 ± 0.10 fold increase above basal levels of 295 ± 2 d.p.m. mg⁻¹ wet wt., $n = 3$ experiments). At 10 μM, the highest concentration used, ET-1 produced similar levels of [³H]-InsP accumulation (7.03 ± 0.55 fold above basal levels, $n = 5$) to that produced by a maximally effective concentration of carbachol (10 mM; 7.97 ± 0.31 fold increase above basal levels, $n = 4$). ET-1-induced accumulation of [³H]-InsPs was not significantly affected by indomethacin (5 μM), nordihydroguaiaretic acid (NDGA, 10 μM), WEB 2086 (10 μM) or phosphoramidon (10 μM).

4   ET-1 also produced concentration-dependent contractions of epithelium-denuded rat tracheal ring preparations. The mean concentration of ET-1 producing 50% of the maximum contractile response to carbachol (EC_{50}) was 31 nM (95% confidence limits, 20–49 nM, $n = 12$). The presence of an intact tracheal epithelium, indomethacin (5 μM), WEB 2086 (10 μM) and phosphoramidon (10 μM) had no significant effect on the mean EC_{50} for ET-1-induced contraction ($n = 5$). In contrast, NDGA (10 μM) inhibited ET-1-induced contractions (4.0 fold increase in mean EC_{50}, $P < 0.001$, $n = 5$). However, this effect of NDGA did not appear to be related to inhibition of leukotriene synthesis via lipoxygenase since the leukotriene antagonist SKF 104353 did not affect ET-1-induced contractions ($n = 5$) and moreover, leukotriene C_4 and leukotriene D_4 did not contract rat isolated tracheal smooth muscle preparations ($n = 4$).

5   The threshold concentrations of ET-1 that produced increases in smooth muscle contraction and [³H]-InsPs accumulation were similar, although the EC_{50} for [³H]-InsP accumulation was 2.9 fold greater than that for smooth muscle contraction. For carbachol, the EC_{50} for [³H]-InsP accumulation (mean $EC_{50} = 5.0$ μM, 1.2–21 μM, $n = 4$) was 25 fold greater than that for smooth muscle contraction (mean $EC_{50} = 0.20$ μM, 0.17–0.24 μM, $n = 12$).

6   It seems likely that ET-1 has a direct effect on InsP generation in rat tracheal smooth muscle and that this is largely responsible for the spasmogenic actions of this peptide.

Keywords: Endothelin-1; inositol phosphates; rat tracheal smooth muscle

Introduction

The 21-amino acid peptide endothelin-1 (ET-1) was first isolated from porcine aortic endothelial cells by Yanagisawa and coworkers (1988). In addition to producing potent vasoconstriction, ET-1 has been shown to produce numerous other actions including a positive inotropic effect in atria (Ishikawa *et al.*, 1988) and release of atrial natriuretic peptide (Fukuda *et al.*, 1988).

Relatively little is known of the airway effects of ET-1, although recent evidence suggests that ET-1 is synthesized within the lung and may exert an influence on its function. *In situ* hybridization and immunohistochemical studies indicate that ET-1 is synthesized and released from airway epithelial cells (MacCumber *et al.*, 1989; Black *et al.*, 1989; Rozengurt *et al.*, 1990). However, a growing body of data suggests that airway smooth muscle may be a primary target for this peptide. For example, quantitative autoradiographic studies

of human and animal central airways have demonstrated high concentrations of specific binding sites for ET-1 in airway smooth muscle (Turner *et al.*, 1989; Henry *et al.*, 1990). *In vitro* functional studies suggest that stimulation of such sites produces a long-lasting and slowly reversible contraction of airway smooth muscle (Uchida *et al.*, 1988; Turner *et al.*, 1989; Henry *et al.*, 1990; Hemsen *et al.*, 1990; McKay *et al.*, 1991). Furthermore, ET-1 produces a significant bronchoconstrictor response in anaesthetized guinea-pigs *in vivo* (Payne & Whittle, 1988; Lagente *et al.*, 1989; Macquin-Mavier *et al.*, 1989; Pons *et al.*, 1991).

The mechanism(s) through which ET-1 produces contraction of airway smooth muscle are not clear. In some studies, a dependence on extracellular calcium was reported (Turner *et al.*, 1989; Advenier *et al.*, 1990), although others have shown ET-1-induced contraction in the absence of extracellular calcium (Hay, 1990; McKay *et al.*, 1991). In addition, calcium-channel blocking drugs, such as nicardipine have been reported to inhibit ET-1-induced contractions of guinea-pig and human airway smooth muscle preparations (Uchida *et al.*,

