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Article

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Albuterol-induced downregulation of Gsα accounts for pulmonary β_2-adrenoceptor desensitization in vivo

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Introduction

β_2-Adrenoceptor agonists are the most effective bronchodilators currently available and exhibit efficacy irrespective of the mediator(s) evoking bronchospasm. However, there has been concern that regular use of this group of drugs may render susceptible individuals tolerant to their beneficial effects in asthma. Lipworth and colleagues (1, 2) have reported that 4 weeks of treatment of asthmatic subjects with inhaled formoterol produced tachyphylaxis to the bronchodilator effects of these drugs. In addition, the results of several studies with terbutaline and albuterol have demonstrated a loss of protection against various bronchoconstrictor challenges (3, 4). This effect renders the airways twice as sensitive to allergen and exercise and increases the late-phase asthmatic response and associated inflammation (5) and may be relevant to the reduced asthma control seen with the regular use of high doses of inhaled β_2-adrenoceptor agonists (6, 7). The cause(s) of this effect is unclear. It has been reported that airways resected from asthmatic patients fail to relax normally to isoproterenol, supporting a possible defect in β-adrenoceptor function (8–10), although it is unknown whether this is the result of treatment or a consequence of the disease process itself. Nevertheless, downregulation of β-adrenoceptor number in lung and airways smooth muscle has been reported in animals given isoproterenol and norepinephrine chronically by infusion (11–13) and is associated with a reduced functional responsiveness toward β-adrenoceptor agonists ex vivo (12, 13). Thus, it is possible that the loss of protection against various bronchoconstrictor challenges in humans is due, at least in part, to pulmonary β_2-adrenoceptor desensitization.

Two major molecular mechanisms have been elucidated in isolated cells that result in β_2-adrenoceptor desensitization. One of these promotes short-term homologous refractoriness and involves the uncoupling of the agonist-occupied form of the receptor from the stimulatory guanine nucleotide binding protein, Gs, by mechanisms that require phosphorylation of serine and threonine residues at the COOH-terminus of the receptor (14, 15). This reaction can be catalyzed by at least three members of the G protein–coupled receptor (GPCR) kinase (GRK) superfamily (GRK-2, GRK3, GRK5) (14, 15). The subsequent binding of β-arrestin, a soluble protein that prevents further coupling to Gs (16), then halts signaling through

the receptor. The β_2-adrenoceptor is similarly desensitized by cyclic adenosine monophosphate–dependent (cAMP-dependent) protein kinase (PKA) following phosphorylation of serine and threonine residues present within the third intracellular loop of the protein in response to an increase in intracellular cAMP (17). The other established process, which promotes prolonged periods of desensitization, involves the physical internalization and subsequent degradation of receptors due to an inhibition of transcription and/or increased post-transcriptional processing of β_2-adrenoceptor mRNA (16, 17). Although the aforementioned processes are believed to account for desensitization of many GPCRs, other mechanisms have also been described, including induction of cAMP phosphodiesterases (PDE) (18) and downregulation of Gsα (19), although the functional significance of these effects has not been rigorously explored.

Although desensitization of GPCRs has been studied extensively, most of the information to date has been gathered from cultured cells, and the extent to which this applies to the in vivo situation is little investigated. Thus, the aim of the present study was to develop an in vivo model of pulmonary β_2-adrenoceptor desensitization and investigate the nature and molecular basis of this phenomenon.

Methods

Animals and surgery. Male Sprague-Dawley rats (Charles River Ltd., Kent, United Kingdom) weighing 275–300 g were housed in an environment maintained at 21°C, with food and water available ad libitum. When required, animals were sedated (0.3 mL/kg of a 2% solution of 300 μg/mL fentanyl citrate and 10 mg/mL fluanisone) and implanted subcutaneously with osmotic minipumps (Alzet model 2001; Alzo Co., Palo Alto, California, USA). Briefly, a 1.5-cm incision was made in the skin between the scapulae, and a small pocket was formed by spreading apart the subcutaneous connective tissues with a hemostat. The pump was inserted into the pocket with the flow moderator pointing away from the head, and the skin was closed with sutures. The rate of infusion of fluid from the minipump was approximately 1 μL/h delivering 40 μg/kg/h of albuterol or PGE_2 or their respective vehicles (saline and saline in 5% ethanol respectively). Rats were left for 7 days before use.

Instrumentation of rats for measurement of airway mechanics. Rats were anesthetized with urethane (12 mL/kg intraperitoneally of a 25% [wt/vol] solution in saline) and placed on a pad maintained at 37°C. The left carotid artery and left jugular vein were cannulated for measuring changes in blood pressure and for the injection of drugs, respectively. Blood pressure was monitored by use of an indwelling Portex cannula filled with heparin-saline (10 U/mL) and linked to a pressure transducer (Druck Ltd., Leicestershire, United Kingdom), which was connected to a multichannel recorder. The trachea was cannulated and the animal ventilated at a constant volume (1 mL/100 g body weight) using a pump operating at 75 strokes per minute. Changes in respiratory insufflation pressure were measured using a modification of the method of Konzett and Rössler (20) using a differential pressure transducer (Farnell Electronic Components Ltd., Leeds, United Kingdom).

Assessment of pulmonary β_2-adrenoceptor desensitization in vivo. Rats were given ACh (500 μg/kg intravenously) repeatedly to establish a constant degree of bronchoconstriction. ACh was selected as stimulus because increased cholinergic tone is an important mechanism of bronchoconstriction in asthma, particularly nocturnal asthma. When airway function had normalized (15 minutes after last dose of ACh), albuterol (100 μg/kg intravenously) was administered, and the magnitude of ACh-induced bronchoconstriction was reassessed 5 minutes later. An identical protocol was used to assess the potential bronchoprotective effects of PGE_2 (300 μg/kg intravenously), IBMX (300 μg/kg intravenously), and forskolin (300 μg/kg intravenously). Preliminary studies established that the dose of ACh was submaximal (Figure 1a) in both groups of animals and that after preparations had stabilized, repeated administration of ACh (500 μg/kg) over a period of 45 minutes did not alter the magnitude of bronchoconstriction (Figure 1b). The resting mean arterial blood pressure (saline: 74.1 ± 4.8 mmHg [n = 32]; albuterol: 78.1 ± 6.1 mmHg [n = 31]; $P > 0.05$) and the peak reduction in blood pressure effected by ACh (saline: 30.1 ± 1.8 mmHg [n = 32]; albuterol: 33.1 ± 2.4 mmHg [n = 31]; $P > 0.05$) were identical in saline- and albuterol-treated rats and could not account for changes in lung function.

