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Prostaglandin E_2 increases cyclic AMP and inhibits endothelin-1 production/secretion by guinea-pig tracheal epithelial cells through EP_4 receptors

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1 Prostaglandin E_2 (PGE_2) increased adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation in tracheal epithelial cells and concomitantly decreased the production/secretion of immunoreactive endothelin (irET).

2 Naturally occurring prostanoids and selective and non-selective EP receptor agonists showed the following rank order of potency in stimulating cyclic AMP generation by epithelial cells: PGE_2 (EP-selective) > 16,16-dimethyl PGE_2 (EP-selective) > 11-deoxy PGE_2 (EP-selective) > > > iloprost (IP/EP_1/EP_3-selective), butaprost (EP_2-selective), PGD_2 (DP-selective), $PGF_{2\alpha}$ (FP-selective). The lack of responsiveness of the latter prostanoids indicated that the prostanoid receptor present in these cells is not of the DP, FP, IP, EP_1, EP_2 or EP_3 subtype.

3 Pre-incubating the cells with the selective TP/EP_4-receptor antagonists AH23848B and AH22921X antagonized the PGE_2-evoked cyclic AMP generation. This suggested that EP_4 receptors mediate PGE_2 effects. However, in addition to any antagonistic effects at EP_4-receptors, both compounds, to a different extent, modified cyclic AMP metabolism. The selective EP_1, DP and EP_2 receptor antagonist (AH6809) failed to inhibit PGE_2-evoked cyclic AMP generation which confirmed that the EP_2 receptor subtype did not contribute to the change in cyclic AMP formation in these cells.

4 The PGE_2-induced inhibition of irET production by guinea-pig tracheal epithelial cells was due to cyclic AMP generation and activation of the cyclic AMP-dependent protein kinase since this effect was reverted by the cyclic AMP antagonist Rp-cAMPS.

5 These results provide the first evidence supporting the existence of a functional prostaglandin E_2 receptor that shares the pharmacological features of the EP_4-receptor subtype in guinea-pig tracheal epithelial cells. These receptors modulate cyclic AMP formation as well as ET-1 production/secretion in these cells.

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Abbreviations: Cyclic AMP, adenosine 3′:5′ cyclic monophosphate; ET-1, endothelin-1; G protein, guanine nucleotide-binding regulatory protein; irET, immunoreactive endothelin; Rp-cAMPS: (Rp)-adenosine cyclic 3′,5′-phosphorothioate

Introduction

Airway epithelial cells have been shown to produce endothelin-1 (ET-1) (Laporte *et al*., 1996; Pelletier *et al*., 1998; Yang *et al*., 1997), which has several effects on the airways such as broncho- and tracheoconstriction (Boichot *et al*., 1991; Inui *et al*., 1994; Uchida *et al*., 1988) and is also an important mediator of pulmonary hypertension (Horgan *et al*., 1991). In addition to its constrictor effect, ET-1 stimulates epithelial cell growth and the synthesis of various autacoids including prostaglandins (Ninomiya *et al*., 1992; Murlas *et al*., 1995; Takimoto *et al*., 1996). On the other hand, ET-1 synthesis and release are stimulated in response to pro-inflammatory, mitogenic and hypertrophic as well as constrictor mediators (Endo *et al*., 1992; Yang *et al*., 1997b; Samransamruajkit *et al*., 2000). By contrast, basal and stimulated ET-1 production has been shown to be inhibited by vaso- and bronchorelaxing hormones or autacoids, namely adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-elevating agents, such as adenosine, β_2-adrenoceptor agonists and forskolin, and the cyclic AMP analogue 8-Br-cyclic AMP (Yang *et al*., 1997a).

Prostaglandin E_2 is a potent modulator of the immune system and a major mediator of inflammation (Goodwin & Ceuppens, 1983). PGE_2 was shown to relax bronchial smooth muscle and to reduce mucus secretion (Goodwin & Ceuppens, 1983; Marom *et al*., 1981; Sweatman & Collier, 1968; Walters & Davies, 1982). It was suggested that PGE_2 could produce its broncho- and tracheoprotective effect through at least two distinct pathways. First, through relaxation of bronchi or trachea by acting directly on smooth muscle cells (Walters & Davies, 1982) and, secondly, through inhibition of bronchoconstrictor mediator

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release and/or synthesis by mast cells and other cell populations of the airway (Thien & Walters, 1995). As a consequence of PGE_2 stimulation, human airway epithelial cells increase their content of intracellular cyclic AMP (Penn *et al*., 1994). This observation, combined with the previous one indicating that cyclic AMP elevating agents inhibit ET-1 production by tracheal epithelial cells, suggested that PGE_2 could regulate the production/secretion of ET-1 through activation of a G_s-coupled receptor and then contribute to a negative feedback regulation loop on ET-1 synthesis.

PGE_2 binds to and activates multiple receptors, namely EP_1, EP_2, EP_3 and EP_4 (Coleman *et al*., 1994a,b. These receptors are members of a large family of receptors and have been classified on the basis of their sensitivity to the five naturally occurring prostanoids. These are TP for thromboxane A_2 (TxA_2), DP for PGD_2, EP for PGE_2, FP for $PGF_{2\alpha}$ and IP for PGI_2 or prostacyclin (Kennedy *et al*., 1982). The development of selective agonists and antagonists led to the identification of the four isoforms of EP receptors. The EP_1-receptor subtype was shown to be a seven transmembrane domain receptor coupled to phosphoinositide turnover and intracellular Ca^{2+} mobilization through a regulatory G_q protein (Coleman & Kennedy, 1985; Creese & Denborough, 1981). The EP_2- and EP_4-subtypes are also seven transmembrane domain receptors coupled to a regulatory G_s protein and activate adenylyl cyclase which increases cyclic AMP formation (De Vries *et al*., 1995; Honda *et al*., 1993; Jumblatt & Paterson, 1991; Nishigaki *et al*., 1995; de Brum-Fernandes *et al*., 1996). The seven transmembrane domain EP_3-receptor subtype is expressed as multiple isoforms as a result of alternative splicings (Breyer *et al*., 1994; Irie *et al*., 1993; Jon *et al*., 1997; Negishi *et al*., 1993). These isoforms, which expressed the same rank order of potency and affinity for the different agonists, were also shown to couple different heterotrimeric G proteins leading to stimulation of various second messenger pathways (Namba *et al*., 1993).

