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Endothelins-induce cyclicAMP formation in the guinea-pig trachea through an ET_A receptor- and cyclooxygenase-dependent mechanism

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1 The non-selective endothelin agonist, endothelin-1 (ET-1), and the selective ET_B receptor agonist, sarafotoxin-S6c (SRTX-c), contracted guinea-pig isolated trachea in a concentration-dependent manner. The EC_{50} value for ET-1 (11 ± 2.1 nM) was significantly higher than that of SRTX-c (3.2 ± 0.21 nM) and the maximal developed tension due to SRTX-c was $42.8 \pm 2.3\%$ higher than that produced by ET-1 ($P < 0.05$).

2 Pretreatment with the ET_A antagonist, BQ-610, appreciably enhanced the developed tension due to ET-1 but not SRTX-c. Likewise, the cyclo-oxygenase inhibitor, indomethacin, markedly potentiated the contractile responses to ET-1, but not to SRTX-c. Combining BQ-610 with indomethacin was not more effective than either of them in augmenting ET-1-evoked tension.

3 ET-1 significantly increased cyclic AMP formation in the trachea in concentration- and time-dependent manners. A $t_{1/2}$ value of 4.3 min, an EC_{50} value of 20 ± 3 nM and a maximal cyclic AMP increment of 124% above the basal level, were obtained for ET-1. Similarly but less effectively, ET-3 (0.1 μM) increased cyclic AMP level ($35 \pm 3.7\%$ compared to $94 \pm 7.8\%$ for the same concentration of ET-1). By contrast, SRTX-c did not alter the cyclicAMP level when applied in concentrations up to 1 μM.

4 Pre-incubation of the trachea with BQ-610 (1 μM) or indomethacin (1 μM) prevented cyclicAMP formation by either ET-1 or ET-3.

5 The results of the present study indicate a negative regulatory role mediated by the ET_A receptor on the ET_B-triggered mechanical response. This effect is likely to be mediated by activation of adenylate cyclase through a cyclo-oxygenase-dependent mechanism.

Keywords: Endothelin receptors; ET_A antagonists; cyclicAMP; cyclo-oxygenase inhibitors; guinea-pig trachea

Introduction

Endothelins (ETs) and sarafotoxins (SRTXs) were originally isolated from vascular endothelial cells (Yanagisawa *et al.*, 1988) and from the venom of the snake *Atractaspis engaddensis* (Kloog *et al.*, 1988). These peptides trigger their actions by activating specific cell-surface receptors that belong to the heptahelical G-protein-coupled superfamily of receptors (Sakurai *et al.*, 1992; Arai *et al.*, 1993). In mammalian tissues, two major ET-receptors have been identified, namely ET_A and ET_B. The ET_A receptors have higher affinity for ET-1 and ET-2 than ET-3 (Arai *et al.*, 1990), whereas ET_B receptors are virtually equisensitive to the three ET isopeptides (Sakurai *et al.*, 1991). Whereas a selective ET_A agonist has been lacking, SRTX-c and IRL-1620 are known as selective ET_B agonists in various tissues and isolated cells (Williams *et al.*, 1991; Takai *et al.*, 1992; EL-Mowafy & Abdel-Latif, 1994).

Several studies revealed the synthesis and release of ET-1 from airway epithelium (Black *et al.*, 1989; Hay *et al.*, 1993; Ninomiya *et al.*, 1995). Moreover, the presence of specific ET binding sites in airway muscles has been documented (Turner *et al.*, 1989; Rozengurt *et al.*, 1990; Hay *et al.*, 1993). ET-1, on a molar basis, is a less potent constrictor in the airways than in vascular tissues. Nevertheless, it is the most powerful airway constrictor ever isolated (Battistini *et al.*, 1992; McKay *et al.*, 1992; Hay *et al.*, 1993). However, relaxant effects to ET-1 have been shown in pre-contracted rabbit trachea (Grunstein *et al.*, 1991) and in guinea-pig trachea following anaphylactic reaction (Saotome *et al.*, 1991). Additionally, ET-1 was shown to activate nitric oxide production, thereby producing a concentration-dependent relaxation of guinea-pig trachea (Filep *et al.*, 1993).