Quantification of β-adrenoceptor number in lung membranes. Lung membranes were prepared as described previously (11, 21), frozen in liquid N_2, and stored at –80°C. When required, membranes (10 μg/tube) were incubated at 37°C in buffer A (25 mM Tris-HCl [pH 7.4], 154 mM NaCl, 1 mM ascorbic acid) containing [^{125}I]iodocyanopindolol ([^{125}I]ICYP) (3–100 pM) in a final volume of 250 μL. The density of the β_1- and β_2-adrenoceptor subtype was estimated by including ICI 118551 (100 nM) or CGP 20712A (100 nM) in the assay cocktail, respectively. Reactions were terminated after 120 minutes by rapid vacuum filtration through Whatman GF/C glass fiber filters that had been presoaked in ice-cold buffer B (25 mM Tris HCl [pH 7.4]). Filters were washed with ice-cold buffer B, and retained radioactivity was detected by γ-counting. Specific binding was determined experimentally from the difference between [^{125}I]ICYP bound in the absence and presence of a large molar excess of unlabeled (–)-isoproterenol (200 μM). Saturation binding isotherms were constructed from which the equilibrium dissociation constant (K_d) and maximal binding capacity (B_{max}) were determined by Scatchard analyses.

Ex vivo treatment of lung and tracheae for cAMP and PKA studies. Lung fragments or tracheal strips were equilibrated for 30 minutes at 37°C in oxygenating

(95%O_2/5%CO_2) Krebs-Henseleit (KS) solution (118 mM NaCl, 5.9 mM KCl, 1.2 mM $MgSO_4$•$7H_2O$, 1.2 mM NaH_2PO_4•$2H_2O$, 2.5 mM $CaCl_2$•$6H_2O$, 10 mM glucose, and 25 mM $NaHCO_3$) containing 10 μM indomethacin. Tissue was incubated for 30 minutes in the absence or presence of IBMX (100 μM), and albuterol (10 nM to 100 μM) or PGE_2 (1 μM) was then added for a further 5 minutes. Tissue was removed from the KH solution, blotted on absorbent paper, frozen in liquid N_2, and stored at –80°C.

Measurement of cAMP content and PKA activity. Frozen lung was homogenized in ice-cold 1 M TCA and centrifuged at 2,500 *g* to precipitate particulate material. The cAMP content in the supernatant was measured by RIA as described previously (22, 23). For PKA measurements, frozen lung was homogenized in ice-cold buffer C (5 mM KH_2PO_4/5 mM K_2HPO_4 [pH 6.8], 10 mM EDTA, 10 mM DTT, 500 μM IBMX, 20 mM NaCl, 140 mM KCl) and centrifuged (35,000 *g* for 15 minutes at 4°C). PKA activity in the supernatant was determined by measuring the phosphorylation of Kemptide as described previously (23, 24).

Measurement of cAMP PDE Activity. Frozen lung was homogenized in ice-cold buffer D (20 mM TEA [pH 8], 1 mM EDTA) supplemented with benzamidine (2 mM), leupeptin (50 μM), PMSF (100 μM), bacitracin (100 μM), and soybean trypsin inhibitor (20 μg/mL) and centrifuged (35,000 *g* for 30 minutes at 4°C). The supernatant was used immediately for the estimation of PDE activity as described previously (22, 23). In this study, PDE3 and PDE4 are defined as cAMP hydrolysis inhibited by Org 9935 (30 μM) and rolipram (30 μM), respectively.

Measurement of GRK activity. Cytosolic and particulate GRK was prepared from rat lung according to the method of Benovic and colleagues (25), and GRK activity was determined immediately according to Mayor et al. (26). Assays were performed in triplicate at 30°C and initiated by the addition of 100 μg protein to 120 μL of a reaction mixture containing (final concentration) 20 mM Tris HCl [pH 7.4], 300 pmol urea-treated rod outer segments (ROS) (25), 50 μM [γ-^{32}P]ATP (2–5 cpm/fmol), 8 mM $MgCl_2$, 3 mM EDTA, 5 mM NaF, 12 mM NaCl, 70 μM PMSF, 7 μg/mL leupeptin, 3.5 μg/mL pepstatin, and 7 μg/mL benzamidine in the absence and presence of light. Reactions were quenched after 45 minutes by the addition of 900 μL of ice-cold buffer E (10 mM Tris HCl [pH 7.4], 100 mM NaCl, 10 mM NaF, 2 mM EDTA) and centrifuged (57,000 *g* for 15 minutes at 4°C). The pellet was reconstituted in SDS sample buffer, electrophoresed on a 10% SDS polyacrylamide slab gel, and stained with Coomassie blue. Bands corresponding to phosphorhodopsin were excised from the gel, and the associated radioactivity was determined by liquid scintillation counting. One unit of activity is defined as that amount of GRK that catalyzed the incorporation of 1 pmol phosphate from ATP into ROS in 1 minute per milligram of protein at 30°C.

Western blot analysis. Frozen lung was ground in liquid N_2 and homogenized in ice-cold buffer F (50 mM Tris-HCl [pH 7.4], 1 mM $MgCl_2$) containing 0.5 mM PMSF, 1 mM sodium orthovanadate, 10 μg/mL leupeptin, 100 μg/mL aprotinin, 5 mM NaF, and 10 mM sodium pyrophosphate and centrifuged (1,000 *g* for 15 minutes at 4°C). The suspension was recentrifuged (100,000 *g* for 60 minutes at 4°C), and the pellet was suspended in buffer F. An aliquot of the cytosolic or membrane fraction was mixed with Laemmli sample buffer, boiled, and loaded (10 μg/lane and 300 μg/lane for G-protein subunits and GRK-2m respectively) onto a 10% SDS/Tris polyacrylamide gel. Proteins were size fractionated, transferred onto nitrocellulose, and "blocked" overnight at 4°C in buffer G (10 mM Tris-