EP_1 receptors were characterized mainly by the use of selective agonists because of the lack of selectivity of receptor antagonists (Coleman *et al*., 1994a). Similarly, there are numerous EP_2-selective agonists, such as butaprost, but no highly selective antagonists are yet available (Gardiner, 1986; Nials *et al*., 1993). The same applies for the EP_3-receptor subtype (Coleman *et al*., 1994). As far as the EP_4-receptor subtype is concerned, there are no selective agonists available but there are selective antagonists such as AH23848B and AH22921X, which allow discrimination between EP receptor subtypes (Coleman *et al*., 1994a,b;). EP-receptor subtypes can be distinguished on the basis of PGE_2 action on second messenger pathways (cyclic AMP formation, phosphoinositide turnover and calcium) combined to the rank order of agonist and antagonist potencies. Since the action of PGE_2 on prostanoid receptors could be mediated by the activation of any of the prostanoid receptor subtypes, the pharmacological characteristics of the other prostanoid receptor subtypes have to be taken into account. IP and DP receptors were shown to be coupled to a regulatory G_s protein thus activating adenylyl cyclase and increasing cyclic AMP formation (Hashimoto *et al*., 1990; Ito *et al*., 1990, 1992; Siegl *et al*., 1979; Simon *et al*., 1980). As for the FP and TP receptors, their signalling pathways involve a regulatory G_q protein (Knezevic *et al*., 1993; Shenker *et al*., 1991) and the activation of phospholipase C (Brass *et al*., 1987).

In the present study, the effects of PGE_2 on the cyclic AMP generation and on the production/secretion of irET by tracheal epithelial cells were evaluated. A pharmacological characterization of the prostanoid receptor subtype responsible for the PGE_2 action was also conducted using selective and non-selective receptor agonists and antagonists on the generation of cyclic AMP.

Methods

Animals

Male Dunkin Hartley guinea-pigs (300–350 g) were obtained from Charles River Laboratory (St Constant, Québec, Canada). The animals were killed by cervical dislocation according to the guidelines of the Canadian Council on Animal Care. The trachea was harvested under sterile conditions and dissected in Krebs–Henseleit physiological solution.

Isolation and cell culture

Tracheal epithelial cells were obtained following a 1 h incubation of the trachea at 37°C with a solution of 0.15% protease type XXIV in Krebs–Henseleit buffer, according to a previously described procedure (White *et al*., 1993). The cells were then mechanically removed from the mucosal surface of the trachea by gentle scraping with a rubber policeman. They were centrifuged and washed twice with 5 ml of culture medium, DMEM-F12, containing 10% ($v\,v^{-1}$) foetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (0.08% $w\,v^{-1}$) and fungizone (1% $w\,v^{-1}$). The cells were resuspended in 10 ml medium, counted and seeded at a concentration of $4-5\times10^5$ cells/ml/well in 24-well culture plates. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Cells were cultured for 72 h in the presence and thereafter 48 h in absence of medium before experiments were conducted. All experiments used confluent cells.

ET-1 production/secretion

Before the experiments, the DMEM-F12 medium was aspirated from the 24-well plates and 1ml of fresh medium was added. The cells were incubated with selected pharmacological agents at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. At the end of the incubation period, the culture media were collected and stored at −20°C until measurement of immunoreactive-ETs (irETs). Triton X-100 (0.1% $v\,v^{-1}$; 0.5 ml) was added to each well and, following an overnight incubation at 4°C, total proteins were assayed using the Bio-Rad protein assay.

Measurement of immunoreactive-ET by radioimmunoassay

The concentrations of irET were measured by radioimmunoassay (RIA) using a commercially available kit (RPA 555; Amersham, Ontario, Canada), as previously described

(Laporte *et al*., 1996). The amount of each aliquot was expressed in picograms per milligram of total proteins.

Cyclic AMP formation

Intracellular cyclic AMP levels were determined by measuring the conversion of [^{3}H]-ATP into [^{3}H]-cyclic AMP as described by Salomon (1979). Briefly, after 5 days of culture in 24-well plates, cells were washed and incubated for 2 h in DMEM-F12 medium with 2 μCi [^{3}H]-adenine/ml. The cells were washed twice with HBSS-glucose and pre-incubated for 15 min in a solution of HBSS-BSA containing 10 μM rolipram (a selective type 4 phosphodiesterase inhibitor) in the presence or absence of selected antagonists and inhibitors. PGE_2 and congeners were then added to the incubation medium for various periods of time at 37°C. The reaction was ended by the addition of 100 μl of ice-cold perchloric acid (50% v v^{-1}, final concentration of 5%) and by putting the 24-well plates on ice. Cells were scraped with a rubber policeman and 100 μl of ice-cold solution of ATP and cyclic AMP (5 mM of each) was added to the cellular extract. The samples were vortexed and centrifuged for 15 min at 3000 r.p.m. and 4°C. The supernatants were sequentially chromatographed on Dowex and alumina columns allowing the separation of [^{3}H]-ATP from [^{3}H]-cyclic AMP. Cyclic AMP formation was expressed as per cent conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP and calculated using the following formula $([^3H]\text{-cAMP}/([^3H]\text{-ATP} + [^3H]\text{-cAMP})) \times 100$. Results are expressed as per cent response to 10 μM PGE_2, unless stated otherwise.

Protein assay

Guinea-pig tracheal epithelial cells in each well were disrupted overnight with 0.1% Triton X-100. A 10 μl aliquot was mixed with 200 μl Bio-Rad protein assay reagent and incubated for 15 min at room temperature to evaluate the concentration of cell proteins. The concentration of proteins was determined by measuring the absorbance at 590 nm, using a standard curve of bovine serum albumin (25–400 μg ml^{-1}).