1988; Maggi et al., 1989; Advenier et al., 1990; Hay, 1990), but not those in rat trachea (Turner et al., 1989). Thus, ET-1-induced contraction of airway smooth muscle in some species may involve an action on receptor-operated calcium-channels. In contrast, recent studies in vascular smooth muscle have demonstrated that ET-1-induced contraction may be mediated via the generation of inositol (1,4,5) trisphosphate (Ins(1, 4,5)P₃) which is linked to the mobilization of intracellular calcium stores (Marsden et al., 1989; Xuan et al., 1989; Pang et al., 1989). This possibility has only recently been investigated with respect to ET-1 in airway smooth muscle (Hay, 1990), although this pathway has previously been demonstrated for some other spasmogens of airway smooth muscle including histamine, muscarinic cholinoceptor agonists, tachykinins and leukotrienes (Grandordy et al., 1986; 1988; Madison & Brown, 1988; Mong et al., 1988). In addition to the direct effects of ET-1, several recent studies have shown that ET-1 may release secondary mediators the actions of which may indirectly and variably contribute to the overall spasmogenic effects of ET-1 (Payne & Whittle, 1988; Macquin-Mavier et al., 1989; Battistini et al., 1990a,b).

In the current study, the relationship between ET-1-induced contraction of airway smooth muscle and [³H]-inositol phosphate accumulation was determined in rat isolated tracheal preparations and compared to those effects induced by the muscarinic cholinoceptor agonist, carbachol. Furthermore, we examined the effects of several receptor antagonists and enzyme inhibitors to determine whether other secondary mediators contributed to these ET-1-induced effects.

Methods

Tissue preparation

Male Wistar rats (8–10 weeks) were stunned and killed by cervical dislocation and exsanguination. The trachea was rapidly excised, cleaned of adhering connective tissue and the tracheal epithelium deliberately removed by rubbing with a moist cotton wool swab (Goldie et al., 1986). To determine the extent of [³H]-InsP accumulation in the entire tracheal wall, the trachea was cut into ring segments approximately 2–3 mm long.

We also compared the ability of various structures within the tracheal wall to generate [³H]-InsPs; the airway smooth muscle, the epithelium, the cartilaginous region and inter-cartilaginous region. In these experiments, the trachea was not initially cut into segments. The airway smooth muscle band, which lies between the cartilage horns along the posterior membrane of the trachea, was dissected from the trachea with the aid of an optical magnifier (2-times magnification). The smooth muscle band was then cut transversely to generate 4 segments of similar length. The remainder of the trachea, which contains the alternating cartilaginous and inter-cartilaginous regions was stretched longitudinally and pinned flat. Although these two regions could be easily distinguished visually, it was not possible to dissect readily the inter-cartilaginous tissue from between neighbouring cartilage rings and to form a preparation comprising only intercartilaginous tissue. Therefore, to determine the relative contributions of the cartilaginous and intercartilaginous regions to the generation of [³H]-InsPs, the trachea was cut at intervals, close to the cartilage, to provide two preparations (P1 and P2) which contained different proportions of the two regions. Both P1 and P2 had 2 cartilaginous regions; however, P1 contained a single intercartilaginous region whereas P2 contained three. The relative contributions of the cartilaginous region (x) and the intercartilaginous region (y) to the accumulation of InsPs was then determined by solving equations (1) and (2) simultaneously:

$$2x + y = InsPs(P1) \qquad (1)$$

$$2x + 3y = InsPs(P2) \qquad (2)$$

where InsPs(P1) and InsPs(P2) are the amounts of [³H]-InsPs accumulated from P1 and P2, respectively. The ability of tracheal epithelium to generate [³H]-InsPs was assessed indirectly by comparing tracheal segments that had an intact epithelium with those in which the epithelium had been mechanically removed, as described above.

Assay of [³H]-inositol phosphate

For each experiment, preparations from 8–10 rats were pooled and randomly divided into 20 groups. Preparations were preincubated in 5 ml of Krebs-Henseleit solution (KH) for 30 min at 37°C and then incubated with myo-[2-³H]-inositol (5 µCi) in 1 ml of carbogen-aerated KH for 3 h at 37°C with gentle shaking. Previous work on airway smooth muscle suggests that the presence of agonists such as carbachol during this loading period stimulates the incorporation of [³H]-inositol into a hormone-sensitive phosphoinositide pool, allowing steady-state conditions to prevail during subsequent periods of agonist stimulation (Chilvers et al., 1989). In the current study, addition of 1 µM carbachol for the final 30 min of the loading period produced no noticeable increase in the subsequent accumulation of [³H]-InsPs produced by ET-1. This suggests that the 3 h loading period used was sufficient to enable steady-state conditions to be achieved.

