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β_2-Adrenoceptor agonists inhibit release of eosinophil-activating cytokines from human airway smooth muscle cells

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1 Airway smooth muscle (ASM) is a potential source of multiple pro-inflammatory cytokines during airway inflammation. β-Adrenoceptor agonist hyporesponsiveness is a characteristic feature of asthma, and interleukin (IL)-1β and tumour necrosis factor (TNF)-α are implicated in its cause. Here, the capacity of β-adrenoceptor agonists to prevent release of GM-CSF, RANTES, eotaxin and IL-8, elicited by IL-1β or TNFα, was examined in human ASM cells.

2 Isoprenaline ($\sim EC_{50}$ 150 nM), a non-selective β-adrenoceptor agonist, and salbutamol ($\sim EC_{50}$ 25 nM), a selective β_2-adrenoceptor agonist, attenuated release of GM-CSF, RANTES and eotaxin, but not IL-8 ($EC_{50} > 1$ μM). The maximum extent of attenuation was RANTES $\geqslant$ eotaxin $>$ GM-CSF $>>$ IL-8, and was prevented by either propranolol (1 μM), a non-selective β-adrenoceptor antagonist, or ICI 118511 (IC_{50} 15 nM), a selective β_2-adrenoceptor antagonist.

3 The cyclic AMP-elevating agents, dibutyryl cyclic AMP ($\sim EC_{50}$ 135 μM), forskolin ($\sim EC_{50}$ 530 nM) and cholera toxin ($\sim EC_{50}$ 575 pg ml^{-1}) abolished IL-1β-induced release of GM-CSF, RANTES and eotaxin, but not IL-8.

4 IL-1β (1 ng ml^{-1}) attenuated early increases (up to 1 h) in cyclic AMP formation induced by salbutamol (1 μM), but not by forskolin (10 μM). The cyclo-oxygenase inhibitor, indomethacin (1 μM) prevented later increases (3–12 h) in IL-1β-stimulated cyclic AMP content, but did not prevent the attenuation by salbutamol of IL-1β-induced cytokine release.

5 We conclude in human ASM cells that activation of β_2-adrenoceptors and generation of cyclic AMP is negatively-linked to the release, elicited by IL-1β or TNFα, of eosinophil-activating cytokines such as GM-CSF, RANTES and eotaxin, but not IL-8.
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Keywords: Airway smooth muscle; asthma; β_2-adrenoceptor agonists; eotaxin; GM-CSF; interleukin-1β; interleukin-8; RANTES; Tumour necrosis factor-α

Abbreviations: COX, cyclo-oxygenase; cyclic AMP, adenosine 3′,5′-cyclic monophosphate; EC_{50}, concentration required for half-maximal stimulation; EDTA, ethylene diamine tetra-acetic acid; ELISA, enzyme-linked immunosorbent assay; FBS, foetal bovine serum; GM-CSF, granulocyte-macrophage colony stimulating factor; H-89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulphonamide.2HCl; ICI 118551, (±)-1-(2,3-[dihydro-7-methyl-1*H*-inden-4-yl]oxy)-3-([1-methyl ethyl]-amino)-2-butanol; IL, interleukin; PTX, pertussis toxin; RANTES, regulated upon activation, normal T cell expressed and secreted; TNFα, tumour necrosis factor-α

Introduction

Patients with long-standing and severe asthma often develop decreased bronchodilator responses to β-adrenoceptor agonists. This phenomenon has been reported in both *post mortem* and surgically-removed bronchi from asthmatic patients (Bai, 1991; De Jongste *et al*., 1987; Goldie *et al*., 1996) and in animal models of asthma (Emala *et al*., 1993). The pathogenesis of allergic inflammation is complex and accumulating evidence suggests the involvement of multiple inflammatory cells, mediators and cytokines in both the induction and perpetuation of these processes. Cytokines such as IL-1β and tumour necrosis factor (TNF)-α are increased in the bronchoalveolar lavage fluid of patients with symptomatic asthma compared to normal subjects or patients with asymptomatic asthma (Mattolli *et al*., 1991; Broidie *et al*., 1992). Immunohistochemical studies of bronchial biopsies from asthmatic patients have shown increased expression of IL-1β in the bronchial epithelium and marked increases in IL-1β-producing cells in the asthmatic submucosa (Sousa *et al*., 1996). Studies of alveolar macrophages from asthmatic subjects, have also shown that IL-1β expression is up-regulated (Pujol *et al*., 1990). In addition, several recent reports have shown that both IL-1β and TNFα decrease the β-adrenoceptor agonist responsiveness of various cell types in the airway including the epithelium (Kelsen *et al*., 1995) and bronchial smooth muscle (Emala *et al*., 1993; 1997; Shore *et al*., 1997; Pang *et al*., 1998).

Evidence from our own studies (John *et al*., 1997; Hallsworth *et al*., 1998; McKay *et al*., 2000) and from others (Saunders *et al*., 1997; John *et al*., 1998; Chung *et al*., 1999; Ghaffar *et al*., 1999), suggests that airway smooth muscle cells, which have long been regarded as having predominantly contractile properties in response to inflammatory mediators, can potentially contribute to the pathogenesis of asthma by expressing and secreting multiple pro-inflammatory cytokines and mediators (Johnson & Knox, 1997; Chung, 2000). Studies

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of human airway smooth muscle stimulated with IL-1β and TNFα have shown increased expression and release of chemokines including RANTES (John *et al*., 1997), eotaxin (Chung *et al*., 1999; Ghaffar *et al*., 1999), IL-8 (John *et al*., 1998), and other cytokines such as GM-CSF (Saunders *et al*., 1997; Hallsworth *et al*., 1998), IL-6 (McKay *et al*., 2000) and IL-11 (Elias *et al*., 1997). GM-CSF, eotaxin, RANTES and IL-8 and are important cytokines for activation of eosinophils, critical effector cells in the pathogenesis of asthma. RANTES is a potent chemoattractant for eosinophils as well as for other cell types observed in allergic inflammation including monocytes and memory T lymphocytes (Furie & Randolph, 1995). IL-8, in addition to its action on neutrophils, is also a chemoattractant for activated eosinophils (Warringa *et al*., 1993; Shute, 1994). Eotaxin is a highly selective chemoattractant for eosinophils (Ponath *et al*., 1996). GM-CSF stimulates maturation, surface activation and proliferation of several pro-inflammatory cells and is particularly important for the survival of eosinophils (Lopez *et al*., 1986). Production by airway smooth muscle of RANTES, IL-8 and eotaxin implies a role for these structural cells to participate directly in the inflammatory process through recruitment and activation of eosinophils. In addition to recruitment, enhanced survival of infiltrating eosinophils is also thought to contribute to airway inflammation in asthma (Woolley *et al*., 1996), and we have recently demonstrated that airway smooth muscle cells stimulated with IL-1β enhance the survival of peripheral blood eosinophils in culture by the release of GM-CSF (Hallsworth *et al*., 1998).

β-Adrenoceptor agonists are the most widely used agents in asthma therapy and β_2-adrenoceptors are important negative regulators of airway smooth muscle contractile function (Goldie *et al*., 1986). Activation of β_2-adrenoceptors has also been shown to inhibit mitogen-stimulated proliferation of airway smooth muscle (Tomlinson *et al*., 1994; Stewart *et al*., 1999). Increasing evidence supports the hypothesis that in addition to promoting cytokine release from airway smooth muscle, pro-inflammatory mediators such as IL-1β and TNFα, contribute to impairment of β-adrenoceptor function in asthma and in human and animal airway smooth muscle tissue- and cell-based models. IL-1β, and to a lesser extent, TNFα, causes β-adrenoceptor agonist hyporesponsiveness in various tissues including isolated rabbit airways (Hakonarson *et al*., 1996; 1997) and in human and canine airway smooth muscle cells (Emala *et al*., 1997; Shore *et al*., 1997).

In this study, experiments were performed to characterize the effectiveness of β-adrenoceptor agonists in preventing the release of eosinophil-activating cytokines from human airway smooth muscle cells that were stimulated by cytokines such as IL-1β and TNFα. Our results demonstrate the first direct evidence that activation of β_2-adrenoceptors is negatively-linked to the release of GM-CSF, RANTES and eotaxin, and the production of intracellular cyclic AMP in human airway smooth muscle.

