Mechanism of Cytokine-induced Modulation of β-Adrenoceptor Responsiveness in Airway Smooth Muscle

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Abstract

To elucidate the role of specific proinflammatory cytokines in regulating airway responsiveness, we examined the effects and mechanisms of action of IL-1β, TNF-α, and IL-2 on the β-adrenoceptor– and postreceptor-coupled transmembrane signaling mechanisms regulating relaxation in isolated rabbit tracheal smooth muscle (TSM) segments. During half-maximal isometric contraction of the tissues with acetylcholine, relaxation responses to isoproterenol, PGE_2, and forskolin were separately compared in control (untreated) TSM and tissues incubated for 18 h with IL-1β (10 ng/ml), TNF-α (100 ng/ml), or IL-2 (200 ng/ml). Relative to controls, IL-1β– and TNF-α–treated TSM, but not IL-2–treated tissues, depicted significant attenuation of their maximal relaxation and sensitivity (i.e., −log dose producing 50% maximal relaxation) to isoproterenol ($P < 0.001$) and PGE_2 ($P < 0.05$); whereas the relaxation responses to direct stimulation of adenylate cyclase with forskolin were similar in the control and cytokine-treated tissues. Further, the attenuated relaxation to isoproterenol and PGE_2 was ablated in the IL-1β–treated TSM that were pretreated with either the muscarinic M_2-receptor antagonist, methoctramine (10^{-6} M), or pertussis toxin (100 ng/ml). Moreover, Western immunoblot analysis demonstrated that: (*a*) G_i protein expression was significantly enhanced in membrane fractions isolated from IL-1β–treated TSM; and (*b*) the latter was largely attributed to induced enhanced expression of the $G_i\alpha_2$ and $G_i\alpha_3$ subunits. Collectively, these observations provide new evidence demonstrating that IL-1β and TNF-α induce impaired receptor-coupled airway relaxation in naive TSM, and that the latter effect is associated with increased muscarinic M_2-receptor/G_i protein–coupled expression and function. (*J. Clin. Invest.* 1996. 97:2593–2600.) Key words: interleukin 1 • tumor necrosis factor • guanosine 5′-triphosphate-binding regulatory proteins • muscarinic cholinergic receptors • β-adrenergic receptors

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Introduction

There is increasing evidence that a variety of cytokines, including the pleiotropic inflammatory mediators, IL-1β and TNF-α, play important roles in orchestrating and perpetuating the airway inflammatory response in asthma (1–7). In this regard, a number of studies have demonstrated increased concentrations of various cytokines in the bronchoalveolar lavage fluid isolated from allergic asthmatic individuals (8–10). While the precise role of each cytokine remains to be established, collectively, these agents have been found to exert a diversity of actions including induction of specific IgE production by mononuclear cells (11–12), antigen presentation by macrophages (13), activation of airway structural cells (5, 14–16), altered expression of adhesion molecules (17, 18), induced secretion of various growth factors (19, 20), recruitment of inflammatory cells from the circulation (21–24), and other proinflammatory events.

Apart from their above actions, there is also emerging evidence that specific cytokines, most notably IL-1β and TNF-α, also play an important role in regulating the changes in airway smooth muscle contractility which characterize the asthmatic phenotype. In this connection, exogenous administration of TNF-α to animals in vivo has been shown to induce bronchial constrictor hyperresponsiveness (6, 25), and treatment of isolated guinea pig airways with IL-1β or TNF-α has been found to reduce isoproterenol-mediated relaxation in vitro (26). Moreover, treatment of antigen-sensitized animals with an IL-1 receptor antagonist has been shown to inhibit both their in vivo bronchial hyperreactivity to histamine (2) or substance P (27), as well as the accompanying pulmonary infiltration with leukocytes, including eosinophils and neutrophils (2, 27, 28). Collectively, these recent observations support the compelling concept that certain proinflammatory cytokines may be directly involved in mediating changes in airway contractility. The potential mechanisms underlying such cytokine-induced pertubations in airway smooth muscle responsiveness, however, remain to be identified.

In addressing the above issue, this study examined the effects of specific proinflammatory cytokines on the relative contributions of β-adrenoceptor– and postreceptor-coupled transmembrane signaling mechanisms regulating airway relaxation. The results provide new evidence demonstrating that: (*a*) IL-1β and, to a lesser extent, TNF-α, induce attenuated β-adrenoceptor–mediated airway relaxation; (*b*) the latter is associated with enhanced muscarinic M_2-receptor–coupled activation of the guanine nucleotide binding protein, G_i; and (*c*) the enhanced G_i-coupled function is related to induced increased expression of the $G_i\alpha_2$ and $G_i\alpha_3$ subunits in membranes from cytokine-treated airway smooth muscle.

Methods

Animals. 28 adult New Zealand White rabbits were used in this study which was approved by the Biosafety and Animal Research Commit-

tee of the Joseph Stokes Research Institute at Children's Hospital of Philadelphia. The animals had no signs of respiratory disease for several weeks before the study.

Cytokine pretreatment and preparation of tissues. After anesthesia with xylazine (10 mg/kg) and ketamine (50 mg/kg), the animals were killed with systemic air embolism. The tracheas were removed via open thoracotomy, cleared of loose connective tissue, divided into eight ring segments of 6–8 mm in length, and incubated over 18 h at room temperature in Dulbecco's modified Eagle's medium containing either: IL-1β (10 ng/ml); TNF-α (100 ng/ml); IL-1β (10 ng/ml) and TNF-α (100 ng/ml) together; IL-2 (200 ng/ml); or medium alone containing no cytokine. The medium was aerated with a continuous supplemental O_2 mixture (95% O_2/5% CO_2) during the incubation phase.

Pharmacodynamic studies. After incubation, each airway segment was suspended longitudinally between stainless steel triangular supports in siliconized 20-ml organ baths (Harvard Apparatus, Inc., South Natick, MA). The lower support was secured to the base of the organ bath, and the upper support was attached via a gold chain to a force transducer (FT.03C; Grass Instrument Co., Quincy, MA) from which isometric tension was continuously displayed on a multichannel recorder. Care was taken not to injure the epithelia and to place the membranous portion of the trachea between the supports to maximize the recorded tension generated by the contracting trachealis muscle.

The tissues were bathed in modified Krebs-Ringer solution containing (mM) 125 NaCl, 14 NaHCO$_3$, 4 KCl, 2.25 CaCl$_2$·2H$_2$O, 1.46 MgSO$_4$·7H$_2$O, 1.2 NaH$_2$PO$_4$·H$_2$O, and 11 glucose. The baths were aerated with 5% CO_2 in oxygen, a pH of 7.35–7.40 was maintained, and the temperature was held at 37°C. Passive resting tension of each tracheal smooth muscle (TSM)[1] segment was set at 2.0 g after each tissue had been passively stretched to a tension of 8 g to optimize the resting length of each segment (29). The tissues were allowed to equilibrate in the bath for 45 min, at which time each tissue was primed with a 1-min exposure to 10^{-4} M acetylcholine (ACh). Cholinergic contractility was initially assessed in the TSM by cumulative administration of ACh in final bath concentrations ranging from 10^{-9} to 10^{-3} M. After thorough rinsing, each tissue segment was then half-maximally contracted with individual concentrations of ACh in the absence and presence of the muscarinic M$_2$-receptor antagonist methoctramine (10^{-6} M). Thereafter, in separate studies, the tissues were treated with cumulative administration of isoproterenol (10^{-9} to 10^{-4} M), PGE$_2$ (10^{-9} to 10^{4} M), or forskolin (10^{-9} to 10^{-4} M).