After loading with myo-[³H]-inositol, preparations were washed twice with 5 ml of KH for 15 min and then washed a third time for a further 15 min. When atropine, indomethacin, nordihydroguaiaretic acid (NDGA), WEB 2086 or phosphoramidon were used, each was added after the third wash period (30 min before exposure to an agonist) and were in contact with tracheal tissue for the remainder of the experiment. After washing, segments were incubated for 15 min with 1 ml KH containing 5 mM LiCl to inhibit the breakdown of inositol monophosphate to inositol and thus enhance the subsequent accumulation of InsPs (Berridge et al., 1982). Tracheal segments were then stimulated for 15 min by addition of 20 µl of ET-1 or other agonists and the incubation terminated by the addition of 1.5 ml chloroform: methanol (1:2, v/v) with vigorous shaking. After standing for 15 min, chloroform (0.5 ml) and water (0.5 ml) were added sequentially to the samples to separate it into an aqueous and a lipid phase. The entire upper methanol/water phase was applied to an anion-exchange chromatography column (1 ml of Dowex AG1-X8 in formate form) and [³H]-InsPs fractionated by washing the column with buffers of increasing ionic strength. Inositol was eluted with 10 ml of water and glycerophosphoinositol was eluted with 15 ml of buffer consisting of 5 mM sodium tetra-borate and 60 mM sodium formate (Buffer I).

In some experiments with isolated airway smooth muscle segments, [³H]-inositol monophosphates ([³H]-InsP₁s), [³H]-inositol bisphosphates ([³H]-InsP₂s) and [³H]-inositol trisphosphates ([³H]-InsP₃s) were eluted stepwise with 15 ml of Buffer II (5 mM sodium tetraborate and 150 mM sodium formate), 10 ml of Buffer III (0.1 M formic acid and 0.3 M ammonium formate) and 10 ml of Buffer IV (0.1 M formic acid and 0.75 M ammonium formate) respectively. The [³H]-InsP₃ fraction was not separated further and may therefore have contained a significant amount of the biologically less active [³H]-Ins(1,3,4)P₃ isomer (Chilvers et al., 1990), as well as tetrakis- and higher polyphosphates. Otherwise, Buffer IV was added immediately after Buffer 1 to elute [³H]-InsP₁, [³H]-InsP₂ and [³H]-InsP₃ in a single 10 ml fraction. Three 1 ml aliquots of this eluted fraction was mixed with 10 ml of scintillant (5.8 g l⁻¹ 2,5-diphenyloxazol (PPO) in Triton X100: toluene, 1:2) and radioactivity counted in a Tri-Carb liquid scintillation counter (Packard, Model 1500). Preliminary experiments revealed that total [³H]-InsP accumulation ([³H]-InsP₁ plus [³H]-InsP₂ plus [³H]-InsP₃) estimated by the first elution method (sum of the radioactivities recovered from Buffers II, III and IV) was similar to the estimate obtained by the second elution method (radioactivity in Buffer

IV). Total [^{3}H]-InsP accumulation was expressed as d.p.m. mg^{-1} wet wt. tracheal tissue or as d.p.m./trachea, as indicated.

Functional studies

Rat trachea was isolated as described above. Four tracheal ring preparations (2 mm long segments) were obtained from each trachea and unless otherwise stated, denuded of epithelium (Goldie *et al.*, 1986). These preparations were suspended under a resting tension of 0.5 g and placed in siliconized organ baths containing 3 ml of KH at 37°C, bubbled continuously with 5% CO$_2$ in O$_2$. Changes in isometric tension in tracheal smooth muscle were measured with a Model 7D Polygraph via FTO3 force-displacement transducers (Grass Instruments). Tracheal rings were allowed to equilibrate for 45 min before exposure to a single dose of carbachol (0.3 μM) to assess airway smooth muscle viability (100%). Preparations were washed for 15 min. In the current study, we determined the influence of several receptor antagonists (WEB2086 (10 μM), SKF 104353 (3 μM)) and enzyme inhibitors (phosphoramidon (10 μM), indomethacin (5 μM), NDGA (10 μM)) on carbachol- and ET-1-induced contractions. Preparations were exposed for 30 min to a selected antagonist/inhibitor or solvent (control) and then to cumulatively administered concentrations of carbachol (0.01–10 μM). Tracheal segments were washed and rested for 15 min and then re-exposed for 30 min to the antagonist/inhibitor or solvent, as before. A concentration-effect curve to ET-1 (1–300 nM) was then obtained. Contractile responses to carbachol and ET-1 are expressed as a percentage of the maximum response obtained with 10 μM carbachol (C$_{max}$). For each experiment, EC$_{50}$ values (concentration of spasmogen producing 50% C$_{max}$) were derived directly from the concentration-effect curves by interpolation. Geometric mean EC$_{50}$ values with associated 95% confidence limits were obtained from *n* different animals.

Statistical analyses

Unless otherwise stated, data are presented as mean ± s.e.mean of the indicated number of experiments (*n*) and differences between treatments assessed by analysis of variance followed by a modified *t*-statistic (Wallenstein *et al.*, 1980). *P* values less than 0.05 were considered to be statistically significant.