Methods

Isolation and culture of human airway smooth muscle cells

Human bronchial smooth muscle was obtained from the lobar or main bronchus of 39 patients of either sex (mean age 63 ± 2 years; range 33–74 years; 24 male, 15 female) undergoing lung resection for carcinoma of the bronchus. After removal of the epithelium, portions of the smooth muscle not invaded by the carcinoma were dissected free of adherent connective and parenchymal tissue under aseptic conditions in Hanks' balanced salt solution and placed in culture as previously described (Hallsworth *et al*., 1998). Briefly, finely chopped (approximately 1 mm^3) pieces of smooth muscle were digested overnight in 1 ml Dulbecco's Modified Eagle's Medium (DMEM) (supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 1 × non-essential amino acid mixture, 50 μg ml^{-1} gentamicin and 1.5 μg ml^{-1} amphotericin B) containing 1 μM insulin, 5 μg ml^{-1} transferrin, 100 μM ascorbate, 1 mg ml^{-1} bovine serum albumin (BSA) and 1 mg ml^{-1} collagenase. The resulting cell suspension was centrifuged ($200 \times g$ for 5 min) and the pellet washed in supplemented DMEM containing 10% FBS. Cells were seeded at 5×10^5 viable cells in 25 cm^2 culture flasks and maintained in a humidified atmosphere at 37°C in 5% CO_2, with the medium replaced every 72 h. Using fluorescent immunocytochemistry techniques, growth-arrested cultured human airway smooth muscle cells (passages 1 and 2) stained (>95%) for both smooth muscle α-actin and smooth muscle myosin heavy chain. When examined by light and electron microscopy, these cells displayed all the reported characteristics of viable smooth muscle cells in culture (Hirst, 1996).

Cell stimulation and collection of cell-conditioned medium

Cells at passages 3–6 were used for all experiments and were harvested from 75 cm^2 flasks by treatment with trypsin/EDTA (0.2 mg ml^{-1} of each in phosphate buffered saline) and washed in supplemented DMEM containing 10% FBS as previously described. Cells were then seeded into 24-well plastic tissue culture plates at an initial density of 2×10^4 cells well^{-1}. When the cells approached confluence, growth was arrested by washing (1×0.5 ml well^{-1}) the monolayers with RPMI 1640 for 2 min and then replacing the washing medium with RPMI 1640 supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-ethane sulphonic acid, 2 mM L-glutamine, 100 U ml^{-1} : 100 μg ml^{-1} penicillin/streptomycin (supplemented RPMI) with the addition of 1 μM insulin, 5 μg ml^{-1} transferrin, 100 μM ascorbate and 1 mg ml^{-1} BSA. After 72 h airway smooth muscle cell monolayers were washed (1×0.5 ml well^{-1}) with supplemented RPMI 1640 containing 1 mg ml^{-1} BSA and then cultured in the same medium (0.5 ml well^{-1}) for a further period up to 96 h in the absence or presence of varying concentrations of recombinant human IL-1β or TNFα, and any other agents under investigation. Cell-conditioned medium (0.5 ml well^{-1}) was collected and cell-free supernatants were stored at −70°C until measurement of cytokine levels by ELISA.

Measurement of cytokine levels by ELISA

Cytokine levels in airway smooth muscle cell-conditioned culture medium were determined in duplicate by specific sandwich enzyme-linked immunosorbent assays (ELISA) using matched monoclonal (anti-human) capture and biotinylated anti-human monoclonal (GM-CSF) or polyclonal

(RANTES, eotaxin, IL-8) detection antibody pairs (R&D Systems, Abingdon, U.K.). The manufacturer's instructions were followed throughout. Briefly, 96-well ELISA plates were coated overnight at room temperature with 100 μl anti-human GM-CSF, RANTES, eotaxin or IL-8 capture antibody diluted in phosphate-buffered saline (PBS) (pH 7.2–7.4). Plates were then washed four times with PBS containing 0.05% Tween 20 (PBS-T) and blocked for 1 h at room temperature with 250 μl blocking buffer (PBS containing 1% BSA, 5% sucrose and 0.01% sodium azide). Plates were washed again with PBS-T and 100 μl of recombinant human cytokine standards and study samples were added. Samples were diluted until the level of cytokine was within the limits of the standard curve of the assay and were added in duplicate to individual wells and incubated at room temperature for 2 h. After four washes, 100 μl of biotinylated detection antibodies diluted in dilution buffer (PBS-T containing 0.1% BSA) was added for 2 h. After another four washes, 100 μl of streptavidin-horseradish peroxidase (HRP) conjugate (62 ng ml^{-1}) in dilution buffer was added for 20 min. After four final washes, 100 μl of the substrate buffer containing HRP substrate tetramethylbenzidine dihydrochloride was added for 20 min and colour-developed in proportion to the amount of each cytokine present. The reaction was stopped by adding 100 μl of stop solution (1 M HCl) and the degree of colour generated was determined spectrophotometrically (Anthos HTll; Salzburg, Austria). Levels of cytokines in cell-conditioned medium were initially expressed in ng ml^{-1} before normalization to ng ml^{-1} million^{-1} cells to correct for small differences in cell densities between patients. The lower limits of sensitivity for each of the assays were: GM-CSF <2.8 pg ml^{-1}; RANTES <5 pg ml^{-1}; eotaxin <5 pg ml^{-1}; IL-8 <10 pg ml^{-1} which was consistent with the manufacturer's specifications. According to the manufacturer's guidelines none of the assays showed any cross-reactivity or interference with one another or with IL-1β.

Measurement of intracellular cyclic AMP levels

Airway smooth muscle cells were grown in 24-well tissue culture plates and growth-arrested as previously described. Cells were stimulated with cyclic AMP-elevating agents in the absence and presence of IL-1β for periods up to 24 h at 37°C. Phosphodiesterase inhibitors were not present. After stimulation, supernatants were aspirated and 0.5 ml ice-cold 0.1 M HCl was added to each well and the plate incubated on ice for 10 min. Cell lysates from each well were removed to a 1.5 ml microcentrifuge tube and each well was washed with a further 0.5 ml 0.1 M HCl and pooled into the microcentrifuge tube. Following centrifugation (13,000 r.p.m.) for 5 min to remove cell fragments, supernatants were removed into clean microcentrifuge tubes and stored at -70°C. Prior to the assay, supernatants were evaporated to dryness in a vacuum centrifuge and reconstituted in 300 μl assay buffer and diluted until the level of cyclic AMP was within the limits of standard curve of the assay. Total cyclic AMP content was determined using a commercially available enzyme immunoassay kit (Amersham, Bucks, U.K.). The lower limit of sensitivity of the enzyme immunoassay was 12.5 fmols cyclic AMP well^{-1} and results were expressed as pmols cyclic AMP million^{-1} cells.

Materials and reagents

All chemicals were of analytical grade or higher. Recombinant human IL-1β, TNFα and matched antibody pairs for GM-CSF, RANTES, eotaxin and IL-8 ELISAs were purchased from R&D Systems (Abingdon, U.K.). H-89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulphonamide.2HCl), ICI 118551 ((±)-1-(2,3-[dihydro-7-methyl-1*H*-inden-4-yl]oxy)-3-([1-methyl ethyl]-amino)-2-butanol) and pertussis toxin were purchased from Calbiochem (Nottingham, U.K.). All cell culture medium (DMEM and RPMI 1640), foetal bovine serum and cell culture reagents were obtained from Gibco Life Technologies (Paisley, U.K.). Collagenase (type CLS 1) was obtained from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.). The streptavidin-horseradish peroxidase conjugate and tetramethylbenzidine dihydrochloride ELISA substrate were purchased from Zymed Laboratories (San Francisco, U.S.A.). All cell culture plasticware was purchased from Falcon Labware (Becton Dickinson, Oxford, U.K.). All other chemical reagents were obtained from Sigma (Poole, U.K.).

Statistical analysis

Data in the text and figure legends are expressed as mean ± s.e.mean of observations obtained from airway smooth muscle cells cultured from n patient donors. EC_{50}/IC_{50} values, and where necessary extrapolated maximum responses, were estimated for individual concentration-response curves using non-linear least-squares regression analysis (SigmaPlot; Jandel Scientific, Erkrath, Germany). EC_{50}/IC_{50} values were converted to negative logarithmic values (pD_2) for all statistical comparisons, although for ease of comprehension EC_{50}/IC_{50} values are given in the text. Data were compared using one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* *t*-test to determine statistical differences after multiple comparisons (SigmaStat; Jandel Scientific, Erkrath, Germany). A probability value of less than 0.05 was considered significant.