Chemicals and drugs

The following chemicals and drugs were used: culture medium, serum and antibiotics (penicillin, streptomycin and fungizone) (Gibco, New York, U.S.A.); protease type XXIV, ATP, cyclic AMP, bovine serum albumin, imidazol, adenosine and alumina (Sigma Chemical Co., St Louis, MO, U.S.A.); the Bio-Rad protein assay kit, Dowex 1X8 (100–200 mesh) (Bio-Rad, Mississauga, Canada); [^{3}H]-adenine, endothelin radioimmunoassay kits (RPA 555) (Amersham, Oakville, Ontario, Canada); butaprost (Dr Harold Kluender, Bayer Corporation, West Haven, CT, U.S.A); AH22921X ([1α(Z),2β,5α]-(±)-7-[5-[[(1,1′-biphenyl)-4-yl] methoxy] -2- (4 -morpholinyl) -3- oxocyclopentyl] -5-heptenoic acid), AH6809 (6-isopropoxy-9-oxoxanthine-2-carboxylic acid) and AH23848B ([1α(Z),2β,5α]-(±)-7-[5-[[(1,1′-biphenyl)-4-yl] methoxy]-2- (4-morpholinyl) -3-oxocyclopentyl] 4-heptenoic acid) (Dr R.A. Coleman, Glaxo R&D Ltd, Hertfordshire, U.K.); PGE_2, D_2, $F_{2\alpha}$ and iloprost (Cayman Chemicals, Ann Arbor, MI, U.S.A.); rolipram (RBI, Research Biochemicals International, Natik, CA, U.S.A.); Rp-cAMPS: (Rp)-adenosine cyclic 3′,5′-phosphorothioate (Calbiochem, San Diego, CA, U.S.A.)

Statistical analysis

The results are expressed as the mean ± s.e.mean. The degree of significance of differences between experimental groups was performed by ANOVA followed by Dunnett or Bonferroni post-test analysis using Graph Pad Software version 2.01. P values less than 5% were considered significant.

Results

Effect of prostaglandin E_2 on cyclic AMP formation by tracheal epithelial cells

PGE_2 (1 μM) significantly stimulated (4 fold) the conversion of ATP to cyclic AMP by cultured guinea-pig tracheal epithelial cells during a 5 min incubation period compared to the basal formation. Longer incubation times such as 15, 30 and 60 min in the presence of PGE_2 (1 μM) led to 4.3, 5.7 and 11.4 fold increment in conversion of ATP to cyclic AMP (Figure 1A). Stimulation of the cells with increasing concentrations of PGE_2 (0.01 to 100 μM) produced a concentration-dependent increase in the conversion of ATP to cyclic AMP and the maximal effect was observed using the concentration of 100 μM, where the values reached 5.03 ± 0.90% (Figure 1B). Subsequent experiments using selected agonists and antagonists were performed at 15 min and expressed as the per cent of the response obtained by the stimulation with 10 μM PGE_2 (100%) to minimize the variability between experiments.

Effect of naturally occurring prostaglandins and iloprost on cyclic AMP formation

In contrast to the results obtained with PGE_2, iloprost, PGD_2 and $PGF_{2\alpha}$ (0.1–10 μM) did not stimulate any conversion of ATP to cyclic AMP by guinea-pig tracheal epithelial cells (Figure 2).

Effect of selective and non-selective prostanoid-receptor agonists on cyclic AMP generation

To identify the EP receptor subtype that mediates cyclic AMP generation in tracheal epithelial cells, four PGE_2 analogues with different affinities for the various EP receptors subtype were tested. PGE_2 was the most potent agonist for increasing cyclic AMP in the cells, followed by the non-selective EP receptor agonists 16,16-dimethyl PGE_2 and 11-deoxy PGE_2, whereas the selective EP_2 receptor agonist, butaprost, failed to show a stimulatory effect (Figure 3).

Effect of selective EP_4-receptor antagonists on cyclic AMP generation

The preincubation of tracheal epithelial cells with a selective EP_4-receptor subtype antagonist, AH23848B (10 and 100 μM) (Coleman *et al*., 1994a,b), shifted the concentration–response

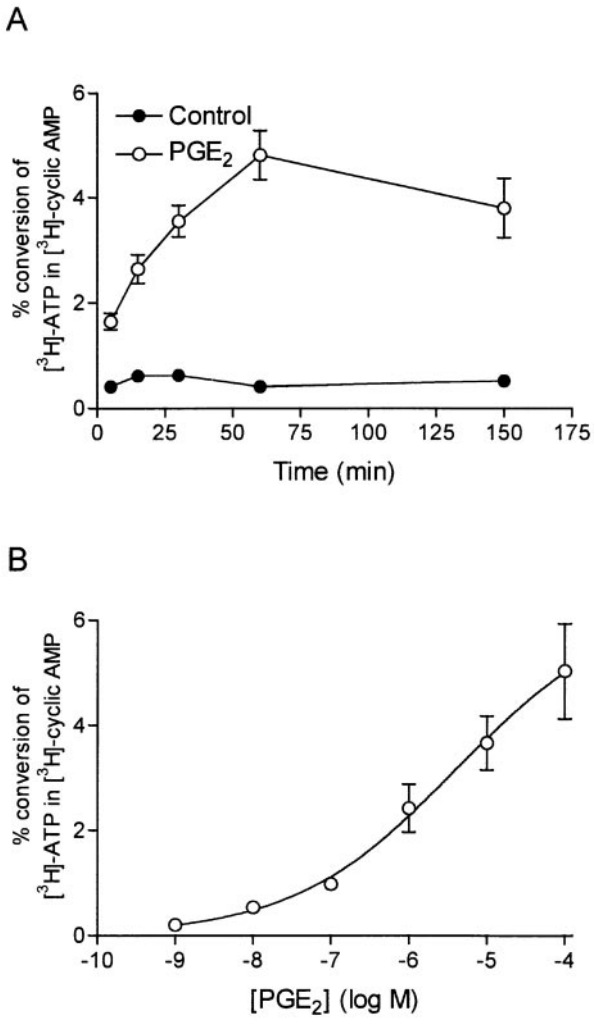

Figure 1 Concentration- and time-dependent effect of PGE_2 on cyclic AMP formation in guinea-pig tracheal epithelial cells. (A) Cells were pre-treated for 15 min with rolipram (10 μM) and incubated in the presence (open circles) or the absence (solid circles) of PGE_2 (1 μM) for 5, 15, 30, 60 and 150 min. The conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP was assayed as described in Methods. (B) Cells were pre-treated for 15 min with rolipram (10 μM) and then incubated for 15 min with increasing concentrations of PGE_2 (1 nM to 100 μM). The points are the means$\pm$s.e.mean of four determinations made with separate cell preparations.