Drugs

Drugs used were: *myo*-[2-^{3}H]-inositol (17.8 Ci mmol^{-1}, Amersham), endothelin-1 (Auspep, Melbourne, Australia), carbamylcholine chloride, indomethacin, nordihydroguaiaretic acid, phosphoramidon, histamine diphosphate (Sigma Chemical Company, St. Louis, USA), WEB 2086 (3-[1,1-dimethyl-ethyl]hexahydro-1,4,7b-trihydroxy-α-methyl-9H-1,7a(epoxy-methanol) - 1H,6aH - cyclapenta[c]furo[2.3 - b]furo[3',2':3,4] cyclopenta[1,2-d]furan-5,9,12[4H]-trione, (Boehringer, Ingelhiem), leukotriene C$_4$, leukotriene D$_4$, SKF 104353 (SmithKline Beecham Pharmaceuticals, King of Prussia, U.S.A.). Indomethacin was dissolved in 0.1 M Na$_2$CO$_3$ and nordihydroguaiaretic acid in ethanol. Stock solutions of endothelin-1 (50 μM) were stored in 0.1 μM acetic acid. All other drugs were dissolved and diluted in 0.9% saline. The composition of the KH solution was (in mM): NaCl 117, KCl 5.36, NaHCO$_3$ 25.0, KH$_2$PO$_4$ 1.03, MgSO$_4$·7H$_2$O 0.57, CaCl$_2$·2H$_2$O 2.5 and glucose 11.1.

Results

Accumulation of [^{3}H]-InsPs in rat tracheal segments

Exposure of *myo*-[2-^{3}H]-inositol-loaded rat tracheal segments to ET-1 caused a significant accumulation of [^{3}H]-InsPs. For example, stimulation with 1 μM ET-1 for 15 min produced a 4.3 ± 0.4-fold increase (*n* = 3 experiments) in total [^{3}H]-InsP accumulation above basal levels (184 ± 12 d.p.m. mg^{-1} wet wt). This was significantly greater than the level of [^{3}H]-InsPs accumulated in response to 1 mM carbachol (3.3 ± 0.2-fold increase; *n* = 4; *P* < 0.05). Following stimulation with 1 μM ET-1, the relative proportions of [^{3}H]-InsPs recovered from elution buffers II, III and IV, which subsequently elute [^{3}H]-InsP$_1$, [^{3}H]-InsP$_2$ and [^{3}H]-InsP$_3$ (plus higher polyphosphates) respectively, was 61 ± 1%:32 ± 2%:7 ± 1% (*n* = 3). These data indicate that approximately 93% of the total accumulated [^{3}H]-InsPs were the major metabolic products of [^{3}H]-InsP$_3$ degradation, namely [^{3}H]-InsP$_1$ and [^{3}H]-InsP$_2$.

Accumulation of [^{3}H]-inositol phosphates in various tracheal wall structures

ET-1 (1 μM) and carbachol (1 mM) induced significant accumulation of [^{3}H]-InsPs in the airway smooth muscle band, the cartilaginous region and the intercartilaginous region of the rat trachea (Figure 1). Approximately half of the [^{3}H]-InsPs accumulated in response to 1 μM ET-1 were generated within the intercartilaginous region of the tracheal wall (Figure 1a). In contrast, the greatest proportion of [^{3}H]-InsPs accumulated in response to 1 mM carbachol were generated within the airway smooth muscle band. When the respective weights of the regions are accounted for (average weight in mg/trachea; smooth muscle, 4.3; intercartilaginous region 13.6 and cartilaginous region, 16.5), the greatest level of [^{3}H]-InsP accumulation produced in response to either ET-1 or carbachol was observed in the airway smooth muscle band (Figure 1b). For example, ET-1-induced accumulation of [^{3}H]-InsPs in the airway smooth muscle band was 3.5 and 1.6 fold greater than that observed in the cartilaginous and intercartilaginous regions, respectively. Similarly, carbachol-induced accumulation of [^{3}H]-InsPs was at least 5.5 fold higher in the airway
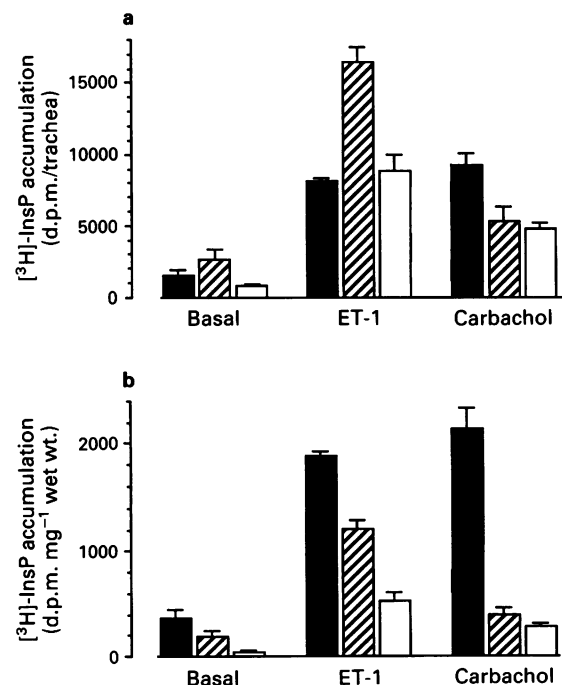