Results

Time-dependent release of eosinophil-activating cytokines from IL-1β-and TNFα-stimulated human airway smooth muscle cells

Levels of GM-CSF, RANTES, eotaxin and IL-8 were determined in the same supernatants. In all cultures examined, up to 96 h, the release of GM-CSF, RANTES and IL-8 by unstimulated cells was less than could be detected by ELISA ($<2.8-10$ pg ml^{-1}). However, constitutive release of eotaxin was observed at 24 h, but did not exceed 2 ng ml^{-1} million^{-1} cells (Figure 1).

Initial experiments showed that the concentrations of IL-1β and TNFα that induced near maximum generation of each of the cytokines were and 10 ng ml^{-1}, respectively ($n=4$, data not shown). These maximally-effective concentrations were used in all subsequent experiments. IL-1β (1 ng ml^{-1}) and TNFα (10 ng ml^{-1}) induced significant release at 24 h of RANTES, eotaxin and IL-8 from human airway smooth

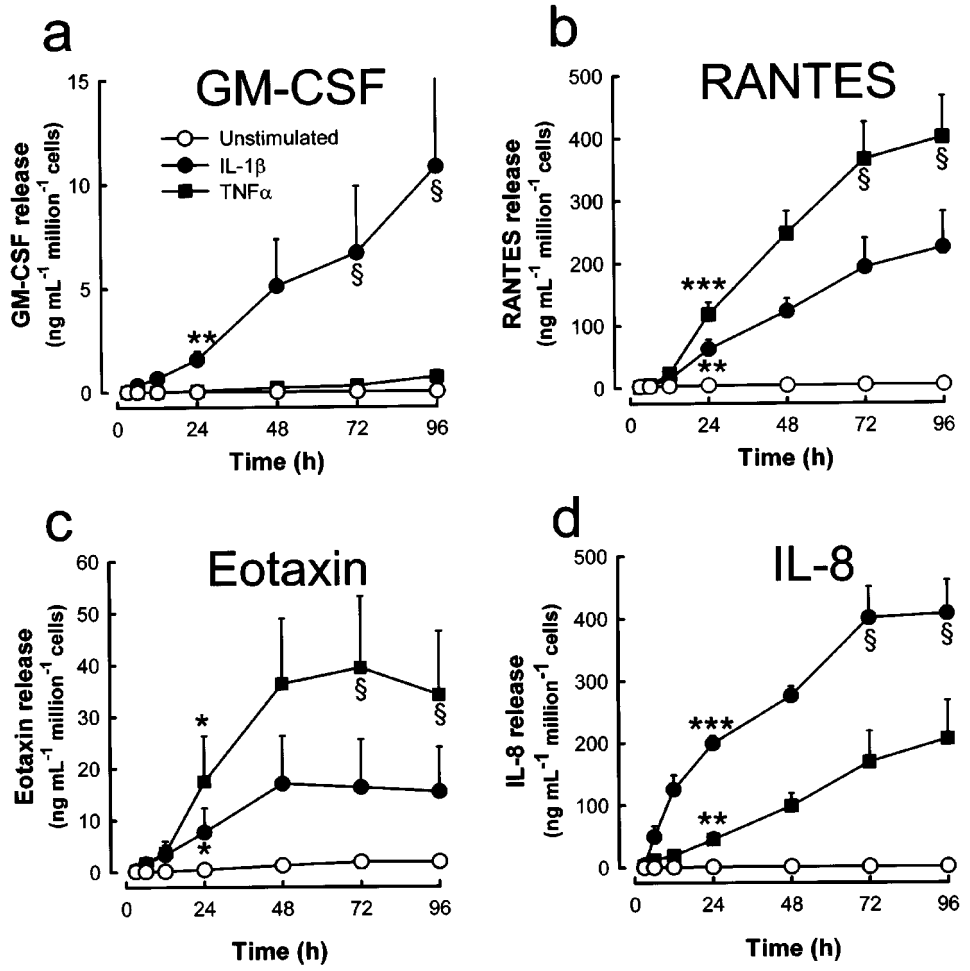

Figure 1 Time course showing relative kinetics and release of (a) GM-CSF, (b) RANTES, (c) eotaxin and (d) IL-8 into the supernatants of human cultured airway smooth muscle cells stimulated with maximally-effective concentrations of either IL-1β (1 ng ml^{-1}) or TNFα (10 ng ml^{-1}). Points represent mean$\pm$s.e.mean of duplicate values from independent experiments using cells cultured from four different donors, cell passages 3–6. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to control at similar time points by Bonferroni's t-test. §$P<0.01$ compared to the other cytokine at similar time points by Bonferroni's t-test.

muscle cells ($P<0.05-0.001$, $n=4$) (Figure 1). Similarly, at 24 h IL-1β also induced significant release of GM-CSF ($P<0.01$, $n=4$). In cells stimulated with TNFα much lower levels of GM-CSF were detected, but were statistically significant at 48 h ($P<0.05$, $n=4$). Maximum release of RANTES, GM-CSF and IL-8 induced by IL-1β and TNFα occurred between 72 and 96 h. Levels of eotaxin increased more rapidly reaching maximum at 48 h. At 72 and 96 h, IL-1β induced significantly higher levels of GM-CSF and IL-8 compared to cells stimulated with TNFα ($P<0.05$, $n=4$); whereas cells stimulated with TNFα produced higher levels of RANTES and eotaxin, compared to IL-1β, but these were not significantly different ($P>0.05$, $n=4$). The rank order for cytokine levels stimulated by IL-1β from human airway smooth muscle cells was IL-8 > RANTES >> eotaxin > GM-CSF. For TNFα, the rank order was RANTES > IL-8 >> eotaxin >>> GM-CSF. Because large differences were present in the absolute levels of each cytokine measured, and in the relative efficacies of IL-1β or TNFα, the data were normalized against the response to either IL-1β or TNFα alone, and this allowed direct comparison of drug effects on each of the cytokines.

Effect of β-adrenoceptor agonists on eosinophil-activating cytokine release from human airway smooth muscle cells

Based on the kinetics of release by human airway smooth muscle cells of each of the eosinophil-activating cytokines, assay of cell-conditioned supernatants at 24 h represented the earliest time point at which clear changes in cytokine release could be determined using ELISA. The capacity of β-adrenoceptor agonists to inhibit the release of eosinophil-activating cytokines by IL-1β and TNFα was therefore determined after 24 h.

The β-adrenoceptor agonists isoprenaline and salbutamol (0.1 nM – 1 μM) did not significantly alter the basal release of any of the cytokines assayed ($P<0.05$, $n=5$) (data not shown). Addition of isoprenaline up to 100 nM to cells stimulated with either IL-1β caused a weak inhibition in GM-CSF, RANTES, eotaxin and IL-8 secretion (Figure 2a). At 1 μM, the highest concentration of isoprenaline studied, up to 75% inhibition of RANTES and eotaxin release occurred in experiments where IL-1β was used to induce cytokine release ($P<0.001$, $n=5$) (Figure 2a). Less than 20% inhibition of

GM-CSF release ($P<0.05$, $n=5$) was found with no attenuation of IL-8 release ($P>0.05$, $n=4$). Concentrations of isoprenaline producing half-maximal attenuation (EC_{50}) of each of the cytokines were 119 ± 15 nM GM-CSF; 159 ± 29 nM RANTES; 154 ± 19 eotaxin; >1 μM IL-8. When TNFα was used to induced cytokine release, the attenuation by isoprenaline was no more than 30% ($P>0.05$, $n=3$) (data not shown).

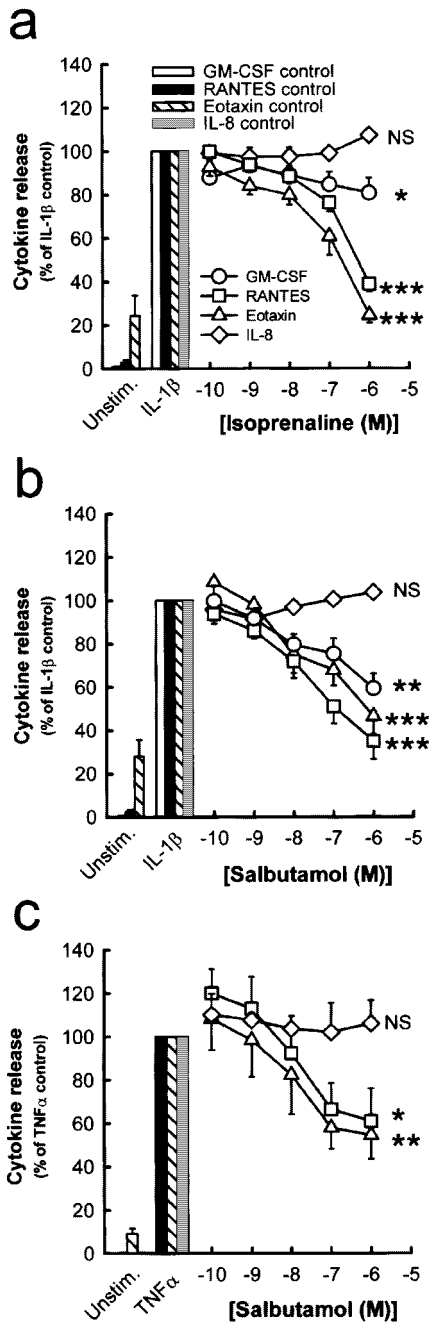

Figure 2 Capacity of the β-adrenoceptor agonists, isoprenaline and salbutamol to inhibit the release of eosinophil-activating cytokines from human airway smooth muscle cells stimulated by either (a,b) IL-1β (1 ng ml^{-1}) or (c) TNFα (10 ng ml^{-1}) for 24 h. Data are expressed as a per cent of the control response to either IL-1β ($n=5-7$) or TNFα ($n=4$) alone and represent mean$\pm$s.e.mean of duplicate values from independent experiments using cells cultured from different donors, cell passages 3–6. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to control by Bonferroni's t-test. NS $P>0.05$ by ANOVA.