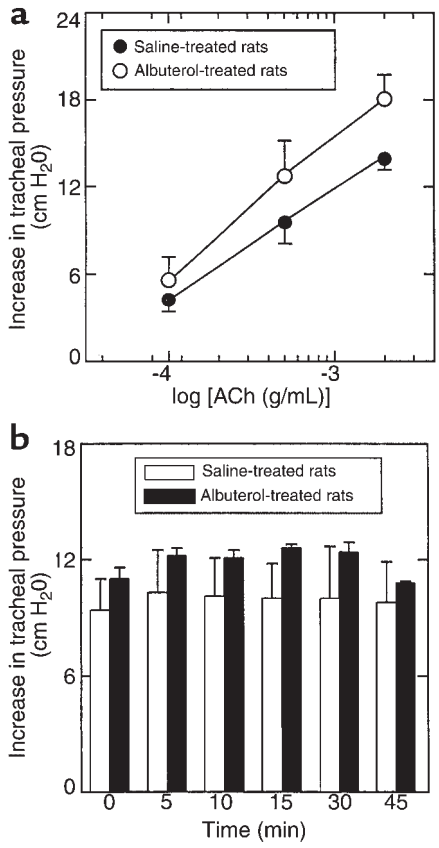

Figure 1
Dose dependence and reproducibility of ACh-induced bronchoconstriction in anesthetized rats treated chronically with albuterol. Rats were implanted subcutaneously with osmotic minipumps delivering saline (filled circles/open bars) or albuterol (open circles/filled bars; 40 μg/kg/h). After 7 days, animals were anesthetized and instrumented for the measurement of lung function. (**a**) ACh (100, 500, and 2,000 μg/kg intravenously) was given and the maximum increase in overflow pressure was measured using a modification of the technique of Konzett and Rössler (20). Three doses of ACh were administered in a randomized fashion to each animal. (**b**) ACh (500 μg/kg intravenously) was given 5, 10, 15, 30, and 45 minutes after the first dose of ACh, and the maximum increase overflow pressure was measured. Data points and bars represent the mean ± SEM of three and four determinations for the dose-dependence and reproducibility study, respectively.

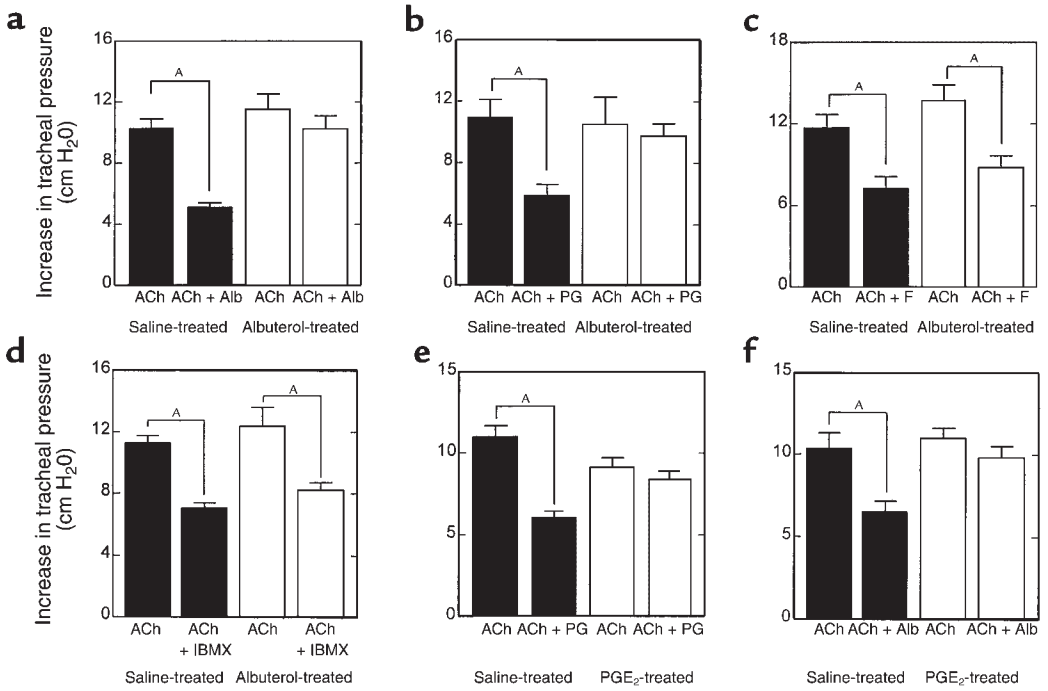

Figure 2
Effect of chronic treatment of rats with albuterol (**a**–**d**) or PGE_2 (**e** and **f**) on lung function in anesthetized rats. Animals were given albuterol, PGE_2 (open bars; both 40 μg/kg/h) or vehicle (filled bars) for 7 days and then instrumented for the measurement of lung function. ACh (500 μg/kg intravenously) was administered and the maximum increase in overflow pressure was measured. When baseline lung function was reestablished, albuterol (Alb; 100 μg/kg; **a** and **f**), PGE_2 (PG; 300 μg/kg; **b** and **e**), forskolin (F; 300 μg/kg; **c**), or IBMX (300 μg/kg; **d**) was given intravenously, and 5 minutes later, ACh was administered again and any change in overflow pressure was noted. Each bar represents the mean ± SEM of four independent determinations. $^AP < 0.05$, significant protection of ACh-induced bronchoconstriction.

base [pH 7.4], 0.05% Tween 20; 5% wt/vol skimmed milk). The nitrocellulose was incubated at 25°C for 1 hour with a rabbit polyclonal antibody specific to either Gsα (NEN/Dupont NEI 805; NEN Life Science Products Inc., Boston, Massachusetts, USA), Gβ (NEN/Dupont NEI 808; NEN Life Science Products Inc.), GRK2 (Santa Cruz *sc* 506; Santa Cruz Biotechnology, Santa Cruz, California, USA), or actin (Santa Cruz *sc* 1615) diluted 1:1,000; 1:1,000; 1:2,000; and 1:500 in buffer G, respectively, washed, incubated (1 h) with a donkey, anti-rabbit horseradish peroxidase–conjugated (HRP-conjugated) secondary antibody (diluted 1:4,000 in buffer G) and then treated with enhanced chemiluminescence (ECL) reagent (Amersham International, Amersham, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. Membranes were exposed to Kodak X-OMAT film (Eastman Kodak Co., Rochester, New York, USA), and the protein bands were quantified by laser-scanning densitometry. G-protein subunits are expressed as a ratio of the "housekeeping" protein actin.

NAD^+-dependent ADP ribosylation of Gsα. Membranes were prepared from lung and tracheal smooth muscle as described for the radioligand binding studies and subjected to cholera toxin-catalyzed (CTX-catalyzed) ADP ribosylation using [α^{32}P]NAD^+ (27). Labeled proteins were resolved on 10% SDS polyacrylamide gels, and the radioactivity associated with each band was quantified by phosphorimaging.

Protein measurement. Protein was measured using a Bio-Rad kit (Bio-Rad, Hemel Hempstead, United Kingdom) according to the manufacturer's instructions.