Figure 1 Accumulation of [^{3}H]-inositol phosphates ([^{3}H]-InsPs) in different regions of the rat isolated, epithelium-denuded trachea under basal conditions or following stimulation with 1 μM endothelin-1 (ET-1) or 1 mM carbachol; airway smooth muscle band (solid columns), intercartilaginous region (hatched columns) and cartilaginous region (open columns). Accumulation of [^{3}H]-InsPs has been expressed in terms of (a) d.p.m. per trachea and (b) d.p.m. mg^{-1} wet wt. region. Data were obtained from three experiments using 12 animals.

smooth muscle band than in the other regions (Figure 1b). Removal of the tracheal epithelium had no significant effect on [^{3}H]-InsP accumulation either under basal conditions or following stimulation with 0.1 μM ET-1 (3.66 $\pm$ 0.19 fold increase above basal accumulation in epithelium denuded segments versus 3.60 $\pm$ 0.18 fold increase in epithelium-intact segments, $n = 3$).

Accumulation of [^{3}H]-inositol phosphates in the airway smooth muscle band

ET-1-induced accumulation of total [^{3}H]-InsPs within the tracheal airway smooth muscle band was time- and concentration dependent (Figure 2 and 3a). ET-1 (1 μM) produced significant accumulation of total [^{3}H]-InsPs within 5 min and the rate of accumulation appeared linear for at least 15 min thereafter. Hence, in all subsequent experiments a 15 min stimulation period was used. Significant [^{3}H]-InsP accumulation was produced with ET-1 concentrations as low as 10 nM
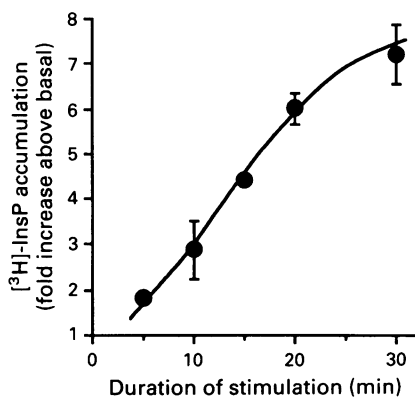

Figure 2 Accumulation of [^{3}H]-inositol phosphates ([^{3}H]-InsPs), above basal levels, produced by increasing periods of stimulation of rat isolated, epithelium-denuded tracheal smooth muscle segments with 1 μM (●) endothelin-1 ($n = 3$ experiments). Basal levels of [^{3}H]-InsP accumulation did not change significantly during the various periods of incubation (mean basal levels = 174 $\pm$ 9 d.p.m. mg^{-1} wet wt.).
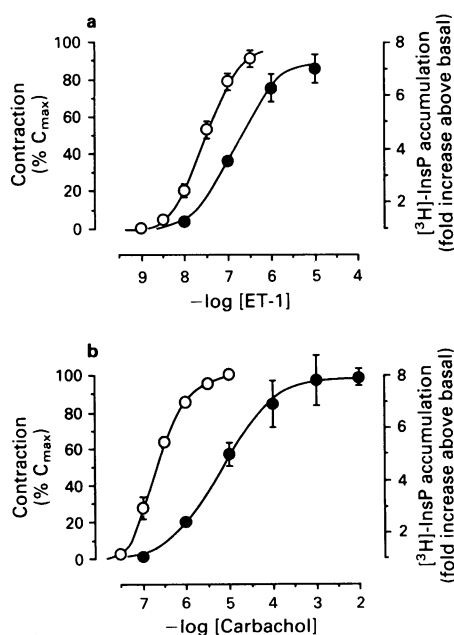

Figure 3 Mean concentration-effect curves for (a) endothelin-1 (ET-1) and (b) carbachol showing agonist-induced changes in isometric tension (O, C$_{max}$ = response to 10 μM carbachol, $n = 12$ preparations from 12 animals) and [^{3}H]-inositol phosphate ([^{3}H]-InsP) accumulation (●, $n = 4$–6 experiments) in rat isolated, epithelium-denuded tracheal smooth muscle preparations.

(1.23 $\pm$ 0.10 fold increase above basal levels of 295 $\pm$ 2 d.p.m. mg^{-1} wet wt, $n = 3$ experiments) and there was a tendency for [^{3}H]-InsP accumulation to plateau after exposure to 10 μM ET-1, the highest concentration used. The mean increase in [^{3}H]-InsP accumulation above basal levels induced with 10 μM ET-1 (7.03 $\pm$ 0.55 fold above basal levels, $n = 5$, Figure 3a) was not significantly different from the mean maximum increase produced in the presence of the cholinoceptor agonist, carbachol (10 mM, 7.97 $\pm$ 0.31, $n = 4$; Figure 3b). The EC$_{50}$ concentration of ET-1-induced accumulation of [^{3}H]-InsPs was 90 nM (95% confidence limits, 47–170 nM, $n = 5$).