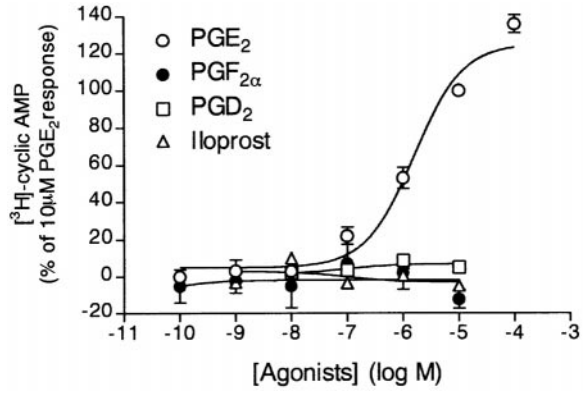

Figure 2 Effects of PGE_2, PGD_2, $PGF_{2\alpha}$ and iloprost on cyclic AMP formation by guinea-pig tracheal epithelial cells. Cells were pre-incubated for 15 min with rolipram (10 μM) and thereafter incubated with increasing concentrations of PGE_2 (open circle), PGD_2 (open squares), iloprost (solid circles) or $PGF_{2\alpha}$(open triangles). The conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP was assayed as described in Methods. The points are the means$\pm$s.e.mean of four determinations made with separate cell preparations.

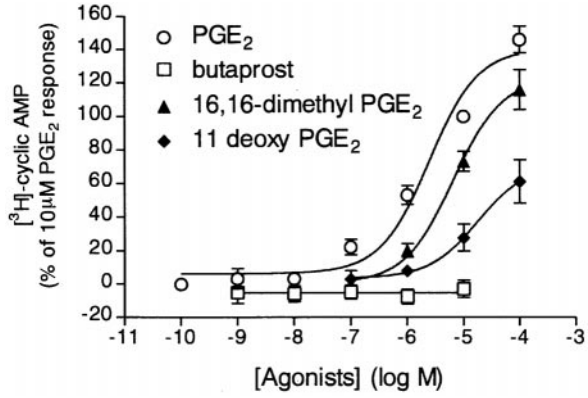

Figure 3 Effects of selective prostaglandin EP receptor agonists on cyclic AMP generation by guinea-pig tracheal epithelial cells. Cells were pre-incubated for 15 min with rolipram (10 μM) and thereafter incubated with increasing concentrations of PGE_2 (open circles), 16,16-dimethyl PGE_2 (solid triangles), 11-deoxy PGE_2 (solid diamonds) or butaprost (open squares). The conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP was assayed as described in Methods. The points are the mean$\pm$s.e.mean of 4–8 determinations made with separate cell preparations.

curve elicited by low concentrations of PGE_2 to the right (Figure 4A). In contrast, in the presence of high concentrations of PGE_2 (10 and 100 μM), AH23848B did not show any antagonist activity. In fact, AH23848B seemed to act synergistically with PGE_2 to increase cyclic AMP formation. However, to support the suggestion that PGE_2 stimulated the cyclic AMP generation through the activation of the EP_4-receptor subtype, the effect of another EP_4-receptor antagonist (AH22921X) (Coleman *et al*., 1994a,b) was investigated. In the presence of 10, 50 and 100 μM AH22921X, basal levels of cyclic AMP were not reduced. The concentration–response curves for PGE_2-induced cyclic AMP accumulation were shifted to the right by AH22921X in a concentration dependent manner (Figure 4B). Shild plots were not constructed since the E_{max} values of the concentration–response curves of PGE_2 in the absence or in the presence of the antagonist were not reached.

Specificity of action of AH23848B and AH22921X

In an attempt to define the specificity of action of AH23848B and AH22921X, their effects on forskolin- and isoprenaline-stimulated cyclic AMP formation were evaluated. Neither compound showed any antagonistic activity against forskolin- or isoprenaline-induced cyclic AMP formation (Figure 5A,B). Surprisingly, AH23848B (100 μM) potentiated forskolin-induced (Figure 5A) but not isoprenaline-induced cyclic AMP formation (Figure 5B). AH22912X slightly

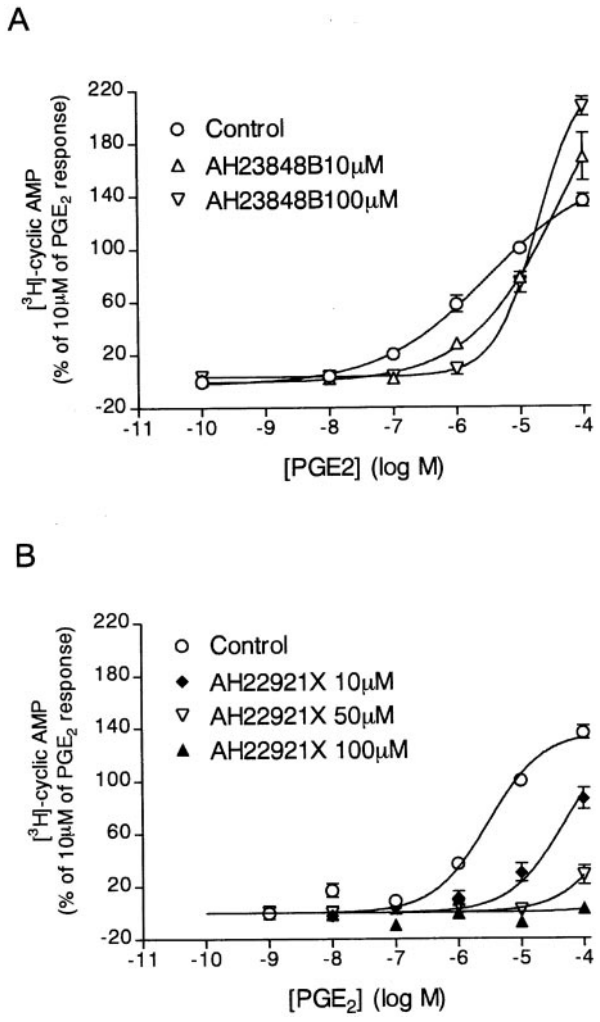

Figure 4 Effect of AH23848B on PGE_2-induced cyclic AMP formation by tracheal epithelial cells. Cells were pre-incubated for 15 min with rolipram (10 μM) plus (A) AH23848B (10 μM (open triangles) or 100 μM (solid triangles)) or medium only (open circles), or (B) AH22921X (10 μM (solid diamonds), 50 μM (open triangles) or 100 μM (solid triangles)) or medium only (open circles) and then incubated for a further 15 min with increasing concentrations of PGE_2 (A and B). The conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP was assayed as described in Methods. The points represents the mean $\pm$ s.e.mean of four determinations made with separate cell preparations.