Salbutamol (0.1 nM – 1 μM) significantly attenuated IL-1β-stimulated GM-CSF (EC_{50} 16 ± 3 nM), RANTES (EC_{50} 25 ± 6 nM) and eotaxin release (EC_{50} 28 ± 3 nM) ($P<0.01-0.001$, $n=7$), but had no effect ($P>0.05$, $n=7$) on the release of IL-8 (EC_{50} >1 μM) (Figure 2b). In cells stimulated with TNFα, a similar attenuation of RANTES (EC_{50} 15 ± 5 nM) and eotaxin (EC_{50} 14 ± 3 nM) was found ($P<0.05-0.01$, $n=4$), and again no effect on IL-8 release was observed (Figure 2c). Maximum inhibition of cytokine release induced by either IL-1β or TNFα occurred at approximately 1 μM. The rank order of the efficacy of salbutamol against IL-1β-stimulated release of the cytokines was RANTES > eotaxin > GM-CSF >>> IL-8. Similarly, for TNFα-stimulated cytokine release the rank order of salbutamol efficacy was RANTES = eotaxin >> IL-8. Induction of GM-CSF by TNFα at 24 h was below the detection limit of the ELISA assay. The anti-oxidant, ascorbate (100 μM), had no effect on the efficacy or potency of either β-adrenoceptor agonist against IL-1β- or TNFα-induced cytokine release ($n=4$, data not shown), suggesting that inactivation of these catecholamines due to oxidation in the culture medium was not a contributing factor in the response.

Attenuation of IL-1β- and TNFα-stimulated cytokine release by β-adrenoceptor agonists was also examined in supernatants harvested at 48 and 72 h. No differences in the efficacy of β-adrenoceptor agonists were found compared to supernatants harvested at 24 h ($n=4$, data not shown).

Effects of β-adrenoceptor antagonists on salbutamol-induced attenuation of IL-1β-stimulated eosinophil-activating cytokine release

Incubation with either the non-selective β-adrenoceptor antagonist, propanolol or the selective β_2-adrenoceptor antagonist, ICI 118551 was examined to determine whether the observed attenuation by salbutamol of cytokine release from human airway smooth muscle cells was receptor-dependent. Pre-incubation for 30 min with the non-selective β-adrenoceptor antagonist, propanolol (1 μM) partially prevented the inhibitory effect of salbutamol (1 μM) on cytokine release in cells that had been stimulated by IL-1β (1 ng ml^{-1}) (Table 1). However, propranolol itself attenuated the stimulant effect of IL-1β alone. Pretreatment with the selective β_2-adrenoceptor antagonist, ICI 118551 (IC_{50} 15 ± 4 nM) prevented the inhibitory effect of salbutamol (1 μM) on IL-1β-stimulated GM-CSF release (Table 2).

Effect of intracellular cyclic AMP-elevating agents on eosinophil-activating cytokine release from human airway smooth muscle cells

To determine whether the effect of IL-1β in up-regulating cytokine release in these cells might also involve attenuation of β-adrenoceptor function, we investigated the efficacy of agents that activate other points in the receptor-G protein-adenylyl cyclase cascade. Dibutyryl cyclic AMP (1 μM – 3 mM), a cell permeable, non-hydrolysable analogue of cyclic AMP, caused concentration-dependent inhibition of IL-1β-stimulated GM-CSF, RANTES and eotaxin release ($P<0.001$, $n=6$), as well as a small (35%), but significant

Table 1 Propranolol inhibits salbutamol-induced attenuation of eosinophil-activating cytokine release from IL-β-stimulated human airway smooth muscle cells

	Cytokine measured			
Treatment	*GM-CSF*	*RANTES*	*Eotaxin*	*IL-8*
IL-1β alone	100	100	100	100
IL-1β + Salb	65 ± 7*	50 ± 14**	54 ± 14*	96 ± 2#
IL-1β + Salb + Prop	79 ± 6*	90 ± 4#	95 ± #	91 ± 3#
IL-1β + Prop	86 ± 7*	107 ± 6#	88 ± 5*	95 ± 3#

Cells were pre-treated with or without propranolol (1 μM) for 30 min before incubation with either IL-1β (1 ng ml^{-1}) alone or IL-1β and salbutamol (1 μM) in combination for 24 h. Cytokine release was measured by ELISA as described in Methods. Data are expressed as a per cent of the control response to IL-1β alone and represent mean ± s.e.mean of duplicate values from independent experiments using cells cultured from four different donors, cell passages 4–6. *$P<0.05$. **$P<0.01$ compared to cells treated with IL-1β alone by Bonferroni's t-test. #$P>0.05$ by ANOVA.

Table 2 ICI 118551 inhibits salbutamol-induced attenuation of GM-CSF release from IL-1β-stimulated human airway smooth muscle cells

		IL-1β + Salb + ICI 118551 (nM)				
IL-1β	*IL-1β + Salb*	*0.1*	*1*	*10*	*100*	*1000*
100	63 ± 8	65 ± 6	69 ± 9	79 ± 9	98 ± 8	102 ± 9

Cells were pre-treated with or without increasing concentrations of ICI 118551 for 30 min before incubation with either IL-1β (1 ng ml^{-1}) alone or IL-1β and salbutamol (1 μM) in combination for 24 h. GM-CSF release was measured by ELISA as described in Methods. Data are expressed as a per cent of the control response to IL-1β alone and represent mean ± s.e.mean of duplicate values from independent experiments using cells cultured from four different donors, cell passages 4–6.

($P<0.05$, $n=6$) inhibitory effect on IL-8 release (Figure 3a). The maximum inhibitory effect of dibutyryl cyclic AMP occurred at 3 mM. EC_{50} values for dibutyryl cyclic AMP against each of the cytokines were similar ($P>0.05$, $n=6$) for GM-CSF (EC_{50} 130 ± 25 μM), RANTES (EC_{50} 139 ± 17 μM) and eotaxin (EC_{50} 140 ± 16 μM), but not for IL-8 (EC_{50} 228 ± 33 μM). Similarly, forskolin (0.1 nM – 100 μM), a direct activator of adenylyl cyclase, significantly inhibited ($P<0.001$, $n=7$) IL-1β-stimulated GM-CSF, RANTES and eotaxin release to baseline levels (Figure 3b). In the same cell-conditioned supernatants induction of IL-8 was unaffected by forskolin ($P>0.05$, $n=6$). EC_{50} values for forskolin against each of the cytokines were similar ($P>0.05$, $n=7$) for GM-CSF (EC_{50} 517 ± 51 nM), RANTES (EC_{50} 491 ± 50 nM) and eotaxin (EC_{50} 579 ± 61 nM), but again not for IL-8 (EC_{50} >100 μM). The maximum inhibitory effect of forskolin occurred at 10 μM. Cholera toxin (1 pg ml^{-1} – 1 μg ml^{-1}), which directly activates the effector $G_s\alpha$ protein subunit for adenylyl cyclase, also significantly inhibited ($P<0.001$, $n=6$) IL-1β-stimulated GM-CSF, RANTES and eotaxin release to unstimulated levels. (Figure 3c). A small (40%), but significant inhibitory effect on IL-8 release was also found ($P<0.01$, $n=6$). The maximum inhibitory effect of cholera toxin occurred at 0.1 – 1 μg ml^{-1}. Cholera toxin was