A growing body of evidence has implicated prostaglandins a mediators in many ET-activated responses such as contraction of canine bronchi and cat iris spincter (Granstam *et al.*, 1991; Uchida *et al.*, 1992), IP_3 formation (Cioffi *et al.*, 1992), cutaneous vasodilatation (Di Maria Gu *et al.*, 1993; Granstam *et al.*, 1993) and luteinizing hormone release (Moretto *et al.*, 1993).

In guinea-pig trachea, the coexistence of ET_A and ET_B has been reported (Ninomiya *et al.*, 1992, Battistini *et al.*, 1994). There is a general agreement that ET-induced contraction in guinea-pig trachea is predominantly mediated by ET_B receptors. However, the exact physiological role of the ET_A receptor in this tissue is not fully defined. Thus, Hay (1992) demonstrated that BQ-123, an ET_A receptor antagonist, attenuated the contractile response to ET-1, suggesting a contribution of ET_A receptors. On the other hand, Battistini *et al.* (1994) showed BQ-123 to potentiate ET-1-induced tension in guinea-pig trachea. Furthermore, other investigators reported no effect for the ET_A receptor antagonists, FR 139317 on tension responses to ET-1 in the trachea and bronchus of the guinea pig (Cardell *et al.*, 1993).

The present study was undertaken to investigate further the role of ET_A receptors in ET-mediated mechanical responses in guinea-pig trachea. Moreover, the possible signal transduction mechanisms underlying these responses were also sought.

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Methods

ET homologue-induced mechanical responses in guinea-pig trachea

Male guinea-pigs (400–500 g) were killed by cervical dislocation. The trachea was removed, placed in ice-cold Krebs-Henseleit buffer (in mM: NaCl 117.6, KCl 5.36, NaH_2PO_4 1.01, $MgSO_4$ 0.69, $CaCl_2$ 2.32, $NaHCO_3$ 25 and glucose 11.1). The trachea was cleaned of surrounding loose connective tissue and cut into rings, 4 to 5 mm wide. The rings were cut open at the cartilaginous side leaving the smooth muscle section in the middle of the resulting tracheal strips. Tracheal strips were mounted in tissue baths containing 10 ml of Krebs-Henseleit solution. The baths were maintained at 37°C and aerated continuously with a 95% O_2 and 5% CO_2 gas mixture. Tension was measured isometrically using a F-03 Grass force displacement transducer (Grass Instruments, Inc., U.S.A.) and recorded on a Grass polygraph.

Before starting experiments, the strips were equilibrated for 50 to 60 min. During this time the bathing solution was removed and replaced with a fresh solution every 8 to 10 min for a total of 6 times, as the strips were stretched to a resting tension of 2 g (the optimal tension for isometric contraction was determined by preliminary experiments). Routinely, tracheal segments were validated by using carbachol (5 μM) and concentration-effect curves to ET-1 or SRTX-c were constructed in a cumulative manner. The time interval between successive additions of the agonists was adjusted to allow the response to be fully developed. Responses were expressed as a percentage of the respective carbachol-induced contraction. In experiments where indomethacin (1 μM) and/or BQ-610 (1 μM) were used, they were allowed to react for 15 min before the addition of ET agonist.

ET homologue-induced cyclicAMP formation in guinea-pig trachea

To equilibrate the tissue, tracheal segments of approximately equal length were incubated for 60 min at 37°C in 1 ml of Krebs-Henseleit solution. At this time, the medium was replaced by fresh one (1 ml) that contained 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) and incubation was continued for 15 min before the addition of an ET homologue. Whenever used, indomethacin (1 μM) or BQ-610 (0.1 μM and 1 μM) were added 15 min prior to exposure to the agonist. Reactions were terminated by the addition of 1 ml 10% (w/v) trichloroacetic acid (TCA). The tissues were homogenized and centrifuged at 3000 r.p.m. for 10 min at 4°C. The excess TCA, in the supernatant, was removed by diethyl ether extraction. The water soluble tissue extract was approximately diluted and its content of cyclicAMP was succinylated and assayed by radioimmunoassay (RIA) as described by Frandsen & Krishna (1977). The pellets were solubilized in 1 N NaOH overnight and its protein content was determined by the method of Lowry *et al.* (1951).