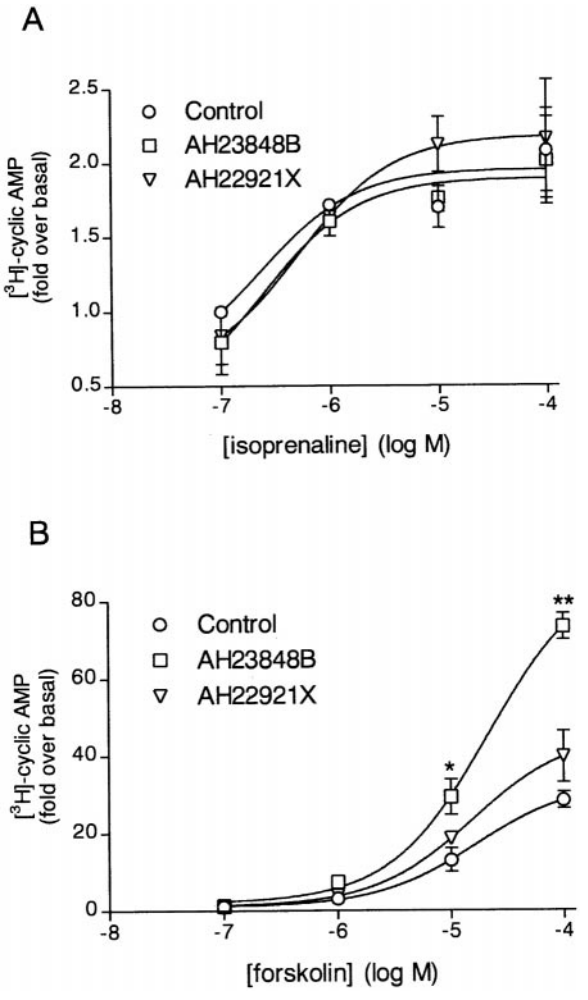

Figure 5 Effects of AH23848B and AH22921X on isoprenaline- and forskolin-induced cyclic AMP generation. Cells were incubated with rolipram (10 μM) in the presence or absence of AH23848B (100 μM) or AH22921X (100 μM) for 15 min and then stimulated for a further 15 min with or without (A) isoprenaline (0.1 – 100 μM) or (B) forskolin (0.1 – 100 μM). The conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP was assayed as described in Methods. The point represents the mean $\pm$ s.e.mean of four determinations made with separate cell preparations.

potentiated forskolin- but not isoprenaline-induced cyclic AMP formation. AH23848B (100 μM) increased by 3 fold the cyclic AMP generation induced by forskolin (100 μM), whereas AH22921X (100 μM) increased it by 1.7 fold (Figure 5B).

Effect of AH6809 on the generation of cyclic AMP evoked by PGE_2

Since PGD_2 and iloprost (EP_1/EP_3/IP receptor agonist) failed to increase cyclic AMP generation in tracheal epithelial cells, AH6809, a DP-, EP_1-and EP_2-receptor antagonist (Coleman *et al*., 1987; Keery & Lumley, 1988; Woodward *et al*., 1995) was used to evaluate the involvement of EP_2-receptors in PGE_2-evoked cyclic AMP generation. Treatment of tracheal epithelial cells with 10 μM AH6809 (Woodward *et al*., 1995) changed neither basal nor PGE_2-induced cyclic AMP formation (Figure 6). Conversely, AH6809 slightly potentiated the stimulatory effect of PGE_2 on the cyclic AMP generation in these cells. However, the leftward shift in the concentration – response curve produced by PGE_2 in presence of AH6809 was not significantly different from the concentration – response curve obtained in absence of this compound.

Effect of PGE_2 on irET production/secretion by tracheal epithelial cells

PGE_2 (0.01 – 100 μM) caused concentration-dependent inhibition of the basal production/secretion of irET from cultured tracheal epithelial cells in a 24 h incubation period (Figure 7). Significant inhibitory effects (35, 44 and 41% from basal levels) were observed at concentrations ranging from 1 to 100 μM.

Effect of Rp-cAMPS on PGE_2- and adenosine-evoked inhibition of irET production/secretion

To confirm that EP_4 receptors, which mediate PGE_2-evoked cyclic AMP generation, are also responsible for the inhibition of irET production/secretion by PGE_2, we evaluated the effects of AH23848B and AH22921X on basal and PGE_2-inhibited production/secretion of the peptide. Unfortunately, AH23848B and AH22921X, like PGE_2, inhibited basal irET production (data not shown). Another approach was then used to evaluate EP_4 receptor involvement in mediating PGE_2-inhibition of irET production/secretion. Since the activation of EP_4 receptors led to an increase in cyclic AMP generation, the role of this second messenger in mediating inhibition of irET production/secretion by PGE_2 was evaluated. Rp-cAMPS, a cyclic AMP antagonist which blocks the activation of the cyclic AMP-dependent protein kinase (Schaap *et al*., 1993), reversed the inhibitory effect of PGE_2 (Figure 8A). The effect of Rp-cAMPS was also tested on the inhibitory effect of adenosine which also activate G_s-coupled receptors in tracheal epithelial cells (Pelletier *et al*., 2000). As observed with PGE_2, Rp-cAMPS reversed the inhibitory effect of adenosine on irET production secretion (Figure 8B).

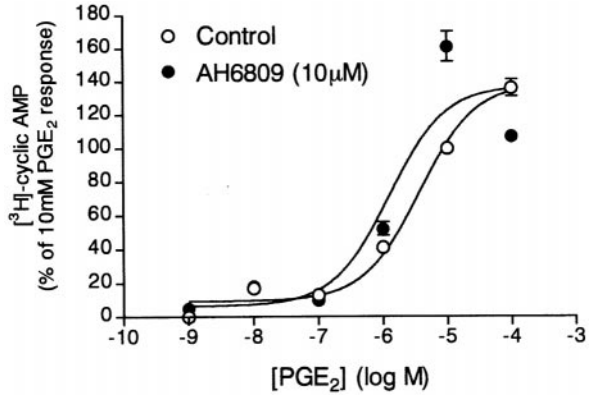

Figure 6 Effect of AH6809, an antagonist at prostanoid EP_1, EP_2 and DP receptors, on the cyclic AMP generation evoked by PGE_2. Cells were pre-incubated for 15 min in the presence of AH6809 (10 μM) and rolipram (10 μM) (solid circles) or in the presence of rolipram alone (10 μM, open circles) and then incubated for a further 15 min with increasing concentrations of PGE_2. The conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP was assayed as described in Methods. The points represents the mean ± s.e.mean of eight determinations made in different cell preparations.