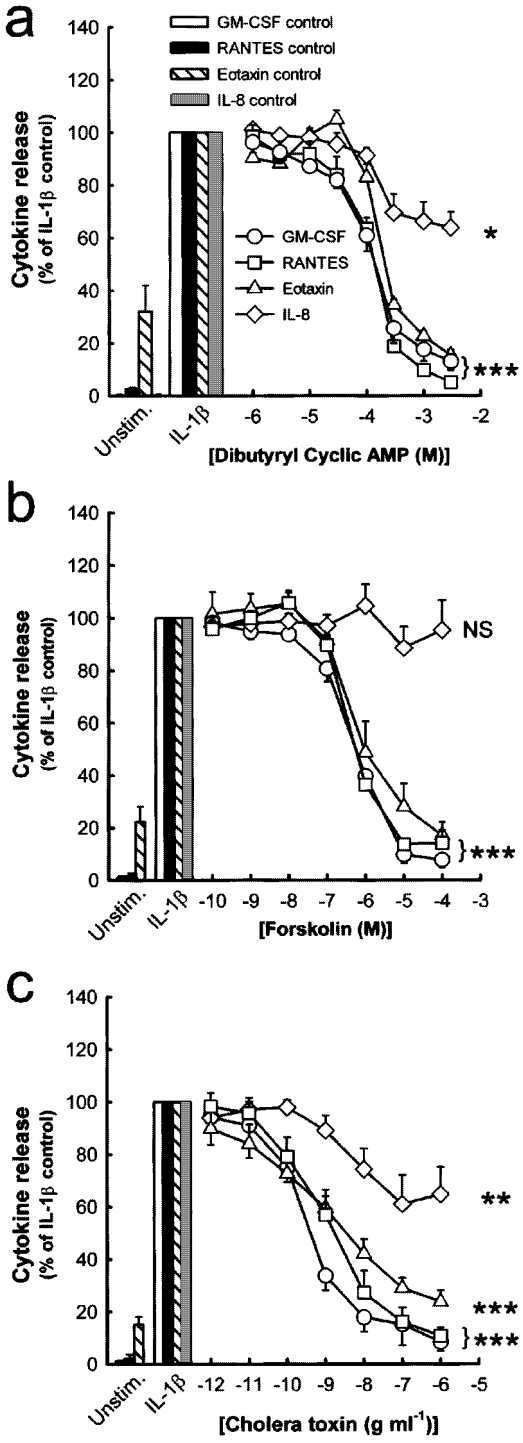

Figure 3 Effect of the cell permeant, non-hydrolysable cyclic AMP analogue (a) dibutyryl cyclic AMP and the cyclic AMP-elevating agents (b) forskolin and (c) cholera toxin on the release of eosinophil-activating cytokines from human airway smooth muscle cells stimulated by IL-1β (1 ng ml^{-1}) for 24 h. Data are expressed as a per cent of the control response to IL-1β alone and represent mean ± s.e.mean of duplicate values from independent experiments using cells cultured from six to seven different donors, cell passages 3–5. *$P<0.05$, **$P<0.01$ ***$P<0.001$ compared to control by Bonferroni's t-test. NS $P>0.05$ by ANOVA.

approximately 2 fold more effective ($P<0.05$, $n=6$) in preventing release of GM-CSF (EC_{50} 391 ± 42 pg ml^{-1})

compared to RANTES (EC_{50} 593 ± 61 pg ml^{-1}) or eotaxin (EC_{50} 738 ± 76 pg ml^{-1}). Approximately 10 fold higher concentrations of cholera toxin were required to prevent IL-1β-stimulated IL-8 release (EC_{50} 6548 ± 381 pg ml^{-1}).

Effect of IL-1β on salbutamol-induced on cyclic AMP accumulation in human airway smooth muscle cells

Changes in cyclic AMP levels were determined at various time points throughout the first 24 h of cytokine generation by the cells (Figure 4). Incubation of human airway smooth muscle cells with a maximally-effective concentration of salbutamol (1 μM) caused a significant 15 fold increase in cyclic AMP formation after 5 min ($P<0.01$, $n=4$). Cyclic AMP levels then fell steadily approaching baseline by 3 h, but remained elevated (approximately 3 fold above basal levels from unstimulated cells) for at least 24 h. IL-1β alone did not alter cyclic AMP formation during the first hour of stimulation (Figure 4a). At 3 h, however, IL-1β induced an increase in cyclic AMP formation, which at its peak at 6 h, was approximately 9 fold above basal cyclic AMP levels ($P<0.05$, $n=4$). Induction by IL-1β of this late increase in cyclic AMP content was completely inhibited by the cyclo-oxygenase inhibitor, indomethacin (1 μM) (data not shown). Increases in cyclic AMP content induced by salbutamol were attenuated by IL-1β by up to 40% within the first hour of stimulation ($P<0.05$, $n=5$). No further attenuation of salbutamol-induced increases in cyclic AMP was evident at 3 h and thereafter.

Forskolin (10 μM) produced a more robust increase in cyclic AMP content (approximately 50 fold above basal levels in unstimulated cells, $P<0.01$, $n=4$) which was over 4 fold greater than the peak salbutamol response. Peak levels of cyclic AMP occurred at 15 min, slightly later than with salbutamol, but were not attenuated by IL-1β (Figure 4b) and remained significantly elevated (approximately 3 fold) at least to 24 h ($P<0.01$, $n=4$). Of interest, cyclic AMP levels between 3 and 24 h in cells stimulated with both forskolin and IL-1β remained markedly elevated ($P<0.01$, $n=4$), much greater than with either agent alone.

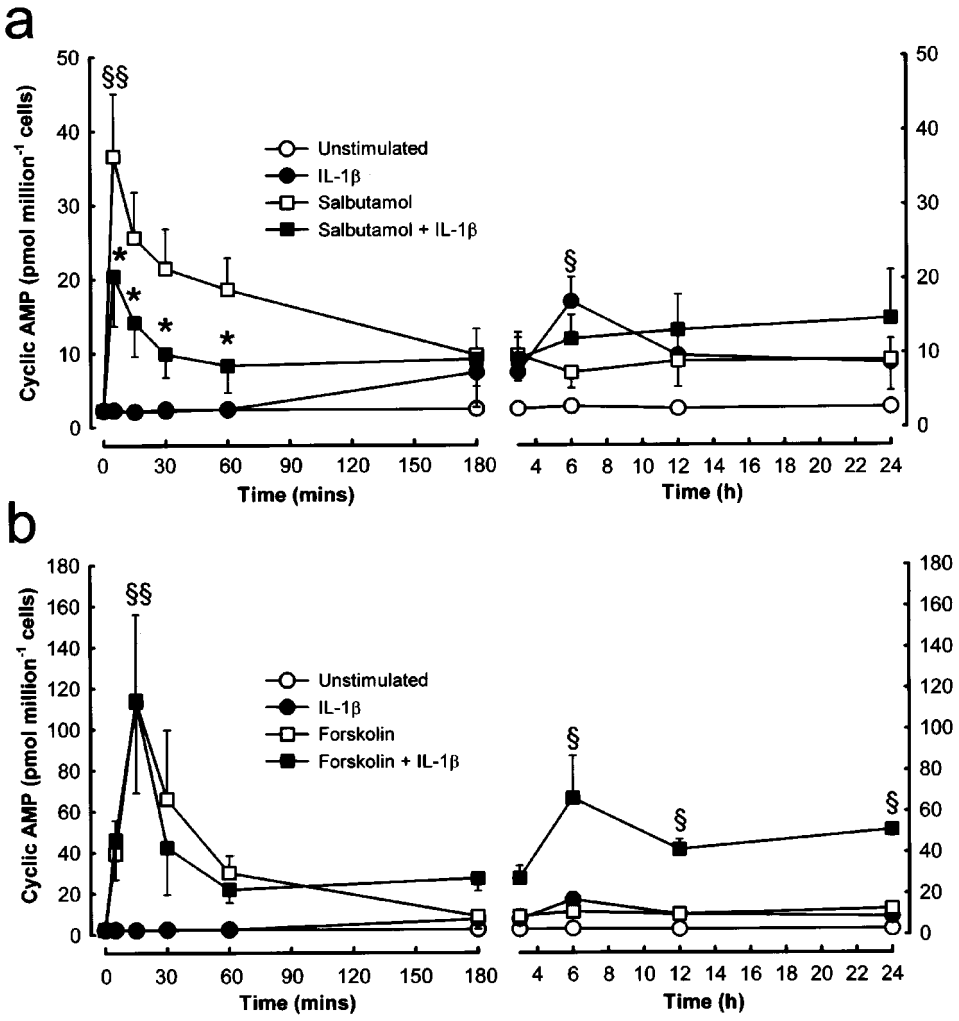

Figure 4 Effect of IL-1β (1 ng ml^{-1}) on (a) salbutamol (1 μM)- and (b) forskolin (10 μM)-stimulated increases in intracellular cyclic AMP levels in human airway smooth muscle cells. Data represent mean$\pm$s.e.mean of duplicate values from independent experiments using cells cultured from four different donors, cell passages 3–4. *$P<0.05$ compared to salbutamol alone at similar time points by Bonferroni's t-test. §$P<0.05$, §§$P<0.01$ compared to unstimulated levels at similar time points by Bonferroni's t-test.