ET-1-induced (0.1 μM) accumulation of [^{3}H]-InsPs in the airway smooth muscle band was not significantly affected by indomethacin (5 μM), NDGA (10 μM), WEB 2086 (10 μM) or phosphoramidon (10 μM) (Figure 4). Similarly, the accumulation of [^{3}H]-InsPs in response to 0.1 μM ET-1 (3.02 $\pm$ 0.19 fold above basal levels, $n = 6$) was not affected by 0.1 μM atropine (3.17 $\pm$ 0.25 fold above basal levels, $n = 3$), whereas accumulation of [^{3}H]-InsPs produced in response to 10 μM carbachol was prevented by atropine (0.84 $\pm$ 0.04 fold above basal levels, $n = 3$). At the concentrations used, none of these inhibitors alone had any significant effect on basal [^{3}H]-InsP accumulation in unstimulated preparations.

Exposure of *myo*-[2-^{3}H]-inositol-loaded rat tracheal smooth muscle preparations to the membrane depolarizing spasmogen, KCl (60 mM) produced a small, but not statistically significant, increase in accumulation of [^{3}H]-InsPs above basal levels (1.36 $\pm$ 0.16 fold, $n = 5$). At a concentration of 100 μM, histamine produced a small accumulation of [^{3}H]-InsPs above basal levels (1.46 $\pm$ 0.14 fold, $n = 5$, $P < 0.05$). This represents only 7.6% of the increase obtained with 10 μM ET-1.

Carbachol- and endothelin-1-induced contractions in rat tracheal segments

Carbachol produced concentration-dependent contraction of epithelium-denuded rat isolated tracheal preparations (mean EC$_{50}$ value, 0.20 μM; 95% confidence limits, 0.17–0.24; $n = 12$; Figure 3b). Carbachol-induced contractions were not significantly affected by the presence of an intact epithelium (mean EC$_{50}$ value, 0.21 μM (0.14–0.31), $n = 5$) or by any of the antagonists/inhibitors tested (Table 1).

ET-1 caused potent concentration-dependent contractions of epithelium-denuded rat tracheal smooth muscle preparations (Figure 3a). Significant contractions to ET-1 were observed with concentrations as low as 10 nM. At the highest concentration of ET-1 used (0.3 μM), the concentration-effect curve had not plateaued but the increase in tone was 91.1 $\pm$ 2.2% C$_{max}$ ($n = 12$). The mean concentration of ET-1 producing 50% C$_{max}$ was 31 nM (95% confidence limits, 20–49 nM). A similar profile was observed in epithelium-intact
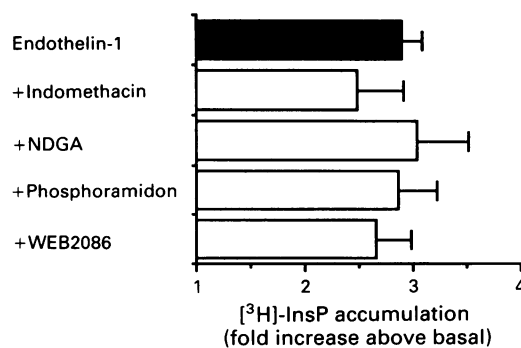

Figure 4 Influence of indomethacin (5 μM), nordihydroguaiaretic acid (NDGA, 10 μM), phosphoramidon (10 μM) and WEB 2086 (10 μM) on [^{3}H]-inositol phosphate ([^{3}H]-InsP) accumulation produced in rat isolated epithelium-denuded tracheal smooth muscle preparations following stimulation with endothelin-1 (0.1 μM). Shown are the mean responses (columns) from 3–4 experiments; horizontal bars show s.e.mean.

Table 1 Contractile potencies of carbachol and endothelin-1 (ET-1) in epithelium-denuded rat isolated tracheal preparations determined in the absence and presence of receptor antagonists and enzyme inhibitors

Antagonist/Inhibitor	n	Mean concentration producing 50% C_{max} (95% confidence limits)	
		Carbachol (μM)	ET-1 (nM)
None (control)	12	0.20 (0.17–0.24)	31 (20–49)
Indomethacin (5 μM)	5	0.14 (0.08–0.26)	31 (15–62)
NDGA (10 μM)	5	0.23 (0.14–0.38)	123** (57–265)
SKF 104353 (3 μM)	5	0.21 (0.16–0.28)	38 (23–63)
WEB 2086 (10 μM)	5	0.27 (0.17–0.43)	32 (12–85)
Phosphoramidon (10 μM)	5	0.26 (0.18–0.38)	33 (26–43)

** Significantly different from control values, $P < 0.001$.
n, number of animals.
For abbreviations, see text.

preparations (0.3 μM ET-1 produced 88.2 $\pm$ 3.6% C_{max} and the mean EC_{50} value was 38 nM (21–67), $n = 5$). ET-1-induced contractions of epithelium-denuded rat isolated tracheal smooth muscle preparations were not influenced by indomethacin, phosphoramidon or WEB 2086 (Table 1). In contrast, 10 μM NDGA produced a 4.0 fold decrease in the potency of ET-1 ($P < 0.001$, $n = 5$). This was apparently not due to the inhibition of leukotriene synthesis or effect since leukotriene C_4 and D_4 did not contract rat isolated tracheal smooth muscle preparations ($n = 4$) and SKF 104353 was without effect on ET-1-induced contractions (Table 1).