In other studies, relaxation dose–response curves to isoproterenol (10^{-9} to 10^{-4} M) and forskolin (10^{-9} to 10^{-4} M) were conducted in paired IL-1β–treated and control tissues in the presence and absence of ADP ribosylation of G$_i$ by pretreatment of the tissues for 18 h with pertussis toxin (PT; 100 ng/ml) (30). Additionally, in separate experiments, relaxation responses to isoproterenol were assessed in TSM segments in the presence and absence of the IL-1 receptor antagonist (150 ng/ml). The relaxant responses to isoproterenol, PGE$_2$, and forskolin were analyzed in terms of percent maximal relaxation (R$_{max}$) from the active cholinergic contraction, and sensitivity to the relaxing agent was determined as the negative logarithm of the dose of the relaxing agent producing 50% of R$_{max}$ (pD$_{50}$) (i.e., geometric mean ED$_{50}$ value).

Determination of G$_i$ protein expression. Expression of the inhibitory G$_i$ protein and its α-subunits was assayed by Western blot analysis of membrane protein samples isolated from both IL-1β–treated ($n = 6$) and control ($n = 6$) rabbit TSM in two separate experiments. The membrane protein samples were prepared as follows. Trachealis muscle was minced and homogenized using a Dounce tissue grinder

(Wheaton Industries, Millville, NJ) in 40 vol of 50 mM Tris-HC1, 150 mM NaCl, 1 mM EDTA (pH 7.4) containing 1 mM PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. Nuclei and large particulates were removed by centrifugation at 100 g for 5 min. The supernatant was then centrifuged at 100,000 g for 1 h to pellet the membrane fractions. The membrane pellet was resuspended in the same Tris-EDTA buffer, and the protein concentration was measured using the Lowry assay. Equivalent amounts (30–50 μg) of membrane protein were fractionated in 11% SDS-polyacrylamide gels followed by transfer to nitrocellulose membranes. The membranes were then blotted overnight at room temperature in 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tergitol NP-40 containing 5% nonfat milk. The rabbit polyclonal anti–G$_i$-common, G$_i\alpha_1$, G$_i\alpha_2$, and G$_i\alpha_3$ antibodies were diluted at 1:1,000, 1:500, 1:500, and 1:500, respectively, and were incubated for 2 h at room temperature. The G$_i$-common antiserum recognizes G$_i\alpha_1$, G$_i\alpha_2$, and G$_i\alpha_3$ equivalently (31). The primary G$_i$ antibodies were the generous gift of Dr. David Manning (University of Pennsylvania). All primary and secondary antibody incubations and washes were done in 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% NP-40 containing 0.50% nonfat milk. The G$_i$ proteins were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL) after a 1-h incubation with a 1:3,000 dilution of an anti–rabbit horseradish peroxidase–linked secondary antibody and subsequent exposure to autoradiography film. Expression levels of common and specific G$_i\alpha$-subunit proteins were quantitated using laser densitometry (Bio Rad Laboratories, Hercules, CA).

Determination of cAMP accumulation. To determine whether β-adrenoceptor–mediated cAMP accumulation was altered by IL-1β, isoproterenol-stimulated time- and dose-dependent cAMP generation was assayed in IL-1β–treated ($n = 6$) and control ($n = 6$) tissues. For the time–response studies, TSM were isolated and prepared as described above. The airway epithelium was removed by scraping and, after resecting the cartilage, the trachealis muscle was divided into separate segments and each segment was exposed to isoproterenol (10^{-5} M) for 0, 0.5, 1, 2, 3, or 5 min. For the dose–response studies, the cAMP level was determined at 1 min after exposure of IL-1β–treated and control TSM to varying concentrations of isoproterenol (10^{-7} to 10^{-4} M). In all experiments, the tissues were treated with the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (10^{-5} M), for 30 min before isoproterenol administration. In these studies, after homogenization of the tissues, cAMP generation was determined with the use of a commercially available radioimmunoassay, with [^{3}H]cAMP as tracer (Amersham). The tissues' protein concentration was assayed using the Lowry method, and the cAMP measurements were expressed in units of picomoles per milligram of tissue membrane protein.

Radioligand binding studies. To identify whether muscarinic receptor maximal binding capacity (B$_{max}$) and dissociation constant (K$_d$) were altered by IL-1β, membrane preparations from IL-1β–treated ($n = 4$) and control ($n = 4$) trachealis tissues were treated with the hydrophilic selective muscarinic radioligand, [N-methyl-^{3}H]scopolamine, in the presence and absence of atropine. The membrane particulate fractions were isolated from TSM, minced with scissors in buffer containing 10 mM Tris, 1 mM EDTA (pH 8.0) at 30°C, and manually homogenized with a glass homogenizer followed by centrifugation at 40,000 g for 15 min. The resulting pellet was resuspended in an appropriate volume of 10 mM Tris buffer. The final assay volume was 1 ml, with a final tissue concentration of 40–70 mg/ml. The protein concentration was determined by the Lowry method, using BSA as the standard. Increasing concentrations of [N-methyl-^{3}H]scopolamine (5–650 pM, i.e., 4× concentration above and below estimated K$_d$) were added, and nonspecific binding was determined in parallel incubations containing 1 mM atropine sulfate to assess for specific binding. Subsequently, ice-cold buffer was added, the samples were filtrated under low vacuum to separate free and bound [N-methyl-^{3}H]scopolamine, the filters were dried, and the retained receptor-bound radioactivity was counted by liquid scintillation. Specific binding was analyzed as a function of free ligand concentration, using an iterative

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1. *Abbreviations used in this paper:* ACh, acetylcholine; B$_{max}$, receptor density; pD$_{50}$ (i.e., $-$log ED$_{50}$); PT, pertussis toxin; R$_{max}$, maximum relaxation response; T$_{max}$, maximum constrictor response; TSM, tracheal smooth muscle.

nonlinear curve fitting program (Ligand) to derive the determinations of K_d and B_{max}.

Statistical analysis. Unless otherwise indicated, results are expressed as mean±SE. Statistical analysis was performed by means of two-tailed paired Student's *t* test. *P* values < 0.05 were considered significant. In the radioligand-binding assays, the K_d and B_{max} of specific [*N*-methyl-^{3}H]scopolamine binding were obtained by Scatchard analysis.

Reagents. IL-1β, TNF-α, IL-2, and IL-1 receptor antagonist were obtained from R&D Systems (Minneapolis, MN). ACh, isoproterenol hydrochloride, PGE$_2$, atropine sulfate, 3-isobutyl-1-methylxanthine, and PT were obtained from Sigma Chemical Co. (St. Louis, MO). Methoctramine tetrahydrochloride was obtained from Research Biochemicals Inc. (Natick, MA). All drug concentrations are expressed as final bath concentrations. Forskolin was dissolved in dimethyl-sulfoxide (Sigma) to prepare a 10^{-4} M solution; and dilutions of 10^{-5} M and lower were prepared in demineralized H$_2$O. Isoproterenol and ACh were made fresh for each experiment, dissolved in normal saline to prepare 10^{-4} M and 10^{-3} M solutions, respectively. PGE$_2$ and methoctramine were dissolved in demineralized H$_2$O to prepare 10^{-4} M and 10^{-6} M solutions, respectively. Addition of these diluents to the organ bath at the appropriate concentrations had no effect on the resting nor half-maximal cholinergic tone of the TSM.