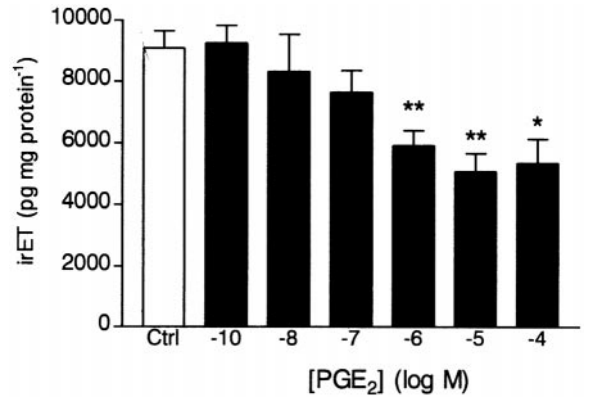

Figure 7 Effect of PGE_2 on the production of immunoreactive ET-1 (irET) by tracheal epithelial cells. Cultured cells were incubated for 24 h with PGE_2 (10^{-10} to 10^{-4} M) or with the medium alone (Control) and irET was measured as described in Methods. Each bar represents the mean ± s.e.mean of 4–12 determinations made with separate cell preparations (*$P<0.05$ and **$P<0.01$).

Discussion

The results presented here show that PGE_2 increases cyclic AMP generation in a concentration-dependent manner in guinea-pig tracheal epithelial cells whereas PGI_2, $PGF_{2\alpha}$ and PGD_2 were ineffective. The rank order of potency of the naturally occurring prostanoids was: $PGE_2 > > > PGI_2$, PGD_2, $PGF_{2\alpha}$. Based on prostanoid receptor classification (Coleman *et al*., 1994b), these results suggest the presence of an EP receptor subtype responsible for the increase in cyclic AMP formation by tracheal epithelial cells. To date, two EP receptor subtypes were shown to be coupled to a regulatory G_s protein and to activate adenylyl cyclase. These two

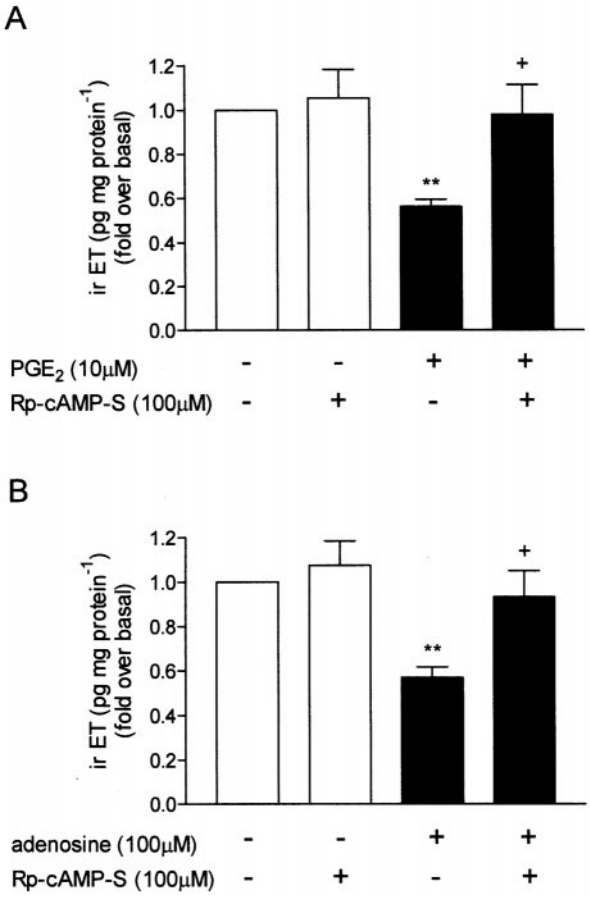

Figure 8 Effect of Rp-cAMPS on the inhibitory effect of G_s-coupled receptor activation on irET production/secretion. Cells were pre-treated for 1 h with Rp-cAMPS (100 μM) or with the medium alone and then stimulated with (A) PGE_2 (1 μM) or (B) adenosine, and then incubated for 24 h at 37°C. IrET was measured as described in Methods. Each bar represents the mean ± s.e.mean of six determinations made with separate cell preparations (**$P<0.01$ *vs* basal level and +$P<0.01$ *vs* PGE_2 or adenosine).

subtypes are classified as EP_2 and EP_4 (Hardcastle *et al*., 1982; Jumblatt & Paterson, 1991; Nishigaki *et al*., 1995). In an attempt to characterize the receptor subtype present on guinea-pig airway epithelium, four non-selective and selective agonists of the EP receptor subtypes were tested on cyclic AMP generation. The rank order of PGE_2 analogues in stimulating cyclic AMP generation was $PGE_2 > 16,16$-dimethyl $PGE_2 > 11$-deoxy PGE_1. Butaprost, a selective EP_2-receptor agonist, and iloprost, a selective $EP_1/EP_3/IP$ receptor agonist failed to increase cyclic AMP generation, which suggested that EP_1, EP_3, IP and EP_2 receptors are not present or coupled to cyclic AMP generation in tracheal epithelial cells. When the cells were incubated with the non-selective EP-receptor agonists, 16,16-dimethyl PGE_2 and 11-deoxy PGE_1, the results showed that 16,16-dimethyl PGE_2 was 10 times less potent than PGE_2 but appeared to be more potent than 11-deoxy PGE_1 in stimulating cyclic AMP generation. These results are different from those observed in a ligand binding assay with the mouse EP_4 receptor expressed in CHO cells where 16,16-dimethyl PGE_2 had a lower affinity than 11-deoxy PGE_1 (Kiriyama *et al*., 1997). This can be explained by the fact that potency is a function of both affinity and efficacy and there is no direct relationship between affinity and potency. Therefore, the value of comparisons of ligand binding studies and functional assays is limited. In bovine chondrocytes, 11-deoxy PGE_1 stimulated the cyclic AMP generation in a concentration–dependent manner and was 10–100 times less potent than PGE_2 (de Brum-Fernandes *et al*., 1996) as observed in our study. However, 16,16-dimethyl PGE_2 was not tested on these cell preparations. Species differences could also explain the discrepant results.