Drugs and materials

ET-1, SRTX-c and BQ-610 (N,N-hexamethylene) carbamoyl-Leu-D-Trp(CHO)-D-Trp-OH, were purchased from Peptides International (Louisville, KY, U.S.A.). Indomethacin was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Succinyl [methyl ester-^{125}I]-cyclicAMP tyrosine (2200 Ci mmol^{-1}) was produced by New England Nuclear-Dupont (Boston, MA, U.S.A.). Antibody to cyclicAMP was obtained from ICN ImmunoBiologicals (Lisle, IL, U.S.A.). All other chemicals were of reagent grade.

Data analysis

Data are presented as mean ± s.e.mean of the indicated number of experiments (n) and the difference between means was assessed by Analysis of Variance (ANOVA) followed by the Student-Newman-Keuls *post hoc* test. A P value less than 0.05 was considered to be statistically significant.

Results

ET homologue-induced mechanical responses in guinea-pig trachea

Both ET-1 and SRTX-c contracted guinea-pig isolated trachea in a concentration-dependent manner. As shown in Figure 1, the increase in tension elicited by SRTX-c was significantly higher than that produced by ET-1 and this was more prominent at higher doses of the peptides (10–100 nM). The EC_{50} values were 11 ± 2.1 and 3.2 ± 0.21 nM for ET-1 and SRTX-c, respectively.

Pre-incubation of the trachea with the ET_A antagonist, BQ-610, significantly enhanced the mechanical response to ET-1 but not SRTX-c (Figure 1a and b). A shift to the left of ET-1 concentration-response curve with a corresponding decrease in EC_{50} value were observed after BQ-610 pretreatment. The potentiation by the ET_A antagonist was best seen at higher concentrations of ET-1.

To investigate whether or not cyclo-oxygenase metabolites

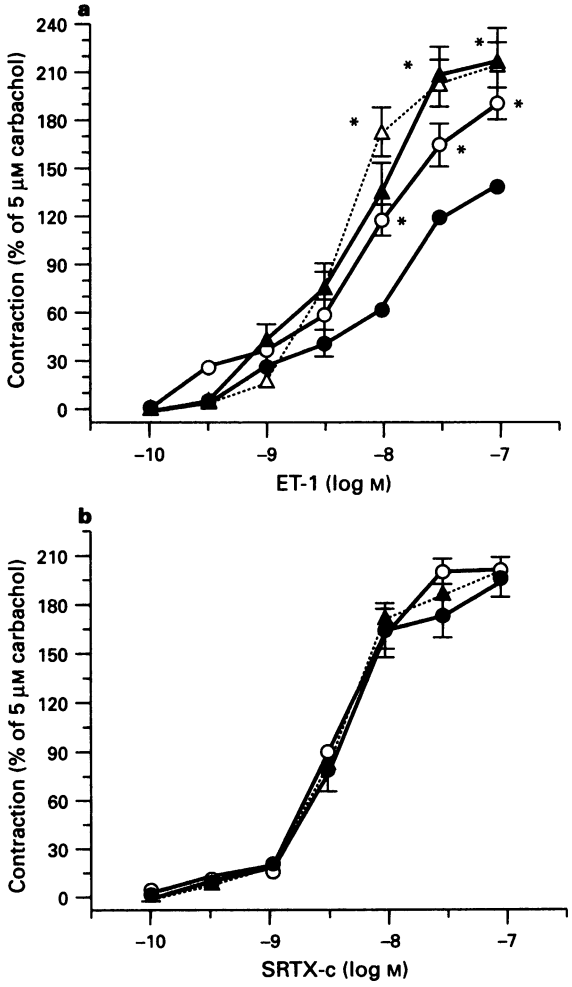

Figure 1 Concentration-contraction curves to ET-1 (a) in guinea-pig isolated trachea in the absence (●) and the presence of 1 μM BQ-610 (○), 0.1 μM indomethacin (▲) and combined BQ-610 and indomethacin (△); and contractions for SRTX-c (b) in the absence (●) and the presence of 1 μM BQ-610 (○), 0.1 μM indomethacin (▲). Data are expressed as means ± s.e.mean of 5–7 experiments. *Significantly different from ET-1.

mediate ET actions in guinea-pig trachea, concentration-response curves for ET-1 and SRTX-c were constructed in the presence of indomethacin. Whereas indomethacin was without effect on SRTX-c-induced increase in muscle tension, it significantly potentiated the contractile responses to ET-1 (Figure 1a and b). Indomethacin shifted the concentration-contraction curve of ET-1 to the left with a concomitant reduction in its EC_{50} (Figure 1a). Co-treatment with BQ-610 and indomethacin failed to effect further potentiation of the ET-1 response compared to that elicited by either of these agents (Figure 1a).