Results

Cytokine effects on cholinergic contractility. To assess the actions of specific proinflammatory cytokines in regulating the TSM constrictor response to cholinergic stimulation, contractile dose–response relationships to ACh were separately compared between IL-1β–, TNF-α–, or IL-2–treated tissues and their respective paired control (untreated) TSM segments. In tissues incubated for 18 h with a maximally effective concentration of IL-1β (10 ng/ml), maximal constrictor responsiveness to ACh was significantly increased, wherein the mean± SE maximal isometric force (T_{max}) amounted to 140.0±6.8 grams/gram of tissue weight, compared with the value of 116.6±5.7 grams/gram of tissue weight obtained in corresponding controls (*P* < 0.01) (Fig. 1 *A*). However, constrictor sensitivity to ACh was similar in the IL-1β–treated and control tissues, wherein the pD$_{50}$ (i.e., −log ED$_{50}$) values amounted to 5.50±0.04 and 5.52±0.05 −log M, respectively. In contrast to IL-1β, neither the T_{max} nor pD$_{50}$ values for ACh were significantly different between the TNF-α– or IL-2–treated tissues and their respective controls. However, when maximally effective concentrations of IL-1β (10 ng/ml) and TNF-α (100 ng/ml) were administered together, the mean T_{max} and pD$_{50}$ values to ACh were significantly synergistically enhanced and amounted to 152.8±10.2 grams/gram of tissue weight and 5.78±0.05 −log M vs. 127.8±6.9 grams/gram of tissue weight (*P* < 0.001) and 5.27±0.16 −log M (*P* < 0.01) in the corresponding controls, respectively.

Cytokine effects on airway relaxation. In separate studies, during comparable levels of initial sustained ACh-induced contractions in IL-1β–treated and control airway segments, averaging 37.9±0.9 and 39.7±1.4% of T_{max}, respectively, administration of the β-adrenergic agonist, isoproterenol, PGE$_2$, or the direct adenylate cyclase activator, forskolin, elicited cumulative dose-dependent relaxation of the precontracted TSM segments. Relative to their respective controls, the R$_{max}$ and pD$_{50}$ to isoproterenol (Fig. 2 *A*) and PGE$_2$ (Fig. 2 *B*) were significantly attenuated in the IL-1β–treated tissues. Accordingly, the mean R$_{max}$ values for isoproterenol amounted to 34.6±2.6 and 55.0±4.0% in the IL-1β–treated and control TSM, respec-
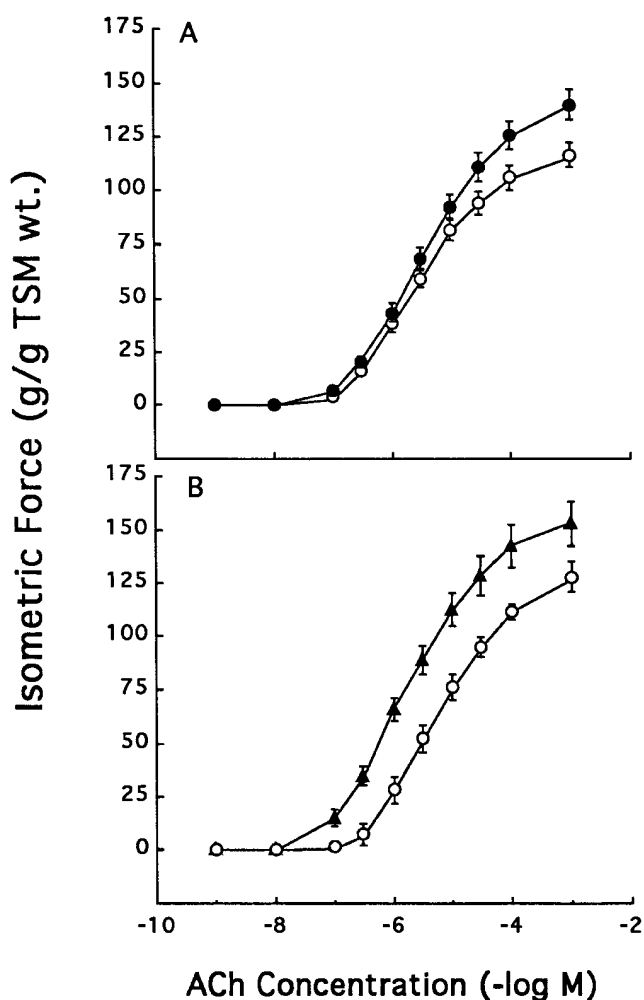

Figure 1. Comparison of contractile dose–response relationships to ACh in (*A*) control (*open circles*) and IL-1β–treated (*closed circles*) TSM; and (*B*) control (*open circles*) and combined IL-1β–and TNF-α–treated (*closed triangles*) TSM segments under initial baseline passive tension. Data represent means±SE from six paired experiments.

tively (*P* < 0.001), with corresponding pD$_{50}$ values of 6.30±0.05 and 6.53±0.05 −log M, respectively (*P* < 0.01). Similarly, the mean R$_{max}$ values of PGE$_2$ averaged 52.6±4.5 and 68.0±6.6% in the IL-1β–treated and control tissues, respectively (*P* < 0.05), with corresponding pD$_{50}$ values of 5.70±0.68 and 6.03±0.09 −log M, respectively (*P* < 0.01). In contrast, there were no differences in the relaxation responses to forskolin between the tissue groups, wherein the mean R$_{max}$ values averaged 92.0±2.1 and 95.0±2.8% in IL-1β–treated and control TSM segments, respectively (*P* = 0.85), and the corresponding pD$_{50}$ values were similar at 6.29±0.11 and 6.36±0.07 −log M, respectively (*P* = 0.64). Thus, whereas reversal of ACh-induced airway constriction with isoproterenol and PGE$_2$, both of which are associated with receptor/G protein–coupled activation of adenylate cyclase, was attenuated in IL-1β–treated tissues, the relaxant effectiveness of forskolin, a diterpene that directly activates the catalytic unit of adenylate cyclase, was unaltered in the tissues treated with IL-1β. Of note, the above IL-1β–induced changes in β-adrenoceptor re-