Effects of a cyclic AMP-dependent protein kinase antagonist on salbutamol-induced attenuation of IL-1β-stimulated eosinophil-activating cytokine release

Incubation with H-89 was used to determine whether the observed attenuation by salbutamol of IL-1β-stimulated cytokine release from human airway smooth muscle cells was cyclic AMP protein kinase (PKA)-dependent. Pre-treatment for 30 min with H-89 (2 μM) prevented the inhibitory effect of salbutamol (1 μM) on GM-CSF, RANTES and eotaxin release in cells that had been stimulated by 1 ng ml^{-1} IL-1β (Table 3). H-89 caused a small increase in the response to IL-1β alone, though this did not reach statistical significance for any of the cytokines measured ($P>0.05$, $n=5$) (Table 3). Changes in IL-8 levels were not examined since attenuation by β-adrenoceptor agonists was not found in previous experiments (Figure 2).

Effect of indomethacin on the efficacy of salbutamol-induced attenuation of cytokine release from human airway smooth muscle cells

To investigate the possible contribution of cyclo-oxygenase products in β-adrenoceptor dysfunction following stimulation with IL-1β, the effect of a selective cyclo-oxygenase inhibitor, indomethacin was examined on the efficacy of salbutamol-induced attenuation of cytokine release from human airway smooth muscle (Figure 5). Indomethacin (1 μM) significantly ($P<0.05$, $n=5$) increased IL-1β-stimulated release of GM-CSF (Figure 5a). In the same cell-conditioned supernatants, levels of RANTES, eotaxin and IL-8 were unchanged ($P>0.05$, $n=5$). The presence of indomethacin did not subsequently alter either the efficacy or potency of salbutamol in attenuating IL-1β-induced cytokine release (Figure 5).

Effect of pertussis toxin on salbutamol-induced attenuation of cytokine release from human airway smooth muscle cells

Incubation with pertussis toxin (PTX) was examined to determine whether activation of the inhibitory G protein (G_i) by IL-1β contributed to the low efficacy of salbutamol in preventing cytokine release. Pre-treatment with PTX (50 ng ml^{-1}) for 18 h enhanced IL-1β-stimulated eotaxin release by approximately 1.5 fold ($P<0.05$, $n=4$). In contrast, PTX had no effect on GM-CSF or RANTES release ($P>0.05$, $n=4$), but reduced IL-8 release by up to 20% ($P<0.05$, $n=4$) (Figure 6). PTX did not affect the baseline release of any of the cytokines assayed ($n=4$, data not shown).

When examined on the attenuation of IL-1β-stimulated cytokine release induced by a threshold concentration of salbutamol (10 nM), PTX reduced GM-CSF and RANTES release by approximately 15–20% (Figure 6). However, these changes were not statistically significant ($P>0.05$, $n=4$) and were not observed for eotaxin release (Figure 6). No significant attenuation of IL-8 release was caused by salbutamol (10 nM) and this was not altered by the presence of PTX.

Table 3 H-89 prevents salbutamol-induced attenuation of eosinophil-activating cytokine release from IL-1β-stimulated human airway smooth muscle cells

	Cytokine measured		
Treatment	*GM-CSF*	*RANTES*	*Eotaxin*
IL-1β alone	100	100	100
IL-1β + Salb	64 ± 7*	43 ± 5**	56 ± 7**
IL-1β + Salb + H89	118 ± 8#	92 ± 7#	96 ± 4#
IL-1β + H89	128 ± 12#	119 ± 7#	96 ± 5#

Cells were pre-treated with or without H-89 (2 μM) for 30 min before incubation with either IL-1β (1 ng ml^{-1}) alone or IL-1β and salbutamol (1 μM) in combination for 24 h. Cytokine release was measured by ELISA as described in Methods. Data are expressed as a per cent of the control response to IL-1β alone and represent mean ± s.e.mean of duplicate values from independent experiments using cells cultured from five different donors, cell passages 3–6. *$P<0.05$, **$P<0.01$ by Bonferroni's *t*-test compared to cells treated with IL-1β alone. #$P>0.05$ by ANOVA.

Discussion

Our results demonstrate that both IL-1β and TNFα induced the simultaneous release from human airway smooth muscle cells of biologically-relevant quantities of several eosinophil-activating cytokines, including GM-CSF, RANTES, eotaxin and IL-8. Release of GM-CSF was regulated primarily by IL-1β; a maximally effective concentration of TNFα induced less than 5% of that induced by IL-1β. In keeping with other published reports from airway smooth muscle, both IL-1β and TNFα were effective stimulators of eotaxin, RANTES and IL-8 release. TNFα produced approximately 2 fold higher levels of RANTES (John *et al*., 1997) and eotaxin (Chung *et al*., 1999), compared to IL-1β; whilst IL-1β induced higher levels of IL-8 (John *et al*., 1998). Our data provide the first direct evidence that the release of GM-CSF, RANTES and eotaxin is negatively linked to increases in intracellular cyclic AMP content following β_2-adrenoceptor activation in human airway smooth muscle.

In the airways β-adrenoceptor agonists mediate their effects primarily by activating β_2-adrenoceptors on airway smooth muscle. The inhibitory effects of salbutamol were prevented by concentrations of propranolol and ICI 118551 that are considered specific for interaction at β_2-adrenoceptors (Hall *et al*., 1992). However, propranolol itself caused a small decrease in IL-1β-stimulated GM-CSF and eotaxin release. The reason for this effect is unclear; it seems unlikely that the release of these cytokines by IL-1β alone involves a β-adrenoceptor component. A similar unexplained effect of propranolol was reported previously on thrombin-stimulated [H^3]-thymidine uptake (Tomlinson *et al*., 1994) and probably reflects a non-specific property of the compound. Nevertheless, complete block of the inhibitory response to the β_2-adrenoceptor agonist, salbutamol by the β_2-adrenoceptor-selective antagonist, ICI 118551 constitutes good evidence that β_2-adrenoceptors mediate the effect. A role for β_1-adrenoceptors in this response cannot entirely be excluded, since a β_1-adrenoceptor-selective antagonist was not examined. However, previous work has shown by the use of selective antagonists (Hall *et al*., 1992) and in binding studies (Green *et al*., 1995) that human airway smooth muscle cells express a single population of β-adrenoceptors of the β_2 subtype.