ET homologue-induced cyclicAMP formation in guinea-pig trachea

Figure 2a and b demonstrates the ability of ET-1 to activate cyclicAMP formation in a time- and concentration-dependent manner. Significant enhancement of cyclicAMP generation started 2.5 min after the addition of ET-1 and reached its maximum after 10 min. A $t_{1/2}$ value of 4.3 ± 0.23 min and an EC_{50} value of 20 ± 3 nM were obtained. A maximal cyclicAMP production of 124% above the basal level was attained with 1 μM ET-1. To explore the ET receptor subtype linked to cyclicAMP production, the differential ability of three ET-homologues (0.1 μM) to generate cyclicAMP, and the effect of the ET_A receptor antagonist, BQ-610, thereon were studied. The rank order of potency for cyclicAMP production was ET-1 > ET-3, while SRTX-c, up to 1 μM, had no effect on cyclicAMP generation (Figure 3).

As shown in Table 1, pre-incubation of the tissue with BQ-610 attenuated ET-1-induced cyclicAMP formation in a concentration-related fashion. Thus, 0.1 μM BQ-610 reduced the cyclicAMP increment to 22% of its original level while 1 μM of the antagonist completely abolished the responsiveness to ET-1. The contribution by cyclo-oxygenase products to ET-evoked cyclicAMP production was assessed. Thus, pretreatment with indomethacin substantially inhibited the increase in cyclicAMP triggered by ET-1. Likewise, the effect of ET-3 on cyclicAMP formation was abolished by pre-treatment with either BQ-610 or indomethacin (data not shown).

Discussion

In their original paper, Yanagisawa and colleagues (1988) introduced ET-1 as a powerful vasopressor peptide. Subsequently, the ability of ET-1 to contract non vascular smooth muscles was evident in intact animals and in isolated tissues (Takua, 1993). Airway smooth muscles (AWSM) and their epithelium were shown to synthesize, release and bind ET isopeptides, suggesting an autocrine and/or paracrine regulatory role for ETs in the airways (Hay *et al.*, 1993; Ninomiya *et al.*, 1995). In eliciting their biological responses in AWSM, ETs may activate a variety of signal cascades that include phospholipase C (PLC), PLD, PLA_2, protein kinase C, tyrosine kinase and ion channels (reviewed by Rubanyi & Polokof, 1994). However, in some preparations, the peptide induced vasodilatation as well as smooth muscle relaxation, a finding which suggested the coupling of ET-receptors to cyclicAMP and/or cyclicGMP formation (Oda *et al.*, 1992; Eguchi *et al.*, 1993; Filep *et al.*, 1993; EL-Mowafy & Abdel-Latif, 1994).

Both radioligand and pharmacological studies provided compelling evidence that the guinea-pig trachea is endowed