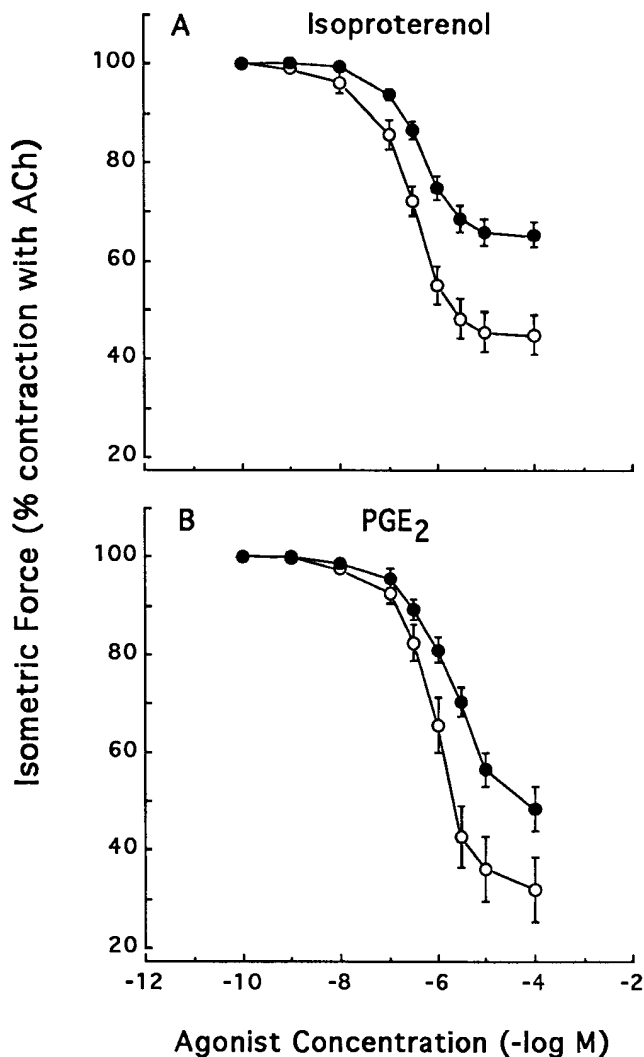Figure 2. Comparison of airway relaxation responses to isoproterenol (A) and PGE_2 (B) in paired control (*open circles*) and IL-1β–treated (*closed circles*) TSM segments half-maximally contracted with their respective ED_{50} doses of ACh. Each data point represents mean±SE from 8–10 paired tissue samples.
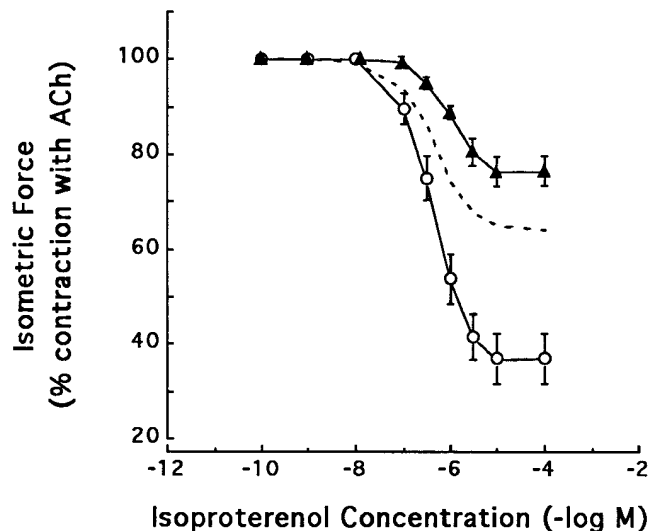

Figure 3. Comparison of airway relaxation responses to isoproterenol in paired control (*open circles*) and combined IL-1β– and TNF-α–treated (*closed circles*) TSM segments half-maximally contracted with their respective ED_{50} doses of ACh. The dashed function represents the relationship obtained in tissues treated with IL-1β alone, as depicted in Fig. 2 A. Each data point represents mean±SE from six paired tissue samples.

sponsiveness were prevented by coincubation of the airway segments with the human recombinant IL-1 receptor antagonist (150 ng/ml), while the latter alone had no effect on either the contractile responses to ACh or relaxation responsiveness to isoproterenol in control tissues (data not shown).

Qualitatively similar changes in β-adrenoceptor responsiveness were observed in TSM treated with TNF-α (100 ng/ml), although their degree of attenuation in relaxation responsiveness to isoproterenol was less pronounced, providing mean R_{max} values of 46.5±2.9 vs. 54.4±3.2% in the corresponding controls ($P < 0.05$). Similarly, there were no differences in the relaxant responses to forskolin between TNF-α–treated and control tissue groups. However, when maximally effective concentrations of IL-1β (10 ng/ml) and TNF-α (100 ng/ml) were administered together, there occurred a synergistic attenuation of the relaxant responsiveness to isoproterenol (Fig. 3), relative to the effect produced by IL-1β alone (dashed function). In the presence of IL-1β and TNF-α, both the R_{max} and pD_{50} were significantly reduced at 23.5±3.2% (vs. 63.0±5.2%

in controls; $P < 0.001$) and 6.01±0.05 −log M (vs. 6.36±0.11 −log M in controls; $P < 0.01$). In contrast to IL-1β and TNF-α, relaxation responsiveness to isoproterenol or PGE_2 was unaffected in IL-2–treated tissues.

In light of the above observations, we next examined whether the IL-1β–induced changes in agonist-mediated reversal of ACh-induced airway constriction were related to altered muscarinic M_2-receptor–coupled modulation of agonist-induced airway relaxation. Accordingly, relaxation–response curves to isoproterenol, PGE_2, and forskolin were compared in paired IL-1β–treated and control tissues wherein one of each tissue pair was pretreated for 45 min with the muscarinic M_2-receptor antagonist, methoctramine (10^{-6} M). The latter significantly potentiated the airway relaxation responses to isoproterenol in IL-1β–treated tissues half-maximally precontracted with ACh (Fig. 4 B). In contrast, methoctramine had relatively little effect on the relaxant responsiveness to the β-adrenergic agonist in control tissues (Fig. 4 A). Moreover, it should be noted that the magnitude of enhancement in β-adrenoceptor responsiveness in the IL-1β–treated tissues that were preexposed to methoctramine yielded R_{max} and pD_{50} values for isoproterenol (i.e., 52.3±4.1% and 6.63±0.08 −log M, respectively) that were not significantly different from those obtained in corresponding control TSM segments ($P = 0.65$). Similarly, in the presence of methoctramine, PGE_2-induced airway relaxation was also selectively enhanced in the IL-1β–treated tissues, yielding R_{max} and pD_{50} values for PGE_2 which were similar to those obtained in corresponding methoctramine-treated control airway segments. In contrast, relaxant responsiveness to forskolin was unaltered by methoctramine in either the IL-1β–treated or control tissues (data not shown).

Given the above findings with methoctramine, and that muscarinic M_2-receptor activation is known to be associated
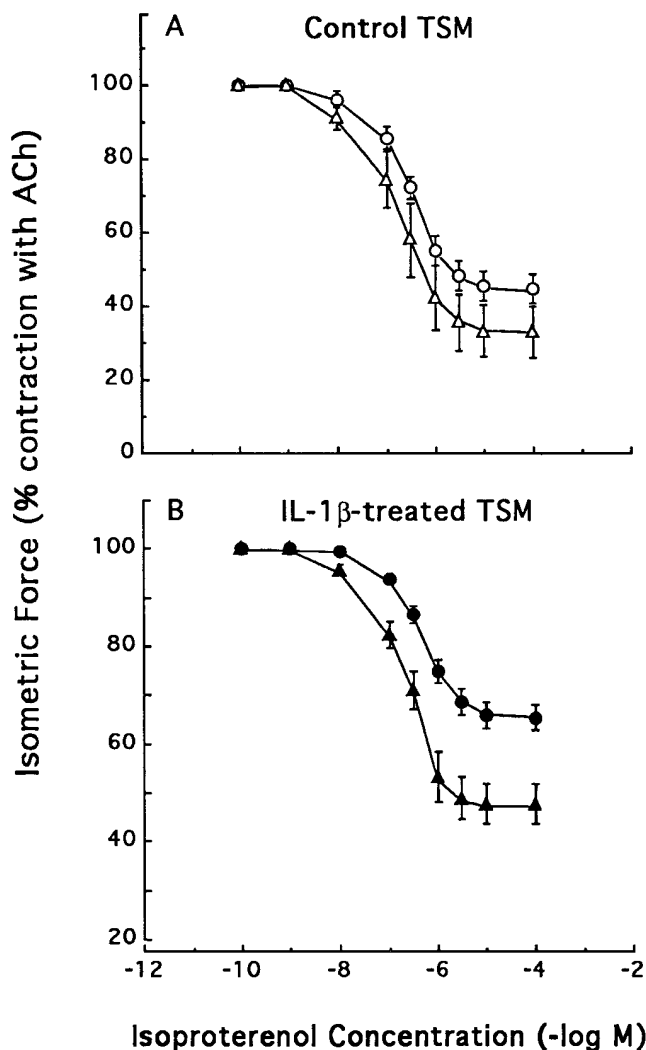
Figure 4. Comparison of airway relaxant responses to isoproterenol in control (*A*) and IL-1β–treated (*B*) TSM segments half-maximally contracted with their respective ED_{50} doses of ACh in the absence (*circles*) and presence (*triangles*) of methoctramine (10^{-6} M). Each data point represents the mean±SE from 8–10 paired tissue samples.