The threshold concentrations of ET-1 producing contraction and accumulation of total [^{3}H]-InsPs in airway smooth muscle were similar (Figure 3), although the mean EC_{50} value for total [^{3}H]-InsP accumulation was 2.9 fold higher than that for smooth muscle contraction. For carbachol, the mean EC_{50} value for [^{3}H]-InsP accumulation (5.0 μM) was 25 fold greater than that for airway smooth muscle contraction (0.20 μM).

Discussion

The current study revealed that several regions within the tracheal wall, including airway smooth muscle, the cartilaginous region and the intercartilaginous tissue, generate InsPs in response to ET-1 and carbachol. Of these regions, the airway smooth muscle band was clearly the most responsive (on a wet weight basis) to the [^{3}H]-InsP generating actions of both ET-1 and carbachol. However, it is also noteworthy that the region generating the greatest amounts of [^{3}H]-InsPs in response to ET-1 was the intercartilaginous region of the tracheal wall. The particular cell type(s) within the intercartilaginous region that generated InsPs in response to ET-1 cannot be determined from the current study. However, in view of the recent reports that the tissue between adjacent cartilage rings contains a dense, highly ordered vascular network (McDonald, 1988) and that blood vessels may generate InsPs in response to ET-1 (Marsden et al., 1989; Xuan et al., 1989), it is possible that ET-1-induced accumulation of InsPs in this region includes a vascular component. Confirmation of this may require additional autoradiographic studies (Hwang et al., 1990). Similarly, the presence of blood vessels such as terminal arterioles, which traverse the cartilage, may have contributed to the smaller, but significant amount of [^{3}H]-InsPs generated within the cartilaginous region following stimulation with ET-1. The contribution of the tracheal cartilage ring to ET-1-induced generation of tracheal [^{3}H]-InsPs is likely to be minor in view of the relatively small overall contribution of the cartilaginous region to the observed effect and recent autoradiographic evidence that tracheal cartilage has

only a very sparse population of ET-1 binding sites (Henry et al., 1990). Although airway epithelial cells have been shown to generate [^{3}H]-InsPs when stimulated with an appropriate agonist (McCann et al., 1989; Bainbridge et al., 1989), in the present study mechanical removal of rat tracheal epithelium had no significant effect on the levels of ET-1-induced accumulation of [^{3}H]-InsPs. Furthermore, quantitative autoradiographic studies in airway preparations from a number of animal species including the rat, revealed that the density of specific ET-1 binding sites, presumably necessary for ET-1-induced [^{3}H]-InsP generation, was very low over the epithelium when compared with the numbers present in the tracheal smooth muscle band (Henry et al., 1990).

Exposure of [^{3}H]-inositol-loaded rat tracheal smooth muscle tissue to ET-1 produced a time- and concentration-dependent accumulation of [^{3}H]-InsPs. In addition, ET-1-induced [^{3}H]-InsP accumulation and contraction in rat tracheal airway smooth muscle occurred at similar threshold concentrations. Furthermore, ET-1 and carbachol produced similar maximal increases in both [^{3}H]-InsP accumulation and smooth muscle contraction. In isolation, these results do not necessarily imply a causal relationship between ET-1-induced [^{3}H]-InsP accumulation and contraction. However, recent studies indicate that phosphatidylinositol(4,5)bisphosphate is the major and possibly exclusive phosphoinositide hydrolysed by hormone-sensitive phospholipase C in airway smooth muscle (Takuwa et al., 1986; Chilvers & Nahorski, 1990). Thus, the accumulation of these [^{3}H]-InsPs provides an indirect index of phosphatidylinositol(4,5)biphosphate hydrolysis and may quantitatively reflect the turnover of the intracellular calcium-mobilizer, $Ins(1,4,5)P_3$. These events have been closely linked to contraction (Hashimoto et al., 1985; Twort & van Breemen, 1989) and suggest that ET-1-induced contraction of airway smooth muscle may be mediated via the generation of $Ins(1,4,5)P_3$ and mobilization of intracellular calcium. Our findings are in accord with those of Hay (1990) who found that ET-1-induced contraction of guinea-pig isolated tracheal smooth muscle also involved the stimulation of phosphoinositide turnover and utilization of intracellular calcium. However, mechanisms in addition to phosphoinositide metabolism may be involved because several recent studies have suggested a role for extracellular calcium (Turner et al., 1989; Advenier et al., 1990) and dihydropyridine-sensitive calcium channels (Uchida et al., 1988; Maggi et al., 1989; Advenier et al., 1990) in ET-1-induced contraction of airway smooth muscle.