Analysis of the agonist profile suggested that the receptor expressed in tracheal epithelial cells was the EP_4-receptor subtype. The antagonist profile suggested a similar conclusion. Both selective EP_4-receptor antagonists used, AH23848B and AH22921X, inhibited in a concentration–dependent (AH22921X) or independent manner (AH23848B, see below) the cyclic AMP generation evoked by PGE_2 in tracheal epithelial cells. Since the maximal effect was not observed in the absence and in the presence of AH22921X, the Schild slope was not calculated and the nature of its antagonistic effect was not defined. Coleman *et al*. (1994a) showed that these two antagonists (AH23848B and AH22921X) have similar pA_2 values at EP_4 receptors. However, in our study, pA_2 values were very different: AH22921X was much more potent than AH23848B and showed a clear concentration-related inhibition of PGE_2-induced cyclic AMP formation whereas AH23848B did not. This discrepancy could have been explained by the agonist activity of AH23848B observed in many EP_4-receptor preparations (Lydford *et al*., 1996; Meja *et al*., 1997; Wise, 1998). However, in tracheal epithelial cells, AH23848B (10–100 μM) did not show any agonist activity on cyclic AMP accumulation. It acted in synergy with PGE_2 only when high concentrations of PGE_2 (10–100 μM) were used (Figure 4A) or possibly when a marked accumulation of cyclic AMP was observed. This synergistic effect was also observed in forskolin-induced cyclic AMP formation and only when high concentrations (10 and 100 μM) of forskolin were used (Figure 5B). Interestingly, this synergistic effect was not observed in isoprenaline-induced cyclic AMP formation (Figure 5A). These observations suggested that, at concentrations used in these experiments, AH23848B could act as an antagonist at EP-receptor (EP_4) and was probably specific since isoprenaline-induced cyclic AMP formation was not affected in its presence. These effects of AH22921X on forskolin-induced cyclic AMP were less marked than those observed with AH23848B. At the present time, there is no obvious explanation for the difference in potency, but it is clear that these compounds need further studies before being used as selective antagonists of EP_4 receptors.

To better characterize the EP receptor present in this cell preparation, another EP antagonist was used. AH6809, which is selective for EP_1 and DP receptor subtypes (Coleman *et al*., 1987; Keery & Lumley, 1988) was also shown to block PGE_2-evoked cyclic AMP accumulation in COS-7 cells expressing recombinant human EP_2 receptors (Woodward *et al*., 1995). In our study, treatment of tracheal epithelial cells with AH6809 did not affect the generation of cyclic AMP induced by PGE_2. These results suggested that the EP_1-, DP- and EP_2-receptors were not responsible for mediating the action of PGE_2 on tracheal epithelial cells. This is consistent with the lack of efficacy of butaprost on this cell preparation. The small potentiation of PGE_2-evoked cyclic AMP generation by the AH6809 is still unexplained. However, this compound was found to inhibit phosphodiesterase (Keery & Lumley, 1988), which could explain its small potentiating effect on cyclic AMP generation induced by PGE_2 in our model.

Comparisons with other EP_4 receptors or EP receptors positively coupled to adenylyl cyclase

Among the various EP receptor agonists, PGE_2 was shown to exhibit the highest affinity and to be the most potent for stimulating the EP_4 receptor subtype in both ligand binding assay and functional assay (Coleman *et al*., 1994a; de Brum-Fernandes *et al*., 1996; Kiriyama *et al*., 1997; Marshall *et al*., 1997; Milne *et al*., 1994). In all EP_4 receptor systems studied, 11-deoxy PGE_1 appeared to be less potent than PGE_2 but more potent than 16,16-dimethyl PGE_2 (de Brum-Fernandes *et al*., 1996; Kiriyama *et al*., 1997), except in our study and in human monocytes (Milne *et al*., 1994), where they had the same potency. The selective EP_2 receptor agonist, butaprost, was the least potent agonist in all studies reported on the EP_4 receptor-subtype (Coleman *et al*., 1994a) and was not active at concentrations up to 10 μM in bovine chondrocytes (de Brum-Fernandes *et al*., 1996). Furthermore, it had a very low affinity in binding studies on human EP_4-receptor subtype expressed in *Xenopus* oocytes (Marshall *et al*., 1997) and on the mouse EP_4-receptor expressed in CHO cells (Kiriyama *et al*., 1997). Iloprost showed very little activity in many studies including ours. In other studies, selective-EP_1 receptor agonists also showed very poor affinity on the EP_4-receptor subtype (Coleman *et al*., 1994a; de Brum-Fernandes *et al*., 1996; Marshall *et al*., 1997).

AH23848B and AH22921X were shown to be of low affinity but selective for EP_4 receptors (Coleman *et al*., 1994a). To our knowledge, AH22921X has only been tested on piglet saphenous vein, where the pA_2 value was 5.3 (Coleman *et al*., 1994a). In contrast, AH23848B was more widely used to discriminate the effect produced by the EP_2 or EP_4-receptor subtypes (Coleman *et al*., 1994a; de Brum-

Fernandes *et al*., 1996; De Vries *et al*., 1995; Feoktistov & Biaggioni, 1997; Kiriyama *et al*., 1997; Lydford *et al*., 1996; Marshall *et al*., 1997; Milne *et al*., 1995; Wise, 1998). In all these experiments, a high concentration (10 μM) of this compound was used to inhibit the effect of PGE_2. Other studies reported the presence of a receptor positively coupled to adenylyl cyclase that responded to PGE_2 but not to butaprost in bovine chondrocytes and in Jurkat T-cell line (de Brum-Fernandes *et al*., 1996; De Vries *et al*., 1995). In these last two studies, AH23848B antagonized the effect of PGE_2 in a concentration-dependent manner but the nature of the antagonism was unclear. The first report describing the effect of AH23848B presented this compound as a competitive antagonist on PGE_2-induced relaxation of piglet saphenous vein (Coleman *et al*., 1994a). In contrast, De Vries *et al*. (1995) described compound AH23848B as a non-competitive antagonist. Based on these data, they postulated that a new EP-receptor subtype was present in Jurkat cells. De Brum-Fernandes *et al*. (1996) reported that the EP receptor expressed in bovine chondrocytes possessed some characteristic features of the EP_4-subtype. AH23848B was also used to determine the receptor subtype responsible for the inhibition of LPS-induced TNFα generation in blood monocytes (Meja *et al*., 1997).