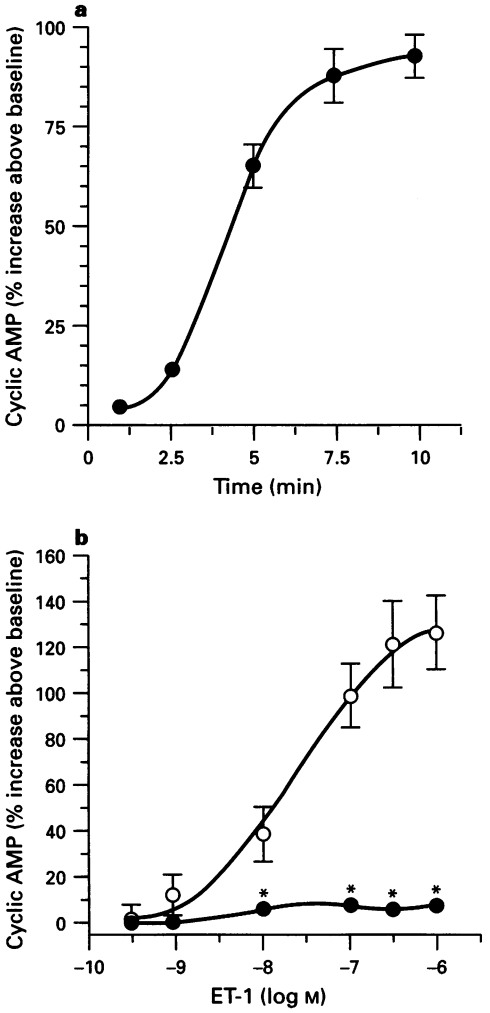

Figure 2 (a) Time course for ET-1 (0.1 μM)-induced cyclicAMP formation in guinea-pig trachea; (b) effect of different concentrations of ET-1 on cyclicAMP formation in the absence (○) and presence (●) of indomethacin (1 μM). Data are expressed as means ± s.e.mean of (6–8) determinations. *Significantly different from ET-1 value.

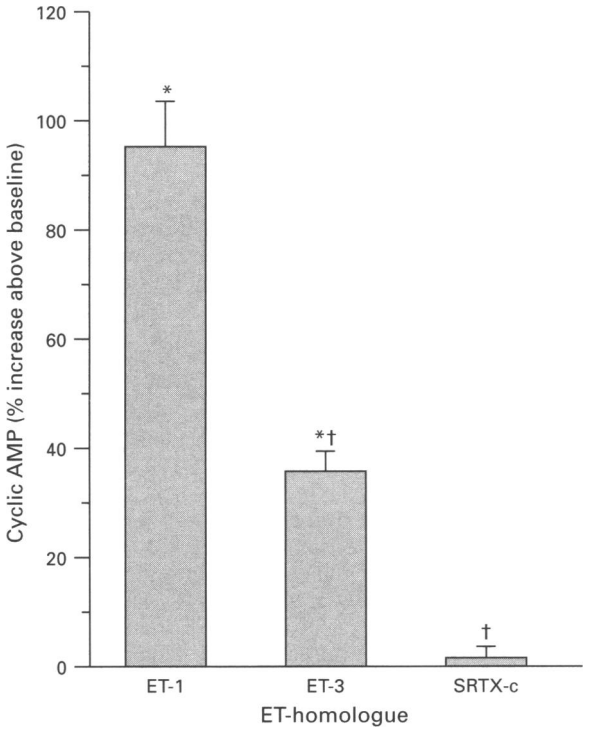

Figure 3 Effect of endothelin homologues (0.1 μM) on cyclicAMP formation in guinea-pig trachea. Data are expressed as means ± s.e.mean of (6–8) determinations. *Significantly different from baseline value; †significantly different from ET-1.

Table 1 Effect of BQ-610 on ET-1-induced cyclic AMP formation in guinea-pig trachea

Treatment	*Cyclic AMP* (pmol mg^{-1} protein)
Basal	45.8±2
BQ-610:	
0.1 μM	46.3±4
1.0 μM	47.6±7
ET-1 (0.1 μM):	
No antagonist	93.3±6*
BQ-610 (0.1 μM)	58±4*†
BQ-610 (1 μM)	44.9±5†

The equilibrated tissues were pre-incubated with or without antagonist for 15 min prior to exposure to ET-1 for 10 min. Data are means±s.e.mean of 6–8 determinations. *Significantly different from basal level; †Significantly different from ET-1.

with both ET_A and ET_B receptors (Tschirhart *et al.*, 1991; Ninomiya *et al.*, 1992; Battistini *et al.*, 1994). ET_A receptors constitute 15% of total ET-receptor population (Ihara *et al.*, 1992). The role of ET_A receptor in this muscle is poorly understood. A controversy revolved around whether this receptor is functionally linked to muscle contraction (Hay *et al.*, 1993) or is associated with attenuation of the contractile response triggered by the ET_B receptor (Battistini *et al.*, 1994).

The results of this study suggest that ET_A receptors are functionally linked to adenylate cyclase activation via a cyclo-oxygenase-dependent pathway and that these receptors function to regulate negatively the contractile responses induced by ETs. Pharmacological and biochemical roles for ET_A and ET_B receptors were assigned based on differential agonist activation, the use of indomethacin and the selective ET_A receptor antagonist, BQ-610.