Drugs and analytical reagents. ECL reagent, HRP-conjugated secondary antibody, and the radioisotopes [γ^{32}P]ATP, [α^{32}P]NAD^+, and [^{125}I]ICYP (specific activities 20–40 Ci/mmol, 30 Ci/mmol and >3,000 Ci/mmol, respectively) were supplied by Amersham International. GF/C glass fiber filters were obtained from BDH (Leicester, United Kingdom) and polyacrylamide (acrylamide/bisacrylamide, 37.5:1; 40% solution) was from Bio-Rad. Rolipram, Org 9935, ICI 118551, and CGP 20712A were donated by Schering AG (Berlin, Germany), Organon Laboratories (Lanarkshire, United Kingdom), Calbiochem (Nottingham, United Kingdom), and Novartis AG (Basel, Switzerland), respectively. All other reagents were obtained from Sigma (Poole, United Kingdom).

Statistical analysis. Data points and bars represent the mean ± SEM of a given number of independent observations (n). When appropriate, data were analyzed using Student's paired t test or analysis of covariance (ANCOVA) as indicated. The null hypothesis was rejected when $P < 0.05$.

Results

Effect of chronic treatment of rats with albuterol on the ability of albuterol, PGE_2, forskolin, and IBMX to protect against ACh-induced bronchoconstriction. Intravenous administration of albuterol (100 μg/kg) or PGE_2 (300 μg/kg) to saline-treated rats significantly reduced (by 52% and 47%, respectively) the magnitude of ACh-induced bronchoconstriction, whereas in the albuterol-treated group of animals, no significant protection was observed with either agonist (Figure 2, a and b), indicating that the desensitization was heterologous. In contrast, forskolin (300 μg/kg intravenously) and IBMX (300 μg/kg intravenously) inhibited, equally, ACh-induced bronchoconstriction in both groups of rats (Figure 2, c and d), suggesting that the intracellular site(s) of desensitization was upstream of adenylyl cyclase. In none of the experiments did albuterol, PGE_2, forskolin, or IBMX affect resting bronchial tone.

Effect of chronic treatment of rats with PGE_2 on the ability of PGE_2 and albuterol to protect against ACh-induced bronchoconstriction. Figure 2 shows the results of experiments designed to determine whether chronic treatment of rats with PGE_2, an agonist at Gs-coupled receptors of the EP_2- and EP_4-subtype, also promoted heterologous desensitization. Intravenous administration of PGE_2 (300 μg/kg) to saline-treated animals significantly reduced (by 48%) the magnitude of ACh-induced bronchoconstriction, whereas in PGE_2-treated rats, no significant protection was observed (Figure 2e). Identical results were obtained when the bronchoprotective effect of albuterol (100 μg/kg intravenously) was examined in saline- and PGE_2-treated animals (Figure 2f).

Effect of chronic treatment of rats with albuterol on β-adrenoceptor density in lung. Chronic treatment of rats with albuterol (40 μg/kg/h) produced a significant reduction in β-adrenoceptor density when compared with naive animals, without affecting the affinity of the nonselective ligand, [^{125}I]ICYP (Table 1). This effect was selective for the β_2-adrenoceptor subtype and resulted in a 30% reduction in B_{max}, which lowered the β_2/β_1 subtype ratio from 2:1 in saline-treated rats to 1.45:1 in animals given albuterol (Table 1).

Effect of chronic treatment of rats with albuterol on the ability of albuterol and PGE_2 to increase the cAMP content and activate PKA ex vivo. To determine whether downregulation of β_2-adrenoceptor number was accompanied by compromised signal transduction, the ability of albuterol to increase cAMP and activate PKA in lung parenchyma was assessed ex vivo. Lung taken from saline-treated animals responded to albuterol with a robust increase in the cAMP content (EC_{50} = 4.1 ± 1.7 μM). This effect was concentration-dependent and resulted, maximally, in a sixfold increase in cAMP over the baseline level (Figure 3a). In contrast, albuterol failed to elevate the cAMP content in lung excised from rats given albuterol chronically for 7 days at any concentration examined (Figure 3a). No significant difference in basal cAMP content in lung excised from saline- and albuterol-treated rats was found. Consistent with the cAMP results already described here, the resting PKA activity ratio in lung taken from saline- and albuterol-treated rats was not significantly different (Figure 4a). However, the ability of albuterol to activate PKA in lung excised from rats desensitized to the bronchoprotective effect of albuterol was markedly impaired when compared with lung taken from saline-treated animals (Figure 4a). Albuterol-induced cAMP accumulation was also blunted in tracheae taken from albuterol-treated rats (data not shown), indicating that β_2-adrenoceptors were desensitized in the relevant tissue. No difference in the basal cAMP content was detected.

Consistent with the in vivo results, the desensitization of β-adrenoceptor–mediated cAMP accumulation produced in rats treated chronically with albuterol was heterologous in that the ability of PGE_2 to elevate the cAMP content in lung also was profoundly impaired (Figure 4b).

Effect of chronic treatment of rats with albuterol on the ability of CTX to increase the cAMP content in lung ex vivo. A pharmacologic approach was adopted to determine whether pulmonary β_2-adrenoceptor desensitization and compromised cAMP formation and PKA activation in lung were associated with any change in the ability of Gs to couple to adenylyl cyclase. This was achieved by making use of CTX, which directly activates Gs by catalyzing the NAD^+-dependent ADP-ribosylation of the α-subunit. As shown in Figure 5, CTX elicited concentration-dependent cAMP accumulation in lung taken from saline-treated animals with an EC_{50} of 1.83 ± 0.17 μg/mL. CTX also increased cAMP in lung from desensitized rats, but the

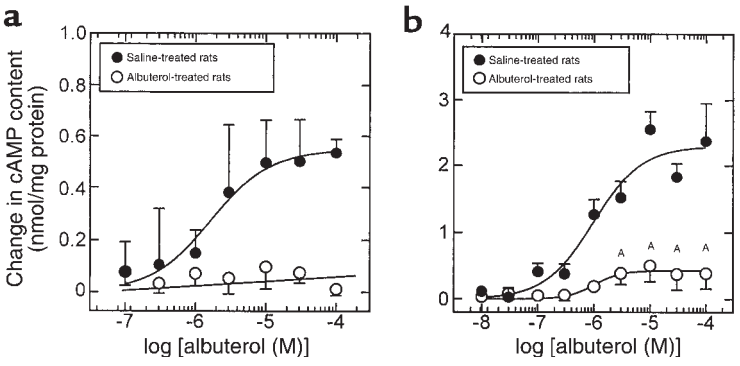

Figure 3
Effect of chronic treatment of rats with albuterol on albuterol-induced cAMP accumulation ex vivo. Rats were given saline (filled circles) or albuterol (open circles; 40 μg/kg/h) for 7 days, and the ability of albuterol to increase the cAMP content in lung was determined in the absence (**a**) or presence (**b**) of IBMX (100 μM). Each data point represents the mean ± SEM of eight determinations. Basal cAMP levels were 81 ± 75 and 57 ± 36 pmol/mg protein in saline- and albuterol-treated rats, respectively, in the absence of IBMX, and 803 ± 57 and 891 ± 78 pmol/mg protein in saline- and albuterol-treated rats, respectively, in the presence of IBMX. $^AP < 0.05$, significant increase in cAMP formation induced by isoproterenol in IBMX-treated tissue.