Prostaglandin E_2 and endothelin-1

The results presented here show that, in addition to increasing cyclic AMP generation, PGE_2 inhibits irET production/secretion by tracheal epithelial cells. To confirm that activation of EP_4 receptor which increases cyclic AMP generation was the mechanism by which inhibition of irET production/secretion occurred, the effects of AH22921X and AH23848B on irET production/secretion were also studied. Unfortunately, both compounds inhibited basal irET-production/secretion, in common with PGE_2. It was, therefore, very difficult to evaluate the role of the EP_4 receptor subtype in mediating the inhibition of irET production/secretion using these tools. This inhibitory effect could be due to their activity on cyclic AMP metabolism. Therefore, another approach was used to answer this question. The ability of a cyclic AMP antagonist (Rp-cAMPS) to inhibit the effect of PGE_2 on irET production/secretion was tested. At the concentration of 100 μM, Rp-cAMPS clearly reversed the inhibitory effect of PGE_2 on irET production. Furthermore, the ability of this compound to reverse the inhibitory effect of adenosine (acting through the A_{2b}-adenosine receptor which is also coupled to a G_s-protein; Pelletier *et al*., 2000), was evaluated on irET production. The results showed that Rp-cAMPS (100 μM) almost completely reversed the effect of adenosine. These results suggested that PGE_2 and adenosine are able to inhibit basal production of irET by activating the cyclic AMP-adenylyl cyclase system. The results also suggested that the EP_4 receptor subtype mediated PGE_2 effects since it is were the only one that increased cyclic AMP generation in guinea-pig tracheal epithelial cells. In addition, a strong correlation between the cyclic AMP generation and the inhibitory effect of PGE_2 on the production/secretion of irET was observed. Linear regression analysis of the responses observed on cyclic AMP generation and inhibition of irET production/secretion gave a slope of 0.91 and a correlation coefficient (r) of 0.95 (data not shown).

Interestingly, Prins *et al*. (1994) showed that PGE_2 and PGI_2 decreased ET-1 production/secretion by endothelial cells in a concentration and time-dependent manner. In their study, PGE_2 and PGI_2 increased cyclic GMP formation and activated cyclic GMP-dependent protein kinase leading to a decrease of ET-1 gene expression, thus resulting in a diminution of the ET-1 production/secretion. The data also showed that the signalling pathway was independent of the activity of a cyclic AMP-dependent protein kinase which suggested that cyclic AMP was not involved in the regulation of ET-1 production/secretion. However, this group did not clearly demonstrate that cyclic AMP was not involved in the inhibition of endothelin secretion.

The involvement of cyclic AMP in the regulation of the expression and/or the production/secretion of ET-1 has already been reported in rat mesangial cells where the production of ET-1 was reduced by stimulation with a β-adrenoceptor agonist (isoprenaline) and by forskolin, a direct activator of adenylyl cyclase (Sakamoto *et al*., 1992). These authors reported that a cyclic AMP-dependent protein kinase appeared to be involved in the signalling pathway. In the two previous studies reported here (Prins *et al*., 1994; Sakamoto *et al*., 1992), the implication of cyclic AMP-dependent protein kinase was investigated on the inhibition of ET-1 secretion. In the first study, Prins *et al*. (1994) tested the effect of a cyclic AMP-dependent protein kinase inhibitor, Rp-cAMPS, and a selective cyclic GMP-dependent protein kinase inhibitor, KT5823, and found that the inhibitory effect of PGE_2 and PGI_2 on ET-1 production/secretion was unchanged when cells were incubated in the presence of the Rp-cAMPS whereas the PG-induced inhibition of ET-1 production/secretion was reversed in the presence of the KT5823. In the second study, H-8, a non-selective cyclic AMP-dependent and cyclic GMP-dependent protein kinase inhibitor, was used and blocked forskolin- and isoprenaline-induced inhibition of ET-1 production/secretion by rat mesengial cells. Thus, Sakamoto *et al*. (1992) suggested that a cyclic AMP-dependent protein kinase was involved in the inhibition of ET-1 production/secretion whereas Prins *et al*. (1994) suggested a cyclic GMP-dependent protein kinase mechanism. These two studies suggested different intracellular pathways involved in the regulation of the production of ET-1 by these two cell types. In fact, a different interpretation of the results obtained by Sakamoto *et al*. (1992) could suggest that, considering that the H-8 compound was recognized as a non-selective cyclic AMP- and cyclic GMP-dependent protein kinase inhibitor (Hagiwara *et al*., 1987; Hidaka *et al*., 1984), the inhibitory effect of forskolin and isoprenaline on ET-1 production secretion was mediated through a cyclic GMP-dependent protein kinase. Studies from our laboratory have already demonstrated that compounds able to bind G_s-coupled receptors in tracheal epithelial cells reduced the production/secretion of ET-1 (Yang *et al*., 1997a). Furthermore, inhibition of ET-1 secretion by activation of G_s-coupled receptors was observed by Durieu-Trautman *et al*. (1993) in brain microvessel endothelial cells, where isoprenaline inhibited ET-1 secretion. Biphasic effects of cyclic AMP analogues were also observed on ET-1 secretion by brain microvessel endothelial cells. Micromolar concentrations of 8-Br-cyclic AMP reduced ET-1 production/secretion by cultured cells whereas high concentrations stimulated the production and secretion of the

peptide (Durieu-Trautman *et al*., 1993). These and our findings suggested that the activation of a G_s-coupled receptor was able to decrease ET-1 production/secretion but the mechanism involved needs further elucidation. More recently, we reported that adenosine can also inhibit ET-1 production by tracheal epithelial cells by activating an A_{2b} adenosine receptor which is coupled to a regulatory G_s-protein (Pelletier *et al*., 2000). This study also suggested an important role for cyclic AMP in regulating ET-1 production.

In conclusion, the results of the present study suggest that guinea-pig tracheal epithelial cells express an EP_4 receptor positively coupled to adenylyl cyclase that inhibits the production/secretion of irET. This receptor subtype may play a major protective role by controlling the release of ET-1, a potent bronchoconstrictor agent which may have a deleterious effect in asthma and other lung diseases.

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