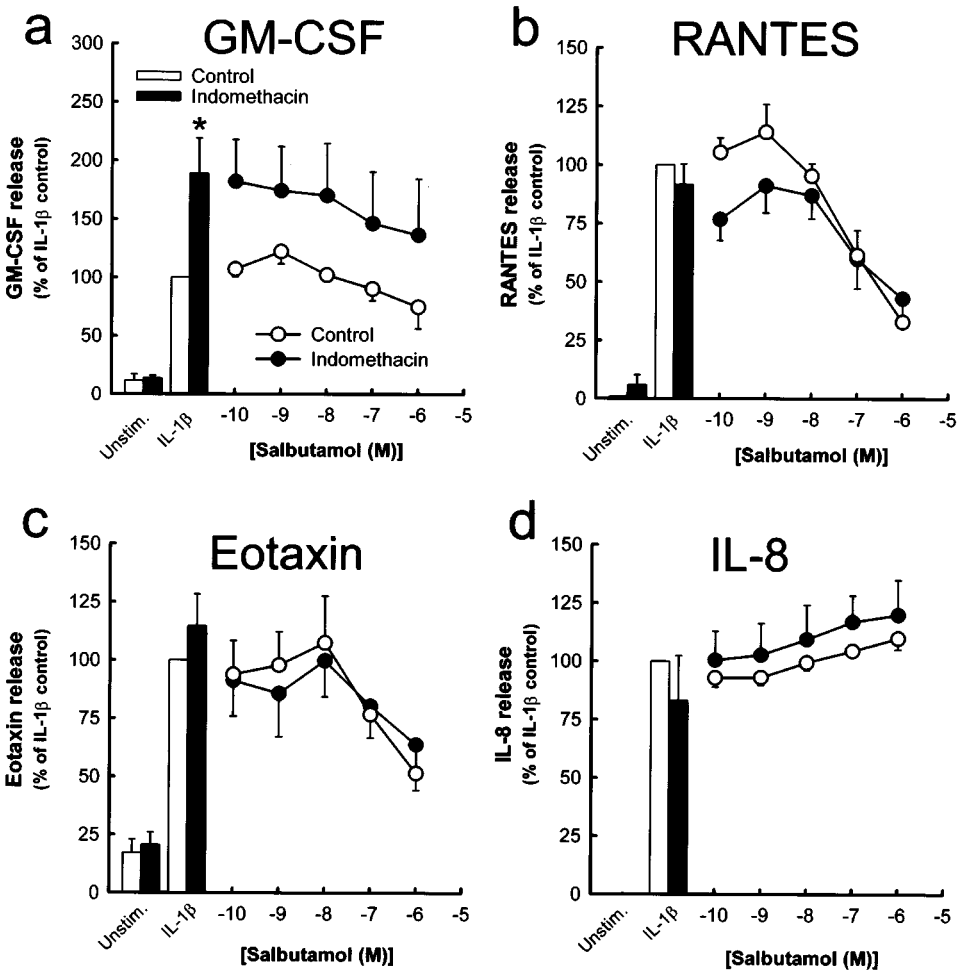

Figure 5 Effect of indomethacin (1 μM) on the attenuation by salbutamol of (a) GM-CSF, (b) RANTES, (c) eotaxin and (d) IL-8 release from human cultured airway smooth muscle cells stimulated with IL-1β (1 ng ml^{-1}) for 24 h. Data are expressed as a per cent of the control response to IL-1β alone and represent mean$\pm$s.e.mean of duplicate values from independent experiments using cells cultured from five different donors, cell passages 3–5. *$P<0.05$ compared to control by Bonferroni's t-test.

In most cell systems activation of β_2-adrenoceptors promotes activation of the adenylyl cyclase-linked G-protein, G_s, an increase in intracellular cyclic AMP levels and activation of the cyclic AMP-dependent protein kinase (PKA). Consistent with the involvement of PKA, the observed attenuation by salbutamol of IL-1β-stimulated cytokine release was prevented by H-89, a highly potent inhibitor of PKA. This effect occurred at concentrations that have been reported to be selective for PKA in other cell systems (Chijawa *et al*., 1990). However, in a very recent report by Penn *et al*. (1999) it was shown at concentrations similar to that used in the present study, that H-89 may also prevent ligand binding to β_2-adrenoceptors. Thus, caution must be exercised when implicating a direct role for PKA in the signal transduction processes in these cells. In many tissues and cells isoprenaline and salbutamol have similar efficacies at the β_2-adrenoceptor and are considered to be full agonists (Goldie *et al*., 1986). In human airway smooth muscle, isoprenaline and salbutamol have the capacity to reverse completely both agonist-induced contraction (Goldie *et al*., 1996) and mitogenesis (Tomlinson *et al*., 1994). This was not the case, however, against cytokine release where maximally-effective concentrations of salbutamol or isoprenaline achieved only a modest inhibitory effect against GM-CSF, RANTES and eotaxin release, and were without effect against IL-8. In the treatment of asthma, normal therapeutic doses may therefore be sub-maximal for the direct inhibitory effect of salbutamol on GM-CSF, RANTES and eotaxin release from airway smooth muscle, and may be completely ineffective against IL-8 release. One explanation for this discrepancy may lie in the choice of agonists (i.e. IL-1β and TNFα) that were used to induce eosinophil-activating cytokine release. We postulated that in addition to induction of cytokine release, IL-1β and TNFα may also cause impaired responsiveness to β-adrenoceptor agonists limiting their therapeutic value and efficacy against the release of smooth muscle-derived cytokines. Further, Shore *et al*. (1997) have previously reported that IL-1β significantly inhibits the ability of human airway smooth muscle cells to decrease their stiffness responses (proposed as a surrogate for contraction in cultured cells) to isoprenaline. A similar effect of IL-1β and TNFα on impairment of β-adrenoceptor agonist responsiveness has been demonstrated for relaxation of isolated rabbit airways (Hakonarson *et al*., 1996). Similar findings were reported by Emala *et al*. (1997) who showed that TNFα reduced adenylyl cyclase activity in canine airway smooth muscle cells. However, in all of these

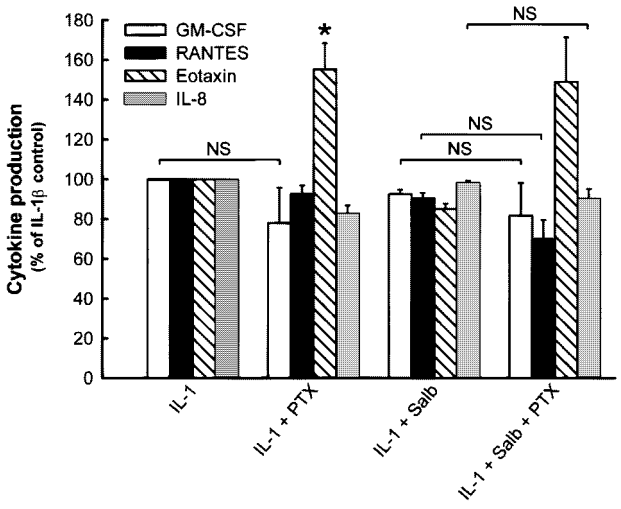

Figure 6 Effect of pertussis toxin pretreatment (PTX, 50 ng ml^{-1} for 18 h) on the attenuation by salbutamol of eosinophil-activating cytokine release from human cultured airway smooth muscle cells stimulated with IL-1β (1 ng ml^{-1}) for 24 h. Data are expressed as a per cent of the control response to IL-1β alone and represent mean$\pm$s.e.mean of duplicate values from independent experiments using cells cultured from four different donors, cell passages 4–6. *$P<0.05$ compared to IL-1β alone by Bonferroni's t-test. NS $P>0.05$ by ANOVA.

systems the impairment was overcome when direct activators of adenylyl cyclase such as forskolin, or cell permeable, non-hydrolysable, phosphodiesterase-resistant, cyclic AMP analogues such as dibutyryl cyclic AMP were examined, suggesting that the effect of IL-1β lay upstream of the effects of cyclic AMP production. Consistent with this explanation, we observed that complete inhibition of GM-CSF, eotaxin and RANTES release occurred when by-passing the β-adrenoceptor with dibutyryl cyclic AMP, forskolin or cholera toxin. In addition, we found that IL-1β impaired cyclic AMP formation due to a maximally-effective concentration of salbutamol, but not to forskolin, consistent with earlier reports in human cultured airway smooth muscle cells (Pang *et al*., 1998; Moore *et al*., 1999). Taken together, our data support some aspects of the hypothesis suggested by others for attenuated airway smooth muscle relaxation (Hakonarson *et al*., 1996; Shore *et al*., 1997; Laporte *et al*., 1998; Pang *et al*., 1998), that IL-1β does not alter the ability of cyclic AMP to activate the downstream targets which ultimately mediate cytokine release, but more instead involves the up-stream targets which lead to cyclic AMP formation. Our observation that cholera toxin also completely inhibited cytokine release suggests that any effect of IL-1β in attenuating the response to isoprenaline or salbutamol lies up-stream of the adenylyl cyclase-linked G-protein, G_s. Several groups have reported that in both rabbit and human airway smooth muscle neither IL-1β or TNFα cause significant changes in β_2-adrenoceptor number or binding affinity for isoprenaline or salbutamol (Hakonarson *et al*., 1996; Shore *et al*., 1997; Laporte *et al*., 1998), or in the expression of $G_s\alpha$ (Laporte *et al*., 1998). Thus, the locus for the effects of IL-1β or TNFα may involve uncoupling β_2-adrenoceptors from G_s-induced activation of cyclase, as suggested by others examining changes in airway smooth muscle contraction (Hakonarson *et al*., 1996) or stiffness (Shore *et al*., 1997).