Figure 5. Comparison of airway relaxant responses to isoproterenol in control (*A*) and IL-1β–treated (*B*) TSM segments half-maximally contracted with their respective ED_{50} doses of ACh in the absence (*circles*) and presence (*triangles*) of PT (100 ng/ml). Data represent means±SE.

with G_i protein–coupled inhibition of adenylate cyclase (30, 32–34), in parallel experiments we examined whether ADP-ribosylation of G_i with PT also modulates the attenuated airway relaxant responsiveness to isoproterenol in IL-1β–treated tissues. Accordingly, relaxation response curves to isoproterenol, PGE_2, and forskolin were compared in groups of paired control and IL-1β–treated tissues wherein one of each pair was pretreated for 18 h with PT (100 ng/m1). The latter significantly enhanced the relaxation responses to isoproterenol in IL-1–treated TSM (Fig. 5 *B*), but had no effect on the responses in corresponding control tissues (Fig. 5 *A*). Comparable results were obtained with PGE_2-induced airway relaxation and, of note, PT had no effect on the R_{max} and pD_{50} values to forskolin (data not shown). Moreover, the R_{max} and pD_{50} values to isoproterenol obtained after PT exposure of IL-1β–treated tissues (i.e., 57.1±0.2% and 6.40±0.45 −log M, respectively) were similar to those obtained in control TSM ($P =$ 0.38). Thus, these observations are consistent with the pres-
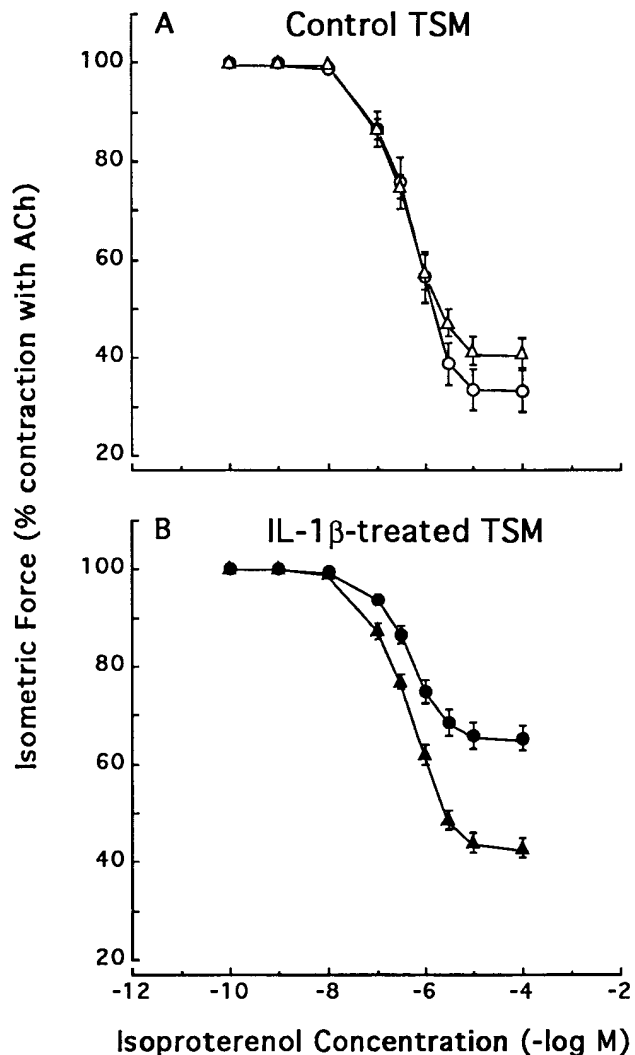
ence of enhanced muscarinic M_2-receptor/G_i protein–coupled inhibition of isoproterenol- and PGE_2-induced relaxation in IL-1β–treated TSM.

cAMP accumulation. To determine whether the enhanced muscarinic M_2-receptor/G_i protein–coupled inhibition of isoproterenol-induced airway relaxation was associated with changes in receptor-mediated accumulation of cAMP in the cytokine-treated tissues, the dose-dependent effects of isoproterenol on cAMP accumulation were compared in IL-1β–treated and control TSM segments. The mean (±SE) resting (baseline) levels of cAMP were similar in unstimulated control and IL-1β–treated TSM, averaging 7.5±1.5 and 7.7±1.2 pmol/mg protein, respectively. In contrast, the peak increases in cAMP accumulation at 1 min after each administered dose of isoproterenol were significantly reduced in the IL-1β–treated tissues, as exemplified by a representative experiment comparing isoproterenol-induced cAMP accumulation in paired control and IL-1β–treated TSM (Fig. 6). Comparable results were ob-
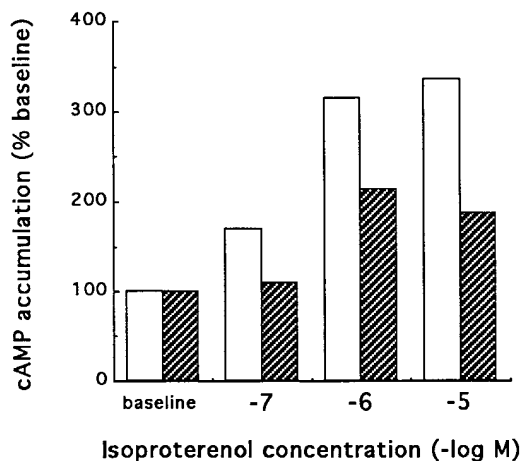
Figure 6. Representative experiment comparing isoproterenol-stimulated cAMP generation in paired control (*white bars*) and IL-1β–treated (*hatched bars*) TSM. Note that basal levels of cAMP were similar in both tissue groups, whereas the cAMP responses to isoproterenol stimulation were reduced in the IL-1β–treated TSM.

tained in the other experiments ($n = 4$), wherein the collective mean±SE maximal increases in cAMP in response to 10^{-5} M isoproterenol amounted to 25.4±2.5 and 17.6±2.3 pmol/mg protein in the control and IL-1β–treated TSM, respectively ($P < 0.01$).

G_i *protein expression.* In view of the above pharmacodynamic evidence of enhanced muscarinic M_2-receptor/G_i protein–coupled attenuation of airway responsiveness to isoproterenol in IL-1β–treated TSM, we next examined whether the expression of G_i and its isoforms is modulated in plasma membranes isolated from IL-1β–treated tissues. Relative to controls, Western immunoblot analyses of G_i-common and specific $G_i\alpha$-subunit expression in membrane homogenates from IL-1β–treated TSM demonstrated increased levels of G_i-common, $G_i\alpha_2$, and $G_i\alpha_3$, the latter qualified by laser densitometry at 251 ($P < 0.01$), 393 ($P < 0.001$), and 303% of control ($P < 0.005$), respectively (Fig. 7). In contrast, $G_i\alpha_1$ expression was not significantly different between control and IL-1β–treated TSM membrane fractions (data not shown).