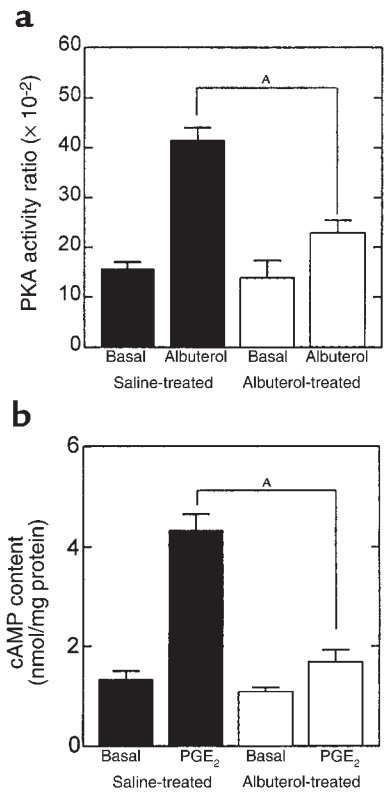

Figure 4
Effect of chronic treatment of rats with albuterol on β_2-adrenoceptor signaling in lung ex vivo. Rats were given saline (filled bars) or albuterol (open bars; 40 μg/kg/h) for 7 days, and albuterol-induced PKA activation (**a**) and PGE_2-induced cAMP accumulation (**b**) in lung were determined. Each bar represents the mean ± SEM of eight determinations. $^AP < 0.05$, cAMP accumulation and PKA activation significantly attenuated in lung from albuterol-treated rats.

concentration-response curve was significantly ($P < 0.05$; ANCOVA) displaced 5.9-fold to the right (EC_{50} = 10.8 ± 4.4 μg/mL) in an apparently parallel fashion without a reduction in the maximum response.

Effect of chronic treatment of rats with albuterol on the expression of Gsα in lung and trachea. Western blotting was performed to determine whether the compromised ability of CTX to elevate the cAMP content in lung taken from albuterol-treated rats was associated with a reduction in the expression of Gsα. A primary antibody, raised against the α-subunit of Gs, identified three bands in membranes prepared from the lung of saline-treated rats that migrated as 42-, 44-, and 52-kDa peptides on SDS polyacrylamide gels. Treatment of rats for 7 days with albuterol significantly reduced the intensity of each of these bands by 40–60% relative to the "housekeeping" protein, actin (Figure 6a). Identical results were obtained with tracheae (data not shown). A primary antibody raised against the common Gβ-subunit, recognized a single 35-kDa band in lung and tracheal membranes prepared from saline-treated rats. However, relative to actin, Gβ was not altered in tissue taken from albuterol-treated animals (data not shown). Neither α nor β subunits of Gs were detected in the cytosolic fraction of tissue taken from either group of animals at 7 days.

Pretreatment of lung membranes with CTX resulted in the [α^{32}P]NAD^+-dependent ADP ribosylation of a single broad 44-kDa band of Gsα (probably reflecting poor resolution of radiolabeled CTX substrates) that corresponded to the predominant species detected by Western blotting. However, a significant ($P < 0.05$) loss of CTX substrate (51.3 ± 12.6%; n = 6) was found in membranes taken from albuterol-treated rats relative to their saline-treated counterparts (Figure 6b).

Effect of chronic treatment of rats with albuterol on cAMP PDE activity in lung. In many cells, agonist-induced desensitization of receptors that couple through Gs is attributable, in part, to upregulation of cAMP PDE (18, 22). To determine the extent to which this effect accounted for the desensitization of β_2-adrenoceptor–mediated cAMP accumulation reported in the current study, the ability of albuterol to elevate cAMP in lung was assessed ex vivo in the absence and presence of the PDE inhibitor, IBMX. As shown in Figure 3, albuterol-induced cAMP accumulation in lung excised from saline-treated rats was markedly potentiated (approximately sixfold) by IBMX (100 μM) with no change in potency (EC_{50} = 1.1 ± 0.3 μM). Significantly, in lung excised from albuterol-treated rats, IBMX conferred sensitivity to albuterol ex vivo. Thus, the cAMP content increased in a concentration-dependent manner with an EC_{50} of 1.3 ± 0.4 μM and a maximum response equivalent to that achieved by albuterol in naive tissue in the absence of IBMX (compare Figure 3, a with b).

cAMP PDE activity was significantly elevated (1.5-fold) in lung tissue harvested from albuterol-treated

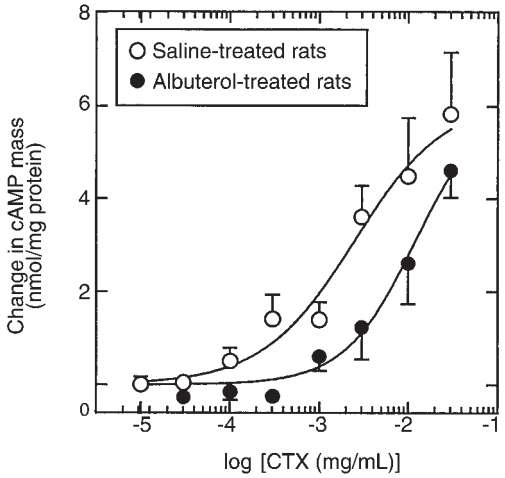

Figure 5
Effect of chronic treatment of rats with albuterol on the ability of CTX to promote cAMP formation in lung ex vivo. Rats were given saline (open circles) or albuterol (filled circles; 40 μg/kg/h) for 7 days, and CTX-induced cAMP accumulation in lung was determined. Each point represents the mean ± SEM of six determinations. Basal cAMP levels were 108 ± 28 and 84 ± 21 pmol/mg protein in saline- and albuterol-treated rats, respectively.