Hakonarson *et al*. (1996) have reported that IL-1β increases expression of the inhibitory G protein alpha ($G_i\alpha$) subunit in rabbit airway smooth muscle. In human airway smooth muscle TNFα was recently shown to up-regulate $G_i\alpha$ with no effect on $G_s\alpha$ proteins (Hotta *et al*., 1999). Adenylyl cyclase is under the dual regulation of both stimulatory G_s proteins and inhibitory G_i proteins. Competition for interaction with the adenylyl cyclase caused by increased $G_i\alpha$ protein in the absence of changes in $G_s\alpha$ expression is postulated to enhance the inhibitory pathway, leading to enhanced coupling of G_i-linked receptors (e.g. muscarinic M_2 receptors), impairment of adenylyl cyclase activation and reduced cyclic AMP levels (Hakonarson *et al*., 1996; Hotta *et al*., 1999). Indeed, activation of M_2 receptors has been shown to inhibit cyclic AMP formation in response to β-adrenoceptor agonists in human airway smooth muscle cells and this effect is blocked by pertussis toxin (PTX) (Widdop *et al*., 1993) which ADP-ribosylates $G_i\alpha$ and inactivates the G_i protein. We reasoned that if a G_i component did contribute to cytokine release, then its removal by pretreatment with PTX might prevent any impairment of adenylyl cyclase activity induced by IL-1β and augment the inhibition of cytokine release by a sub-maximal concentration of salbutamol. Whilst some evidence of this was found for IL-1β-stimulated RANTES, and to an extent with GM-CSF release, the effect was small and was complicated by the unexpected effect of PTX on IL-1β-stimulated cytokine release in the absence of salbutamol; PTX markedly and selectively enhanced eotaxin release, but caused around 10–15% reduction in IL-1β-stimulated GM-CSF, RANTES and IL-8 release. Selective enhancement of eotaxin release suggests a possible role for G_i in tonically regulating IL-1β-stimulated release of this chemokine, though the precise mechanism for this effect requires further investigation. Overall, we conclude that PTX has no marked effect on the ability of IL-1β to attenuate β_2-adrenergic responses, suggesting that activation of G_i is not a major factor in modulating the action of IL-1β in human airway smooth muscle in culture. This contrasts with a previous report by Hakonarson *et al*. (1996) in rabbit tracheal smooth muscle, but is in general agreement with a later study by Shore *et al*. (1997) in human airway smooth muscle cells.

An additional explanation for the poor efficacy of isoprenaline and salbutamol in preventing cytokine release by IL-1β or TNFα may be because these cytokines also activate cyclic AMP-dependent mechanisms in human airway smooth muscle cells, thereby further stimulation by β_2-adrenoceptor agonists can be of little further functional consequence. However, there are features of the response which do not support this explanation. Thus, if IL-1β or TNFα activate these mechanisms it is unlikely that agents that further elevate intracellular cyclic AMP would be so effective in preventing cytokine release. Secondly, although our cyclic AMP data demonstrated that IL-1β did increase cyclic AMP levels, this occurred only at later time points (2–6 h), and was found to be abolished by indomethacin pretreatment. This almost certainly reflected the cyclic AMP elevating activity of cyclo-oxygenase (COX) products as PGE_2, the major arachidonic acid metabolite released by airway smooth muscle following stimulation by IL-1β (Belvisi *et al*., 1997; Pang & Knox, 1997), and produced as a result of induction of the COX-2 isoform (Belvisi *et al*., 1997; Pang &

Knox, 1997). Thus IL-1β, whilst it may attenuate early increases (up to 1 h) in cyclic AMP levels induced by β_2-adrenoceptor agonists such as salbutamol, it also induces a later sustained increase in cyclic AMP levels which potentially could limit the levels of cytokine release and the efficacy of any agonist acting through cyclic AMP-linked receptors, including β_2-adrenoceptors. However, in spite of the effectiveness of indomethacin, a mixed COX-1/COX-2 inhibitor, in abolishing the late cyclic AMP response to IL-1β and selectively increasing IL-1β-stimulated GM-CSF release, it failed to improve the efficacy of either salbutamol or isoprenaline in attenuating IL-1β-stimulated cytokine release, suggesting that while the levels of GM-CSF released by IL-1β-stimulated cells appeared to be tonically regulated by an indomethacin/COX-dependent pathway, the processes by which IL-1β might induce β_2-adrenoceptor hyporesponsiveness for cytokine release are independent of this mechanism in human airway smooth muscle. Furthermore, in the present study salbutamol also attenuated cytokine release induced by TNFα, which does not induce PGE_2 (Belvisi *et al*., 1997) release or induction of COX-2 (Pang & Knox, 1997). These data are in contrast to the proposed COX-dependent pathway reported by Laporte *et al*. (1998) and further investigations are required to resolve this.

In addition to possible mechanisms of heterologous desensitization induced by either IL-1β or TNFα, homologous desensitization of the β_2-adrenoceptor may also be a contributing factor in the limited efficacy of isoprenaline and salbutamol. In human airway smooth muscle cell cultures classical homologous desensitization, characterized by marked reductions in maximal cyclic AMP formation and increases in EC_{50}, occurs after only 30 min exposure of the cells to isoprenaline (Penn *et al*., 1998). Overexpression of the G protein-coupled receptor kinase 2 (GRK2) is reported to enhance isoprenaline-induced desensitization, suggesting that endogenous levels of GRK2 may be important in limiting the magnitude of desensitization in these cells (Penn *et al*., 1998). Further studies are required to examine the possible role GRK2 in the attenuation of cytokine release from human airway smooth muscle cells by β_2-adrenoceptor agonists, as well as its regulation by pro-inflammatory cytokines such as IL-1β or TNFα.

A consistent finding in this study was that β-adrenoceptor agonists did not attenuate IL-8 release from human airway smooth muscle cells stimulated with either IL-1β or TNFα. Distinct adenylyl cyclase subtypes have been characterized in human airway smooth muscle, and types VI and IX appear to be the most abundantly expressed (Billington *et al*., 1999). A possible explanation for the poor efficacy of isoprenaline and salbutamol in preventing IL-8 release, compared to GM-CSF, RANTES and eotaxin is that elevation in intracellular cyclic AMP levels may lead to differential gene regulation through recruitment of specific adenylyl cyclase subtypes. Alternatively, sensitization of specific adenylyl cyclase subtypes may also contribute to selective gene regulation and may explain in part why cyclic AMP levels remained elevated (in the absence of phosphodiesterase inhibitors) in response to both salbutamol and forskolin in cells treated with IL-1β (see Figure 4). Furthermore, in a human transformed bronchial epithelial cell line (16HBE), β_2-adrenoceptor activation was reported to increase TNFα-stimulated IL-8 release and this was mimicked by dibutyryl cyclic AMP (Linden, 1996). Similarly, Pang & Knox (2000) have recently reported that cyclic AMP-elevating agents, including β_2-adrenoceptor agonists and forskolin, release small quantities of IL-8 (up to 2% of the maximal response to TNFα) from human airway smooth muscle cells, though this effect occurred at relatively high concentrations and in the present study we failed to detect a similar increase in basal IL-8 release induced by either salbutamol, isoprenaline or forskolin. In colonic epithelial cells, using nuclear run-on assays, forskolin and PGE_2 have been shown to enhance the stability and not the rate of IL-8 mRNA transcripts, suggesting that regulation of the IL-8 gene by agonists that elevate cyclic AMP may involve a post-transcriptional mechanism (Yu & Chadee, 1998). In keeping with the absence of an inhibitory role for cyclic AMP in regulation of the IL-8 gene, we found that dibutyryl cyclic AMP, forskolin and cholera toxin were much less effective in preventing IL-1β-stimulated IL-8 release, compared to the release of GM-CSF, RANTES and eotaxin measured in the same cell culture supernatants. Thus, IL-8 release served as a useful control in the present study confirming the specific nature of the β-adrenoceptor agonist response and the likely role of cyclic AMP in regulating cytokine release from these cells.

In summary, experiments were performed to characterize the effectiveness of β-adrenoceptor agonists in preventing the release of eosinophil-activating cytokines from human airway smooth muscle cells that were stimulated by cytokines such as IL-1β and TNFα. Our results indicate that activation of β_2-adrenoceptors is negatively-linked to the release of GM-CSF, RANTES and eotaxin, but not IL-8, and that this is dependent on the production of intracellular cyclic AMP and the possible subsequent activation of the cyclic AMP-dependent kinase pathway in human airway smooth muscle. Isoprenaline and salbutamol behaved as partial agonists and this may have resulted from impaired cyclic AMP formation due to functional uncoupling of the β_2-adrenoceptor by IL-1β, which was used to induce release of eosinophil-activating cytokines from these cells. Understanding the cellular mechanisms and intracellular pathways which modulate release of eosinophil-activating cytokines by airway smooth muscle may be important for the development of future therapeutic interventions that are targeted at the airway smooth muscle remodelling process in the airway wall of the diseased lung.

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