Although ET-1-induced contraction and [^{3}H]-InsP accumulation in rat tracheal airway smooth muscle occurred at similar threshold concentrations, the concentration-effect curve for contraction lay slightly (2.9 fold) to the left of the curve describing [^{3}H]-InsP accumulation. A close

concentration-effect relationship for these two phenomena in airway smooth muscle has recently been observed for ET-1, histamine and leukotriene D_4 (Weichman et al., 1982, Grandordy & Barnes, 1987; Mong et al., 1988; Hay, 1990). In contrast, the concentration-effect curve describing carbachol-induced [^{3}H]-InsP accumulation in rat tracheal smooth muscle was displaced markedly (25 fold) to the right of the curve describing contractile responses to this agonist. These results agree with the previously reported effects of muscarinic cholinoceptor agonists on [^{3}H]-InsP accumulation in airway smooth muscle (Grandordy & Barnes, 1987; Meurs et al., 1988; van Amsterdam et al., 1989). Such data have been interpreted as indicating a marked capacity for InsP generation and accumulation in response to muscarinic agonists far beyond that which is required to activate contraction. Alternatively, cholinoceptor agonists such as carbachol may have invoked other transduction systems (Katsuyama et al., 1990) not stimulated by ET-1.

Our results with ET-1 are consistent with studies showing that phosphoinositide metabolism plays a primary role in the pharmacomechanical coupling of receptor-mediated airway smooth muscle contraction for a number of other spasmogens including muscarinic cholinoceptor agonists, histamine, 5-hydroxytryptamine (5-HT), bradykinin, tachykinins and leukotrienes (Grandordy et al., 1986, 1988; Grandordy & Barnes, 1987; Lemoine et al., 1988; Chilvers et al., 1989; Mong et al., 1988). In contrast, KCl failed to alter significantly [^{3}H]-InsP accumulation above basal levels in rat tracheal smooth muscle. These data agree with previous reports that the calcium-mobilizing and spasmogenic actions of KCl in airway smooth muscle occur via activation of voltage-dependent calcium channels and influx of extracellular calcium (Kotlikoff, 1988) rather than via generation of InsPs and release of calcium from intracellular stores (Baron et al., 1984; Grandordy et al., 1986). Histamine ($100 \mu M$) produced only a small increase in [^{3}H]-InsP accumulation above basal levels and this is consistent with its weak spasmogenic activity in rat isolated tracheal smooth muscle (Lulich & Paterson, 1980).

In vivo studies of anaesthetized guinea-pigs have demonstrated that the bronchoconstriction observed following intravenous administration of ET-1 is due, at least in part, to the actions of cyclo-oxygenase products such as thromboxane A_2 and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Payne & Whittle, 1988; Lagente et al., 1989). Furthermore, Battistini and coworkers (1990a) have suggested that ET-1-induced contractions in guinea-pig isolated airways are mediated through the release of thromboxane A_2. However, in the present study in rat isolated trachea, neither ET-1-induced accumulation of [^{3}H]-

InsPs, nor smooth muscle contraction were significantly modulated by the cyclo-oxygenase inhibitor, indomethacin. This is in accord with in vitro studies of isolated airway smooth muscle preparations from rat (Henry et al., 1990) and man (Advenier et al., 1990; McKay et al., 1991) showing that indomethacin had no inhibitory influence on ET-1-induced contractions. Similarly, Pons et al. (1991) report that the bronchoconstriction produced following aerosol administration of ET-1 to guinea-pig isolated lungs occurred through an indomethacin-insensitive process. Whereas ET-1-induced effects were indomethacin-insensitive, the combined cyclo-oxygenase/lipoxygenase inhibitor NDGA produced significant inhibition of ET-1-induced contractions without altering ET-1-induced accumulation of InsPs. Although these results suggest that ET-1-induced contraction may be partly mediated via lipoxygenase products such as the leukotrienes, this explanation is unlikely since the leukotriene antagonist SKF 104353 did not affect ET-1-induced contractions and leukotrienes C_4 and D_4 did not contract rat tracheal smooth muscle. At present, the mechanism through which NDGA inhibits ET-1-induced contraction is not clear although this seems to be independent of effects on InsP accumulation.

Although a recent study has indicated that platelet-activating factor may mediate ET-1-induced contraction in guinea-pig airways (Battistini et al., 1990b), the inability of a platelet-activating factor receptor antagonist, WEB 2086, to affect either ET-1-induced contraction or InsP accumulation in the current study argues against this mechanism operating in rat airways. Overall, our studies utilizing selected receptor antagonists and enzyme inhibitors indicate that both the InsP accumulating and tension generating effects of ET-1 observed in rat isolated tracheal smooth muscle appear to be the result of a direct action on smooth muscle rather than due to the actions of secondary spasmogenic mediators such as prostaglandins, leukotrienes, platelet-activating factor, histamine or thromboxane.

In this study we have established that ET-1 induced significant accumulation of [^{3}H]-InsPs in rat tracheal segments. It seems likely that the generation of [^{3}H]-InsPs is due to a direct effect of ET-1 via specific receptors on the tracheal smooth muscle and is responsible, at least in part, for ET-1-induced contraction in this preparation.

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