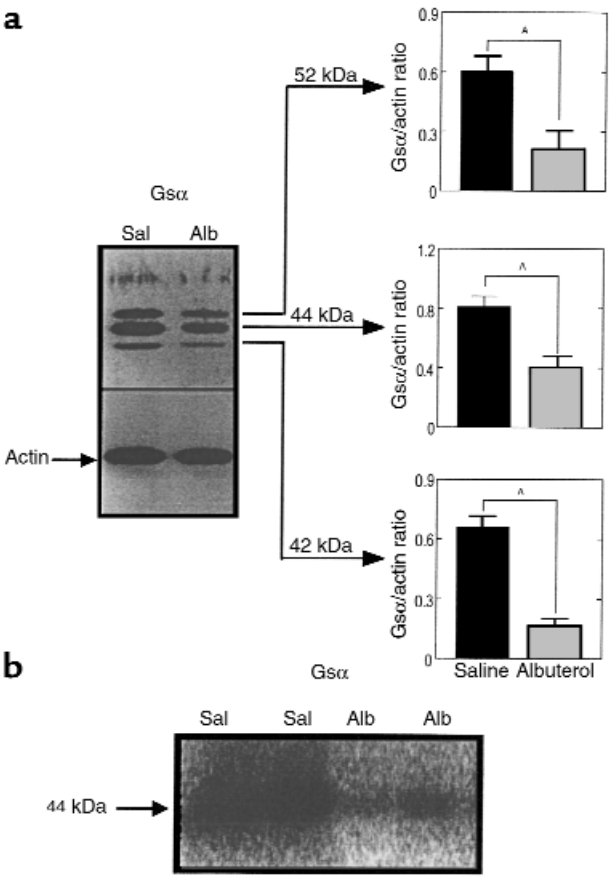

Figure 6
Effect of chronic treatment of rats with albuterol on Gsα expression in lung membranes. Rats were given saline (filled bars) or albuterol (gray bars; 40 μg/kg/h) for 7 days, and the expression of membrane-associated Gsα was determined. (**a**) Representative Western blot and bar charts of the mean (± SEM) results of nine determinations. (**b**) Representative digitized phosphorimage of the amount of Gsα assessed by CTX-catalyzed NAD^+-dependent ADP ribosylation. Equal loading was confirmed by staining of the gels with Coomassie blue. Sal, animals treated with saline; Alb, animals treated with albuterol. $^AP < 0.05$, significant reduction in Gsα expression.

rats when compared with animals that received saline [(B + C + D)/(A + D) in Figure 7]. Rolipram (30 μM) markedly attenuated cAMP hydrolysis in lung excised from both groups of animals by approximately 50%, and quantification of this effect (B/A in Figure 7a) demonstrated a 1.43 increase in PDE4 activity. However, a residual activity remained in albuterol-treated lung in the presence of rolipram (C + D in Figure 7a) that was consistently greater than that seen in control tissue (D in Figure 7a), indicating that a rolipram-insensitive isoenzyme was also upregulated [(C + D)/D in Figure 7a]. This activity was attributable solely to PDE3. Indeed, the PDE3 inhibitor, Org 9935 (30 μM) suppressed cAMP hydrolysis in lung excised from saline- and albuterol-treated rats by 56% and 57%, respectively (A and B in Figure 7b), which equated to a 1.5-fold increase in activity (B/A in Figure 7b). Furthermore, when rolipram and Org 9935 were used in combination, the remaining PDE activity (D in Figure 7c) was the same in lung taken from both groups of animals.

Effect of chronic treatment of rats with albuterol on GRK activity and GRK-2 expression in lung. Chronic infusion of rats with albuterol produced a significant (56%) increase in cytosolic GRK activity in lung parenchyma when compared with saline-treated animals (Figure 8a). This effect was associated with a 1.95-fold increase in the expression of GRK-2 as assessed by Western blotting using an antibody directed against the COOH-terminus of the protein (Figure 8, b and c). In contrast, no change in GRK activity or GRK-2 expression was detected in the particulate fraction of albuterol-treated rats at 7 days (data not shown).

Discussion

Intravenous administration of ACh to saline- and albuterol-treated rats provoked bronchoconstriction that was submaximal, highly reproducible with repeated challenges, and of equivalent magnitude. Given that chronic treatment of rats with albuterol resulted in profound pulmonary β_2-adrenoceptor desensitization, these data suggest that neither sympathetic tone nor endogenous catecholamines regulate baseline airway caliber, which is consistent with the sparse innervation of airway smooth muscle with catecholamine-containing varicosities in this species (28, 29).

An in vivo model of pulmonary β_2-adrenoceptor desensitization was established in rats by administering albuterol continuously to mimic the effect of regular therapy of asthmatic subjects with β_2-adrenoceptor agonists. In these animals, the ability of albuterol, given acutely, to protect against ACh-induced bronchoconstriction was abolished, indicating that functional desensitization had occurred. Although desensitization of β_2-adrenoceptor–mediated relaxation has been demonstrated ex vivo in tracheae and lung taken from rats treated chronically with isoproterenol and norepinephrine (12, 30), this is one of the first reports to our knowledge to demonstrate this phenomenon in vivo.

Albuterol-induced desensitization was studied in more detail by examining the effect of PGE_2, which relaxes rat airways by stimulating adenylyl cyclase through EP_4-like prostanoid receptors (31). The demonstration that the antispasmogenic activity of PGE_2 was also impaired indicates that the desensitization was heterologous. Furthermore, rats rendered tolerant to PGE_2 were also insensitive to albuterol, implying that a common mechanism(s) may account for β_2-adrenoceptor- and EP_4-receptor–mediated cross-desensitization. Although few reports document heterologous desensitization of GPCRs in vivo, the data reported here are consistent with results of Zeiders et al. (32), who demonstrated that chronic treatment of rats with isoproterenol attenuated isoproterenol- and glucagon-stimulated adenylyl cyclase activity in cardiac membranes ex vivo.