Both ET-1 and SRTX-c induced a concentration-dependent contractile response in the guinea-pig trachea. This is consonant with previous studies which showed that contractile responses in guinea-pig trachea are mediated mainly by the ET_B receptor (Cardell *et al.*, 1992; Battistini *et al.*, 1994). However, the response to SRTX-c was significantly higher than that to ET-1 as inferred from both maximal responses obtained by the ETs and their EC_{50} values. Knowing that ET_B receptors are equisensitive to different ET homologues (Sakurai *et al.*, 1991), the lower activity of ET-1 compared to SRTX-c in guinea-pig trachea could be ascribed to ET_A receptor-dependent mechanisms which function to oppose tension development via the ET_B receptor. A similar situation has been reported in the iris sphincter muscle where ET_B receptor generates cyclicAMP that functions to attenuate IP_3 formation and the contractile responses elicited by ET_A receptors (EL-Mowafy & Abdel-Latif, 1994).

To investigate the possible mechanism(s) underlying the observed differences in the actions of ET-1 and SRTX-c, we examined the capacity of ET homologues to activate cyclicAMP formation in guinea-pig trachea. Exposure to ET-1 induced a time- and concentration-dependent cyclicAMP accumulation. ET-3 was less potent than ET-1 in eliciting such responses whereas SRTX-c had no effect on the cyclic nucleotide formation. Such order of potency among ET homologues further implicates the ET_A receptor in transducing cyclicAMP formation in guinea-pig trachea. This finding was confirmed by the use of BQ-610. BQ-610 (1 μM) fully inhibited cyclicAMP formation by ET-1 and ET-3 and significantly potentiated contractile response to ET-1. Similar potentiation of ET-1-induced mechanical responses by the ET_A antagonist, BQ-123, was described by Peachey & Kitchen (1991).

Since the discovery of ET-1, it has been believed that ET_A receptors transduce excitatory (contractile) responses of ETs whereas ET_B are involved in the inhibitory (relaxant) responses of the peptides (reviewed by Davenport & Maguire 1994). Evidently, both receptors were shown to be actively engaged in transducing IP_3 accumulation in rat lung (Cioffi *et al.*, 1992) and in contracting rat trachea (Henry, 1993). In this study, however, it appears that the ET_A receptor is coupled to cyclicAMP formation whereas ET_B triggers muscle contraction. Accordingly, ET receptors seem to possess such a scattered coupling that renders their linkage to a definitive biological response unpredictable.

In smooth muscles, cyclicAMP may function as a relaxant and/or to attenuate muscle contraction, depending on the amount of the cyclic nucleotide generated (Abdel-Latif, 1991; Xuan *et al.*, 1991). Thus, in the guinea-pig trachea, Turner *et al.* (1989) and Battistini *et al.* (1994) reported that contraction to ET-1 was preceded by a transient relaxation. By contrast, such a relaxation was not reported in studies by Cardell *et al.* (1992; Ninomiya *et al.* (1992) and Cardell *et al.* (1993). In the current study, we were unable to detect direct relaxant responses to ETs nor were we able to relax tracheal segments precontracted by carbachol. The lack of such a response to ETs may be related to the relatively small increment found in cyclicAMP production via the ET_A receptor. In guinea-pig airways, ET_A receptors constitute about 15% of total receptor density in the trachea whereas ET_B receptors, which induce phosphatidyl inositol turnover and muscle contraction (Hay, 1989) represent 85% (Ihara *et al.*, 1992). The well-documented reciprocal interaction (cross-talk) between IP_3 and cyclicAMP signals in smooth muscles may additionally explain the magnitude of the cyclicAMP increment and consequently the inability of ET-1 to provoke a prominent relaxation in this smooth muscle. Alternatively, this cyclicAMP signal seems to attenuate the ET_B-evoked contractions.