Muscarinic radioligand binding. Muscarinic-cholinergic receptor binding using the radioligand [N-methyl-^{3}H]scopolamine was assayed in IL-1β–treated and control TSM membrane preparations to determine whether the above augmented
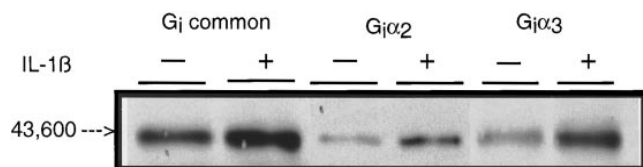

Figure 7. Comparison of Western blots of G_i-common, $G_i\alpha_2$, and $G_i\alpha_3$ subunit expression in membrane homogenates prepared from control ($-$) ($n = 6$) and IL-1β–treated ($+$) ($n = 6$) TSM. Figure represents one of two separate experiments. Note significantly enhanced expression of G_i-common, $G_i\alpha_2$, and $G_i\alpha_3$ in IL-1β–treated compared with control samples (see text for quantitated differences using laser densitometry).
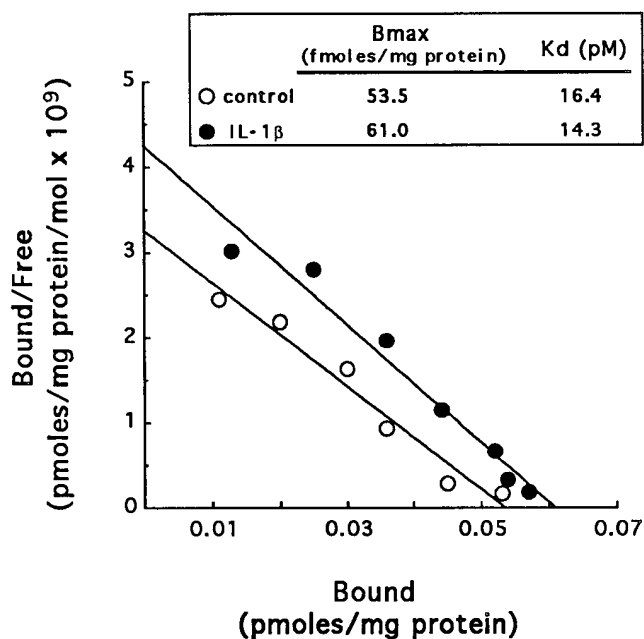

Figure 8. Scatchard plots comparing the binding of the muscarinic radioligand, [N-methyl-^{3}H]scopolamine, in control ($n = 4$) and IL-1β–treated ($n = 4$) TSM. Data represent mean values, with no significant differences between control and IL-1β–treated tissues for B_{max} or K_d.

muscarinic M_2-receptor/G_i protein–coupled expression and function in IL-1β–treated tissues was also associated with altered muscarinic B_{max} and/or K_d. Scatchard analysis of the binding isotherms demonstrated that there were no differences in the B_{max} or K_d values between IL-1β–treated and control tissues, wherein the B_{max} values amounted to 61.0 and 53.5 fmol/mg protein, respectively, with corresponding K_d values of 14.3 and 16.4 pM, respectively (Fig. 8).

Discussion

In recent years, substantial evidence has been accumulated in support of the concept that cytokines are key regulators of the cascade of cellular events associated with airway inflammation in atopic asthma (2, 27, 35–38). In this regard, some studies have demonstrated elevated levels of various cytokines in the bronchoalveolar lavage fluid from asthmatic patients (8–10). Moreover, recent animal studies have shown that, apart from their potential role in regulating airway inflammation in the allergic state, certain cytokines (notably IL-1β and TNF-α) may also contribute to the associated heightened airway constrictor responsiveness (2, 6, 25, 27). Finally, administration of specific cytokines has been shown to induce impairment of β-adrenoceptor–mediated airway relaxation in the isolated guinea pig trachea (26). Thus, taken together, these studies implicate a role for proinflammatory cytokines in mediating certain changes in airway contractility which characterize the asthmatic phenotype. In view of the latter, and to elucidate the mechanism(s) underlying any cytokine-induced modulation of airway responsiveness, the present study examined the actions of specific cytokines on the relative contributions of β-adrenoceptor– and postreceptor-coupled transmembrane signaling mechanisms regulating airway smooth muscle relaxation. Our

results demonstrated that: (*a*) IL-1β alone, and to a lesser extent, TNF-α, produces attenuation of β-adrenoceptor–mediated airway relaxation; (*b*) the latter effect is attributed to cytokine-induced enhanced muscarinic M_2-receptor/G_i protein coupling; and (*c*) when given together, IL-1β and TNF-α exert a synergistic attenuating effect on β-adrenoceptor–mediated airway relaxation.

The observed reduced relaxation responses of IL-1β–treated airways to β-adrenoceptor stimulation were not specific to activation of the β-adrenergic receptor, since attenuated relaxation responses to PGE_2 were also observed in these cytokine-treated tissues (Fig. 2). These observations, together with the finding that the airway relaxant responses to direct activation of adenylate cyclase with forskolin was unaltered in IL-1β–treated tissues, suggest that the cytokine action was associated with attenuation of a receptor-coupled signaling mechanism upstream to adenylate cyclase activation. Additionally, our observations also demonstrated reversal of the attenuated β-adrenoceptor– and PGE_2-mediated airway relaxation by muscarinic M_2-receptor blockade with methoctramine (Fig. 4) and by ADP-ribosylation of G_i protein with PT (Fig. 5), suggesting that the cytokine-induced impairment of airway relaxation was likely attributed to enhanced M_2-receptor/G_i protein–coupled inhibition of adenylate cyclase. Indeed, in related experiments, we found reduced isoproterenol-stimulated cAMP accumulation in IL-1β–treated versus control TSM (Fig. 6). Moreover, in this context, it should be noted that since the relaxation responses to isoproterenol and PGE_2 obtained in IL-1β–treated tissues preexposed to the M_2-receptor antagonist or PT were similar to those generated in control airways (Figs. 4 and 5), our observations further suggest that the attenuated relaxation responses in the IL-1β–treated tissues occurred largely independent of any concomitant significant changes in intrinsic β-adrenoceptor or PGE_2 receptor expression or G_s protein coupling.

Taken together, our present findings largely concur with the known inhibitory action of muscarinic M_2-receptor activation on adenylate cyclase activity in other cell systems (39–42), and agree with the established evidence that the functional responses to both β-adrenoceptor and PGE_2 receptor stimulation are downregulated by G_i protein activation (39–44). Moreover, in this regard, insofar as the TSM relaxation responses to isoproterenol were virtually unaffected by methoctramine or PT in control tissues (Figs. 3 and 4), our findings further suggest that the contribution of the above M_2-receptor/G_i protein–coupled action is relatively insignificant under control conditions. Thus, collectively, the observations support the concept that IL-1β induces muscarinic M_2-receptor/G_i protein–coupled inhibition of β-adrenoceptor– and PGE_2-mediated airway relaxation associated with attenuated cAMP accumulation.

In further examining the above mechanism, we found that G_i protein expression was increased in membranes isolated from IL-1β–treated TSM, a finding associated with specific enhanced expression of the $G_i\alpha_2$ and $G_i\alpha_3$ subunits (Fig. 7). However, expression of the $G_i\alpha_1$ subunit was found to be similar in IL-1β–treated and control TSM membranes. Of interest, these findings agree with our recent observations in atopic passively sensitized rabbit TSM wherein attenuated β-adrenoceptor–mediated airway relaxation was also found to be associated with increased $G_i\alpha_3$ subunit expression (30). Moreover, in this context, it is relevant to note that elevated levels of $G_i\alpha_2$ have been detected in lung membranes from antigen-challenged

guinea pigs (26), and that IL-1β administration was found to induce increased mRNA expression of $G_i\alpha_2$ in guinea pig TSM (45) and in cultured human endothelial cells (46). Thus, given our present observations and those of the above earlier studies, the consideration is raised that while changes in β-adrenergic function in cytokine-treated or atopic sensitized airways may be related to altered G_i protein expression, the latter effect may be species specific with respect to the $G_i\alpha$ subtypes involved, wherein $G_i\alpha_2$ and $G_i\alpha_3$ are both altered in rabbits while a potential more selective effect on $G_i\alpha_2$ expression alone is seen in guinea pig and human tissues.