A clue to the mechanism of pulmonary β_2-adrenoceptor desensitization in vivo was that the antispasmogenic activity of forskolin was preserved in albuterol-treated

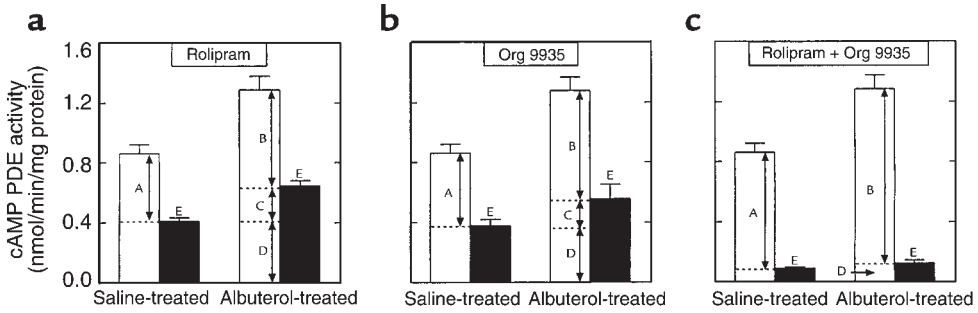

Figure 7
Effect of chronic treatment of rats with albuterol on cAMP PDE activity in lung. The ability of rolipram (30 μM; **a**), Org 9935 (30 μM; **b**), and a combination of rolipram and Org 9935 (**c**) to suppress cAMP hydrolysis was assessed in lung taken from rats treated chronically with saline and albuterol. Open and filled bars show PDE activity in the absence and presence of PDE inhibitor, respectively. In each panel, B/A corresponds to the fold-increase in activity attributable to the PDE isoenzyme(s) defined with rolipram, Org 9935, or rolipram/Org 9935. Total induction is given by (B + C + D)/(A + D). Data represent the mean ± SEM of eight determinations. $^{E}P < 0.05$, significant inhibition of PDE activity.

rats, implying that a defect in signal transduction had occurred upstream of adenylyl cyclase. Several possibilities were considered as follows.

Downregulation of β_2-adrenoceptor number. Treatment of rats for 7 days with albuterol produced a significant (30%) reduction in pulmonary β_2-adrenoceptor number when compared with animals that were given saline. A similar loss in pulmonary β-adrenoceptor density has also been reported in rats after in vivo treatment with isoproterenol (11, 21, 33, 34) and terbutaline (35), and in guinea pigs given norepinephrine (12). Collectively, these findings support the general concept that prolonged in vivo exposure of animals to β_2-adrenoceptor agonists promotes desensitization by stimulating the internalization and degradation of the cognate receptor (17). Thus, such a process could account for the in vivo desensitization seen in the present study. However, this mechanism probably plays a relatively minor role for at least two reasons (15, 36). First, the reduction in β_2-adrenoceptor density was quite small and of questionable functional significance given that a receptor reserve is thought to exist on airways smooth muscle for many β_2-adrenoceptor agonists (5). Second, in vivo treatment of rats with albuterol produced heterologous desensitization.

Activation of GRKs. Another mechanism of desensitization involves phosphorylation of the β_2-adrenoceptor by GRKs, in particular GRK2. Unlike results obtained in other systems (14, 15), GRK activity was unchanged in lung membranes prepared from albuterol-treated rats, which is, perhaps, not unexpected, as translocation of GRK2 to the plasma membrane is rapid and transient, occurs concurrently with receptor phosphorylation, and precedes desensitization and receptor sequestration. Moreover, GRKs show considerable substrate specificity and effect homologous desensitization by phosphorylating only the agonist-occupied form of the receptor (15). Clearly, therefore, this mechanism cannot readily explain why PGE_2-induced responses were also desensitized by albuterol.

It is noteworthy that cytosolic GRK activity was significantly increased in the lung of albuterol-treated rats when compared with those animals that received saline. This effect was associated with increased protein expression suggesting that GRK2 mRNA stability and/or gene transcription had been increased. This interpretation is supported by the finding that long term infusion of mice with isoproterenol increased mRNA, protein and activity of GRK2 in the heart by a mechanism that was attributed to a cAMP-independent increase in GRK2 gene transcription (37).

Table 1
Effect of chronic treatment of rats with albuterol on the density of β-adrenoceptors on rat lung membranes and on the affinity of [^{125}I]ICYP

Treatment	Total β-adrenoceptors		β_1-Adrenoceptors^{A}		β_2-Adrenoceptors^{B}	
	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
	(pM)	(fmol/mg protein)	(pM)	(fmol/mg protein)	(pM)	(fmol/mg protein)
Saline	7.3 ± 1.0	172 ± 9	21 ± 2.7	68 ± 7	5.3 ± 1.1	126 ± 7
Albuterol	9.5 ± 2.7	109 ± 18^{C}	18 ± 3.0	63 ± 11	8.1 ± 1.5	85 ± 9^{C}

Rats were implanted subcutaneously with osmotic minipumps delivering saline or albuterol (40 μg/kg/h). After 7 days, lung membranes were prepared, and total, β_1- and β_2-adrenoceptor density and the affinity of [^{125}I]ICYP were determined as described in Methods. Data represent the mean ± SEM of 12 observations. $^{A}\beta_1$-Subtype defined with the selective β_2-adrenoceptor antagonist, ICI 118551 (100 nM). $^{B}\beta_2$-Subtype defined with the selective β_1-adrenoceptor antagonist, CGP20712A (100 nM). $^{C}P < 0.05$, significant reduction in B_{max} compared with saline-treated rats.

Downregulation of membrane-bound Gsα. One mechanism that could account for the ability of albuterol to promote heterologous desensitization is a reduction in the abundance of Gsα (19). Three molecular weight species of Gsα were detected in lung and tracheal membranes prepared from both groups of rats. Based on electrophoretic mobility on SDS polyacrylamide gels, two of these proteins represent so-called "long" and "short" Gsα variants that arise through alternative mRNA splicing and are ubiquitously expressed (38, 39). The other isoform is probably one of several other splice variants that have a more tissue-specific distribution (40). Significantly, the expression of Gsα (assessed by Western blotting and CTX-catalyzed ADP ribosylation) was reduced (~50%) in the lung of albuterol-treated rats. Moreover, the ability of CTX to increase the cAMP content in lung harvested from albuterol-treated rats was also compromised when compared with animals that received saline. Collectively, these results provide a compelling explanation for heterologous desensitization in this model. Indeed, downregulation of Gsα would blunt the ability of albuterol, and other agonists that utilize the same pool of Gsα, to activate adenylyl cyclase and protect the airways against ACh-induced bronchoconstriction. A similar explanation has been advanced to account for the impaired activation of adenylyl cyclase by isoproterenol and glucagon ex vivo in cardiac membranes purified from rats treated chronically with isoproterenol (32).