The effects of the cyclo-oxygenase inhibitor, indomethacin, on ET-1-induced contraction in guinea-pig trachea have been studied previously. Hay *et al.* (1993) reported a potentiation of ET-1-mediated tension by indomethacin. By contrast, other investigators reported an attenuation of ET-1-induced contractile responses by indomethacin (Ninomiya *et al.*, 1992). Although it is obvious that prostaglandins modulate the responsiveness of guinea-pig trachea to ET-1, their definite role and linkage to a particular ET receptor subtype(s) have been unclear. In the present study, indomethacin shifted the concentration-response curve of ET-1, but not of SRTX-c, to the left and completely prevented the rise in cyclicAMP due to ET-1 and ET-3. These observations imply a negative regulatory role for cyclo-oxygenase products on ET-1-induced muscle tension, a response that is likely to be mediated by ET_A receptors. The full inhibition of cyclicAMP response due to ET-1 or ET-3 by the ET_A antagonist, BQ-610, along with the inability of SRTX-c to induce any rise in cyclicAMP level, excludes a possible role for ET_B in enhancing cyclicAMP formation. On the other hand, the abrogation by indomethacin (1 μM) of ET-induced cyclicAMP formation rules out a contribution by lipoxygenase products in such a response. These findings are substantiated by the reported ability of ET-1 to release prostaglandin E_2 (PGE_2) and prostaglandin D_2 in guinea-pig trachea (Hay *et al.*, 1993) and by the fact that lipoxygenase metabolites are more apt to constrict the airways rather than to induce cyclicAMP formation (Saotome *et al.*, 1991; Dahl'en *et al.*, 1993; Henry, 1994).

In the guinea-pig trachea, removal of the epithelium augments ET-evoked contractions (Ninomiya *et al.*, 1992; Battistini *et al.*, 1994). The relaxant prostanoid, PGE_2 is the predominant cyclo-oxygenase product released by the guinea-pig and feline tracheal epithelial cells, in the absence and presence of ET-1 (Goodwin, 1989; Wu *et al.*, 1993). Besides, ET_A receptors were functionally shown to exist in the guinea-pig epithelium (Battistini *et al.*, 1994). On the other hand, the smooth muscle of guinea-pig trachea was shown to release predominantly PGE_2 and PGD_2 from a non-epithelium source following ET-1 treatment (Hay *et al.*, 1993); and ET_A receptors seem to coexist with those of ET_B in guinea-pig tracheal smooth muscle cells (Inui *et al.*, 1994). Accordingly, the ET_A receptors and PGs that generate cyclicAMP in the present

study could be located in the epithelium and/or the smooth muscle layer. The relative contribution by these receptors in the observed adenylyl cyclase activation and in regulation of the contractile response to ET_B receptors remains to be determined. Our primary concern was to study the ET receptor linkage, the net pharmacological effect and the molecular target of the released prostanoids.

Accumulating evidence revealed species-related differences in the influence of cyclo-oxygenase products on ET-mediated contraction of airway smooth muscle. In bovine bronchi, indomethacin inhibited ET-3-mediated mechanical responses (Nally *et al.*, 1994), whereas it was without effect on ET-1-stimulated responses in human bronchi (Advenier *et al.*, 1990; McKay *et al.*, 1991) and in rat trachea (Peachey & Kitchen, 1991). On the other hand, a potentiation of the ET-1 contractile response was observed in guinea-pig trachea (Peachey & Kitchen, 1991). It seems likely that these variations are associated with the ability of ETs to release different prostaglandins in various airway smooth muscles. Nonetheless, results from this study and others present prostaglandins as potent 'second messenger' modulators of ET signals and biological responses.

The increase in cyclicAMP in response to ET peptides seems not to be peculiar to the guinea-pig trachea. In the rat trachea, ETs induce cyclicAMP formation via ET_A receptors, whereas in the rabbit trachea the rise in cyclicAMP is coupled to ET_B receptors. Both responses, however, were cyclo-oxygenase-dependent (EL-Mowafy & Abou-Mohamed, unpublished observations). In line with this, ETs evoked cyclicAMP formation in cultured embryonic bovine tracheal cells (Oda *et al.*, 1992) and in the iris sphincter smooth muscle of at least 7 different species (Abdel-Latif & Zhang, 1991; EL-Mowafy & Abdel-Latif, 1994). Thus, the slowly-developing tension responses that have been reported exclusively for endothelins in some vascular and non vascular smooth muscles (Masaki & Yanagisawa, 1992; Takua, 1993) can be accounted for, at least in part, by a possible concomitant production of cyclicAMP that tends to counteract the contractile responses to ETs.

In conclusion, the present study demonstrated a positive coupling of the ET_A receptor to adenylate cyclase in guinea-pig trachea through a cyclo-oxygenase-dependent mechanism. The enhanced cyclicAMP production is likely to counteract muscle contraction triggered by the ET_B receptor.

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