Although the mechanism accounting for the cytokine-induced increased G_i protein expression (including altered transcriptional and/or posttranscriptional regulation) remains to be determined, in contrast to changes in G_i protein expression, our extended findings demonstrated that neither muscarinic-cholinergic receptor density nor affinity was altered in IL-1β–treated TSM membranes (Fig. 7). Taken together, these findings along with earlier observations demonstrating both a significant muscarinic receptor reserve (i.e., spare receptors) in TSM (47) and that > 70% of rabbit airway muscarinic receptors are of the M_2-receptor subtype (48) allow for the compelling speculation that the attenuated β-adrenoceptor–mediated relaxation in IL-1β–treated TSM reflects enhanced functional coupling of the increased G_i protein to spare muscarinic receptors. This possibility remains to be systematically addressed in extended studies.

In conclusion, this study examined the role and mechanisms of action of specific proinflammatory cytokines in regulating rabbit airway smooth muscle relaxation. The results demonstrated that: (*a*) during cholinergic stimulation, IL-1β alone, and to a lesser extent, TNF-α (but not IL-2), produces attenuated relaxation of TSM to isoproterenol and PGE_2, but not to direct activation of adenylate cyclase with forskolin; (*b*) the latter combined effects of IL-1β and TNF-α are synergistic; (*c*) the attenuated relaxation to isoproterenol and PGE_2 is ablated in cytokine-treated tissues preexposed to either a muscarinic M_2-receptor antagonist or PT; (*d*) the impaired β-adrenoceptor–mediated relaxation is associated with reduced isoproterenol-stimulated cAMP accumulation in IL-1β–treated TSM; (*e*) the impaired relaxation to isoproterenol is not accompanied by changes in muscarinic-cholinergic receptor binding in IL-1β–treated tissues; and (*f*) the attenuated relaxation to β-adrenoceptor stimulation is associated with IL-1β–induced enhanced G_i protein expression in TSM membrane, attributed to increased expression of both the $G_i\alpha_2$ and $G_i\alpha_3$ subunits. Collectively, these observations provide new evidence implicating enhanced muscarinic M_2-receptor/G_i protein coupling in mediating cytokine-induced impairment of airway relaxation to β-adrenoceptor stimulation, a phenomenon which may account for attenuated airway relaxation in the atopic/asthmatic state.

References

1. Bradding, P., J.A. Roberts, K.M. Britten, S. Montefort, R. Djukanovic, R. Mueller, C.H. Heusser, P.H. Howarth, and S.T. Holgate. 1994. Interleukin-4, -5, and -6 and tumor necrosis factor-α in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am. J. Respir. Cell Mol. Biol.* 10:471–480.

2. Watson, M.L., D. Smith, A.D. Bourne, R.C. Thompson, and J. Westwick. 1993. Cytokines contribute to airway dysfunction in antigen-challenged guinea pigs: inhibition of airway hyperreactivity, pulmonary eosinophil accumulation and tumor necrosis factor generation by pretreatment with an interleukin-1 receptor antagonist. *Am. J. Respir. Cell Mol. Biol.* 8:365–369.

3. Djukanovic, R., W.R. Roche, J.W. Wilson, C.R.W. Beasley, O.P. Twentyman, P.H. Howarth, and S.T. Holgate. 1990. Mucosal inflammation in asthma. *Am. Rev. Respir. Dis.* 142:434–457.

4. Barnes, P.J., K.F. Chung, and C.P. Page. 1988. Inflammatory mediators and asthma. *Pharmacol. Rev.* 40:49–84.

5. Kelley, J. 1990. Cytokines of the lung. *Am. Rev. Respir. Dis.* 141:765–772.

6. Kips, J.C., J. Tavernier, and R.A. Pauwels. 1992. Tumor necrosis factor (TNF) causes bronchial hyperresponsiveness in rats. *Am. Rev. Respir. Dis.* 145:332–336.

7. Hamblin, A.S. 1991. The role of cytokines in asthma. *Ann. NY Acad. Sci.* 629:250–261.

8. Hamid, Q., M. Azzawi, S. Ying, R. Moqbel, A.J. Wardlaw, C.J. Corrigan, B. Bradley, S.R. Durham, J.V. Collins, P.K. Jeffery, D.J. Quint, and A.B. Kay. 1991. Expression of mRNA for interleukin 5 in mucosal bronchial biopsies from asthma. *J. Clin. Invest.* 87:1541–1546.

9. Walker, C., E. Bode, L. Boer, T.T. Hansel, K. Blaser, and J.C. Virchow. 1992. Allergic and non-allergic asthmatics have distinct pattern of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am. Rev. Respir. Dis.* 146:109–115.

10. Broide, D.H., M. Lotz, A.J. Cuomo, D.A. Coburn, E.C. Federman, and S.I. Wasserman. 1992. Cytokines in symptomatic asthma airways. *J. Allergy Clin. Immunol.* 89:958–967.

11. Del Prete, G.F., E. Maggi, P. Parronchi, I. Chreitien, A. Jiri, D. Macchia, M. Ricci, J. Banchereau, J.E. De Vries, and S. Romagnani. 1988. IL-4 is an essential co-factor for IgE synthesis induced in vitro by human T-cell clones and their supernatants. *J. Immunol.* 140:4193–4198.

12. Vercelli, D., H.H. Jabara, B.W. Lee, N. Woodland, R.S. Geha, and D.Y. Leung. 1988. Human recombinant interleukin 4 induces FcεRII/CD23 on normal human monocytes. *J. Exp. Med.* 167:1406–1416.

13. Arai, K., F. Lee, A. Miyajima, S. Miyatake, N. Arai, and T. Yokota. 1990. Cytokines: coordinators of immune and inflammatory responses. *Annu. Rev. Biochem.* 59:783–836.

14. Mantovani, A., F. Bussolino, and E. Dejana. 1992. Cytokine regulation of endothelial cell function. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 6:2591–2599.

15. Mantovani, A., and E. Dejana. 1989. Cytokines as communication signals between leukocytes and endothelial cells. *Immunol. Today.* 10:370–375.

16. Mizel, S.B. 1989. The interleukins. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3:2379–2384.

17. Masinovsky, B., D. Urdal, and W.M. Gallatin. 1990. IL-4 acts synergistically with IL-1β to promote lymphocyte adhesion to microvascular endothelium by induction of vascular cell adhesion molecule-1. *J. Immunol.* 145:2886–2895.

18. Thornhill, M.H., S.M. Wellicome, D.L. Mahiouz, J.S.S. Lanchbury, U. Kyan-Aung, and D.O. Haskard. 1991. Tumor necrosis factor combines with IL-4 or IFN-α to selectively enhance endothelial cell adhesiveness for T-cells. The contribution of vascular cell adhesion molecule-1-dependent and independent binding mechanisms. *J. Immunol.* 146:592–598.

19. Li, J., M.A. Perrella, J.C. Tsai, S.F. Yet, C.M. Hsieh, M. Yoshizumi, C. Patterson, W.O. Endege, F. Zhou, and M.E. Lee. 1995. Induction of vascular endothelial growth factor gene expression by interleukin-1β in rat aortic smooth muscle cells. *J. Biol. Chem.* 270:308–312.

20. Swades, D., E.T. Zelazny, J.F. Souhrada, and M. Souhrada. 1993. Interleukin-1β stimulates the proliferation of cultured airway smooth muscle cells via platelet-derived growth factor. *Am. J. Respir. Cell Mol. Biol.* 9:645–651.