Albuterol could downregulate Gsα by at least three mechanisms: decreased Gsα gene transcription, destabilization of preexisting Gsα mRNA transcripts, or redistribution/degradation of membrane-associated Gsα. McKenzie and Milligan (41) have reported that downregulation of Gsα after treatment of NG108-15 cells with PGE_1 is not associated with a reduction in Gsα mRNA or prevented by cycloheximide. Thus, the decrease of Gsα may not reflect a reduction in transcription or in mRNA stability. Concordant with this interpretation is that isoproterenol promotes a rapid translocation of Gsα from the membrane to a cytosolic pool in a number of different cells (42, 43) that is followed, after chronic exposure, by degradation of the protein (19). In this study, Gsα was not detected in the cytosol of lung or tracheae taken from albuterol-treated rats, suggesting that degradation of Gsα may have occurred. It is unclear exactly how albuterol reduces the abundance of Gsα at the membrane but it might involve rapid depalmitoylation at Cys^3, which prevents the association of Gsα with the membrane (44, 45). Indeed, isoproterenol-induced depalmitoylation of Gsα correlates with a redistribution of the G-protein from membrane to cytosol (44), which is consistent with the finding that that membrane-bound, but not cytosolic, Gsα contains palmitic acid (46). The molecular basis of this depalmitoylation is unknown, but it is independent of cAMP (45).

It is unclear whether a 50% reduction in Gsα reduced signaling through pulmonary β_2-adrenoceptors in this study. However, agonist-induced activation of adenylyl cyclase is attenuated in GH_3 rat pituitary tumor cells transfected with a plasmid-encoding antisense Gsα mRNA under conditions in which Gsα protein was reduced by approximately 50% (47). It is likely, therefore, that the loss of membrane-bound Gsα in lung and tracheae accounts for compromised β_2-adrenoceptor signaling in rats treated chronically with albuterol. Furthermore, the magnitude of Gsα downregulation in GH_3 cells is agonist-dependent, indicating differences in the efficiency at which GPCRs utilize the available pools of Gsα for the stimulation of adenylyl cyclase (47). This finding could have important clinical implications for the design of new β_2-adrenoceptor agonists that produce less desensitization, as depalmitoylation of Gsα is positively related to the intrinsic activity of the agonist (45). Thus, given that a large receptor reserve exists in airway smooth muscle for most β_2-adrenoceptor agonists (5), a drug with low intrinsic activity would produce less downregulation of Gsα and so limit β_2-adrenoceptor desensitization without significantly affecting bronchodilator activity.

Another mechanism that can produce heterologous desensitization is upregulation of cAMP PDE (18). In leukocytes, β_2-adrenoceptor agonists increase PDE

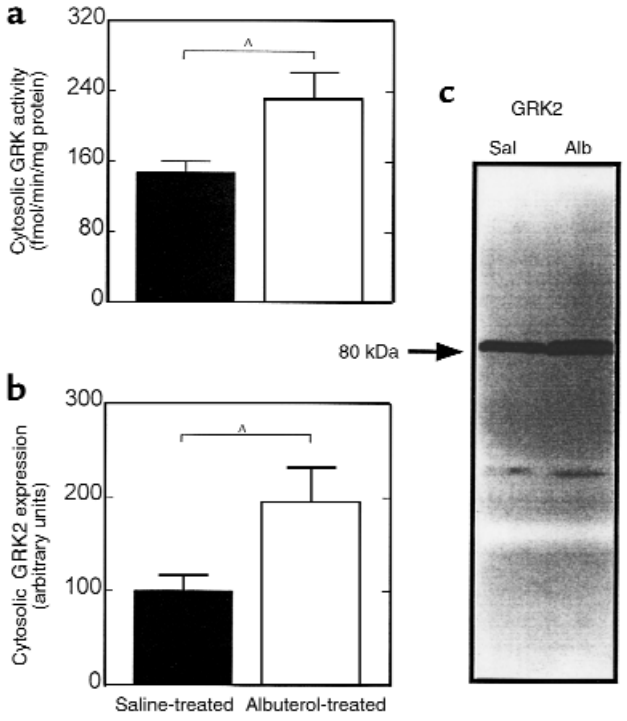

Figure 8
Effect of chronic treatment of rats with albuterol on GRK activity and GRK2 expression in lung. Rats were given saline or albuterol (40 μg/kg/h) for 7 days, and GRK activity and the expression of GRK2 protein in lung were determined by measuring the phosphorylation of rhodopsin (**a**) and by Western analysis (**b** and **c**), respectively. Each bar represents the mean ± SEM of 12 determinations. $^A P < 0.05$, significant increase in GRK2 activity and expression.

activity through the induction of specific PDE isogenes, thereby compromising signaling through all GPCRs that couple positively to adenylyl cyclase (18, 22). Although the in vivo results obtained with forskolin in albuterol-treated rats imply that the site of desensitization was upstream of adenylyl cyclase, the activity of PDE3 and PDE4 was elevated in lung taken from these animals. It is possible, therefore, that induction of PDE occurred over a time frame when albuterol-induced adenylyl cyclase activation was not impaired. The additional finding that IBMX partially restored albuterol-induced cAMP accumulation in "desensitized" lung implies that upregulation of PDE conferred this effect. However, whether the enhanced rate of cAMP metabolism contributed to the loss of antispasmogenic activity of albuterol and PGE_2 is unclear given that the protection afforded by forskolin against ACh-induced bronchoconstriction was preserved in both groups of animals. Therefore, we conclude that the increase in PDE in the lung is either functionally irrelevant at 7 days or occurred in nonairway smooth muscle cells. Alternatively, as forskolin can evoke large increases in cAMP, the upregulation of PDE may be insufficient to alter PKA activity.

In conclusion, the results of this study demonstrate that chronic treatment of rats with albuterol produced pulmonary β_2-adrenoceptor desensitization. Although a number of processes could contribute to this effect, the heterologous nature of the desensitization and the finding that the antispasmogenic activity of forskolin was preserved suggest that the primary molecular etiology is a reduction in the abundance of membrane-associated Gsα. It is important to appreciate that these studies were performed in healthy rats and that the applicability of the model to patients with asthma, in whom the airways are inflamed and hyper-reactive, is unknown. However, it has been reported that segmental allergen challenge of asthmatic subjects does not impair β_2-adrenoceptor function in epithelial cells obtained by bronchoscopic brushings, suggesting that the disease itself does not predispose to desensitization (48). Thus, downregulation of Gsα may have direct relevance to the treatment of asthma in circumstances in which susceptible individuals become tolerant to the beneficial effects of regular, high-dose β_2-adrenoceptor agonists.

Acknowledgments

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