21. Schleimer, R.P., S.A. Sterbinsky, J. Kaiser, C.A. Bickel, D.A. Klund, K. Tomioka, W. Newman, F.W. Luscinskas, M.A. Gimbrone, B.W. McIntyre, and B.S. Bochner. 1992. IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium: association with expression of VCAM-1. *J. Immunol.* 148:1086–1092.

22. Lukacs, N.W., R.M. Strieter, S.W. Chensue, and S.L. Kunkel. 1994. Interleukin-4-dependent pulmonary eosinophil infiltration in a murine model of asthma. *Am. J. Respir. Cell Mol. Biol.* 10:526–532.

23. Wang, J.M., A. Rambaldi, A. Biondi, Z.G. Chen, C.J. Sanderson, and A. Mantovani. 1989. Recombinant interleukin 5 is a selective eosinophil chemoattractant. *Eur. J. Immunol.* 19:701–705.

24. Brown, J., and W. Konig. 1992. Cytokine-induced (interleukins-3, -6 and -8 and tumour necrosis factor-β activation and deactivation of human neutrophils. *Immunology.* 75:281–285.

25. Wheeler, A.P., G. Jesmok, and K.L. Brigham. 1990. Tumor necrosis factor's effect on lung mechanics, gas exchange and airway reactivity in sheep. *J. Appl. Physiol.* 68:2542–2549.

26. Wills-Karp, M., Y. Uchida, J.Y. Lee, J. Jinot, A. Hirata, and F. Hirata. 1993. Organ culture with proinflammatory cytokines reproduces impairment of the β-adrenoceptor-mediated relaxation in tracheas of a guinea pig model. *Am. J. Respir. Cell Mol. Biol.* 8:153–159.

27. Selig, W., and J. Tocker. 1992. Effect of interleukin-1 receptor antagonist on antigen-induced pulmonary responses in guinea pigs. *Eur. J. Pharmacol.* 213:331–336.

28. Ulich, T.R., S. Yin, K. Guo, J. Del Castillo, S.P. Eisenberg, and R.C. Thompson. 1991. The intratracheal administration of endotoxin and cytokines. III. The interleukin-1 receptor (IL-1) antagonist inhibits endotoxin- and IL-1-induced acute inflammation. *Am. J. Pathol.* 138:521–524.

29. Tanaka, D.T., and M.M. Grunstein. 1990. Maturation of neuromodulatory effect of substance P in rabbit airways. *J. Clin. Invest.* 85:345–350.

30. Hakonarson, H., D.A. Herrick, and M.M. Grunstein. 1995. Mechanism of impaired β-adrenoceptor responsiveness in atopic sensitized airway smooth muscle. *Am. J. Physiol. (Lung Cell. Mol. Physiol.)* 269:L645–L652.

31. Carlson, K.E., L.F. Brass, and D.R. Manning. 1989. Thrombin and phorbol esters cause the selective phosphorylation of a G protein other than G_i in human platelets. *J. Biol. Chem.* 264:13298–13305.

32. Katada, T., K. Kusakabe, M. Oinuma, and M. Ui. 1987. A novel mechanism for the inhibition of adenylate cyclase via inhibitory GTP-binding protein. *J. Biol. Chem.* 262:11897–11900.

33. Schaefer, O.P., M.F. Ethier, and J.M. Madison. 1995. Muscarinic regulation of cyclic AMP in bovine trachealis cells. *Am. J. Respir. Cell Mol. Biol.* 13:217–226.

34. Taki, R.K., K. Takagi, T. Satake, S. Sugiyama, and T. Ozawa. 1986. The role of phospholipase in reduced β-adrenergic responsiveness in experimental asthma. *Am. Rev. Respir. Dis.* 133:362–366.

35. Mattoli, S., V.L. Mattoso, M. Soloperto, L. Allegra, and A. Dasoli. 1991. Cellular and biochemical characteristics of bronchoalveolar lavage fluid in symptomatic nonallergic asthma. *J. Allergy Clin. Immunol.* 87:794–802.

36. Pujol, J.L., B. Cosso, J.P. Daures, J. Clot, F.B. Michel, and P. Godard. 1990. Interleukin-1 release by alveolar macrophages in asthmatic patients and healthy subjects. *Int. Arch. Allergy Appl. Immunol.* 91:207–210.

37. Schleimer, R.P., S.V. Benenati, B. Friedman, and B.S. Bochner. 1991. Do cytokines play a role in leukocyte recruitment and activation in the lungs? *Am. Rev. Respir. Dis.* 143:1169–1174.

38. Martin, M., and K. Resch. 1988. Interleukin 1: more than a mediator between leukocytes. *Trends Pharmacol. Sci.* 9:171–177.

39. Candell, L.M., S.H. Yun, L.L.P. Tran, and F.J. Ehlert. 1990. Differential coupling of subtypes of the muscarinic receptor to adenylate cyclase and phosphoinositide hydrolysis in the longitudinal muscle of the rat ileum. *Mol. Pharmacol.* 38:689–697.

40. Fernandes, L.B., A.D. Fryer, and C.A. Hirshman. 1992. M₂-muscarinic receptors inhibit isoproterenol-induced relaxation of canine airway smooth muscle. *J. Pharmacol. Exp. Ther.* 262:119–126.

41. Leiber, D., S. Marc, and S. Harbon. 1990. Pharmacological evidence for distinct muscarinic receptor subtypes coupled to the inhibition of adenylate cyclase and to the increased generation of inositol phosphates in the guinea pig myometrium. *J. Pharmacol. Exp. Ther.* 252:800–809.

42. Sankary, R.M., C.A. Jones, M.M. Madison, and J.K. Brown. 1988. Muscarinic cholinergic inhibition of cyclic AMP accumulation in airway smooth muscle: role of a pertussis toxin-sensitive protein. *Am. Rev. Respir. Dis.* 138:145–150.

43. Garrity, M.J., M.M. Reed, and E.P. Brass. 1989. Coupling of hepatic prostaglandin receptors to adenylate cyclase through a pertussis toxin sensitive guanine nucleotide regulatory protein. *J. Pharmacol. Exp. Ther.* 248:979–983.

44. Lerner, R.W., G.D. Lopaschuk, and P.M. Olley. 1992. Prostaglandin E₂ receptors in the heart are coupled to inhibition of adenylyl cyclase via a pertussis toxin sensitive G protein. *Can. J. Physiol. Pharmacol.* 70:77–84.

45. Hirata, F., J.Y. Lee, T. Sakamoto, A. Nomura, Y. Uchida, A. Hirata, and S. Hasegawa. 1994. IL-1β regulates the expression of the $G_i\alpha_2$ gene via lipid mediators in guinea pig tracheal muscle. *Biochem. Biophys. Res. Commun.* 203:1889–1896.

46. Lee, R.T., T.A. Brock, C. Tolman, K.D. Bloch, J.G. Seidman, and E.J. Neer. 1989. Subtype-specific increase in G-protein alpha-subunit mRNA by interleukin 1 beta. *FEBS Lett.* 249:139–142.

47. Gunst, S.J., J.Q. Stropp, and N.A. Flavahan. 1987. Analysis of receptor reserves in canine tracheal smooth muscle. *J. Appl. Physiol.* 62:1755–1758.

48. Mahesh, V.K., L.M. Nunan, M. Halonen, H.I. Yamamura, J.D. Palmer, and J.W. Bloom. 1992. A minority of muscarinic receptors mediate rabbit tracheal smooth muscle contraction. *Am. J. Respir. Cell Mol. Biol.* 6:279–286.