

Antithetic regulation by β -adrenergic receptors of G_q receptor signaling via phospholipase C underlies the airway β -agonist paradox

See the related Commentary beginning on page 495.

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β -adrenergic receptors (β ARs) relax airway smooth muscle and bronchodilate, but chronic β -agonist treatment in asthma causes increased sensitivity to airway constriction (hyperreactivity) and is associated with exacerbations. This paradox was explored using mice with ablated β AR genes (β AR^{-/-}) and transgenic mice overexpressing airway smooth muscle β_2 AR (β_2 AR-OE) representing two extremes: absence and persistent activity of airway β AR. Unexpectedly, β AR^{-/-} mice, lacking these bronchodilating receptors, had markedly decreased bronchoconstrictive responses to methacholine and other G_q -coupled receptor agonists. In contrast, β_2 AR-OE mice had enhanced constrictive responses. Contraction to permeabilization with β -escin was unaltered by gene ablation or overexpression. Inositol phosphate accumulation by G_q -coupled M_3 -muscarinic, thromboxane- A_2 , and 5-HT₂ receptors was desensitized in airway smooth muscle cells from β AR^{-/-} mice and sensitized in cells from β_2 AR-OE mice. Thus, β AR antithetically regulates constrictive signals, affecting bronchomotor tone/reactivity by additional means other than direct dilatation. Studies of signaling elements in these pathways revealed the nodal point of this cross talk as phospholipase C- β 1, whose expression was altered by β AR in a direction and magnitude consistent with the physiologic and cellular responses. These results establish a mechanism of the β -agonist paradox and identify a potential asthma modifier gene (phospholipase C- β 1), which may also be a therapeutic target in asthma when chronic β -agonists are required.

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Introduction

Functional regulation of G protein-coupled receptors (GPCRs) is a dynamic process, such that integration of multiple signals leads to an adaptive cellular response and end-organ homeostasis. Regulatory events, however, can also be maladaptive, contributing to disease pathophysiology, and can limit the therapeutic efficiency of agents targeted to these receptors (1–3). One of the most readily observed regulatory

phenomena of GPCRs is agonist-promoted desensitization (3, 4), which has been extensively investigated with the β_2 -adrenergic receptor (β_2 AR). Early events include phosphorylation of the receptor by GPCR kinases and the second messenger-dependent protein kinase A, while prolonged agonist exposure results in a decrease in receptor expression (termed downregulation). The signals evoked by GPCRs and the accompanying regulatory events, however, do not occur in isolation of other cellular processes or physiologic responses. This propensity for one GPCR signal to alter another has been termed cross talk and serves to further modify receptor function and coordinate multiple signaling events leading to global modification of responses within the cell or organ.

In the treatment of asthma, inhalation of β -agonists is the most common and effective approach for acute relief of bronchoconstriction, and chronic use of β -agonists is not infrequent. The primary target for these agonists is the β AR expressed on airway smooth muscle, which acts to relax constricted muscle and thus bronchodilate. A common physiologic consequence of chronic β -agonist use, however, is an increase

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Nonstandard abbreviations used: G protein-coupled receptors (GPCRs); β -adrenergic receptor (β AR); muscarinic acetylcholine subtype 3 receptor (M_3 R); thromboxane A_2 receptor (TXA_2 R); 5-hydroxytryptamine subtype 2 receptor (5-HT₂R); phospholipase C (PLC); inositol 1,4,5 trisphosphate (IP_3); enhanced pause (Penh); airway resistance (R_{aw}); relative units (RUs); ¹²⁵I-cyanopindolol (¹²⁵I-CYP); β_2 AR overexpression (β_2 AR-OE).

in bronchoconstrictive responses (5–7). Such responses are assessed in humans by measuring airflow after provocation with graded inhalations of agents such as methacholine or histamine. This enhancement of contractile sensitivity observed with chronic β -agonist use has been referred to as hyperresponsiveness. Similarly, the functional antagonism of β -agonists to constrictive stimuli (termed the bronchoprotective effect) also wanes over time with chronic use. These effects on airway contractility are thought to predispose asthmatic patients using β -agonists on a regular basis, and potentially during abrupt withdrawal, to episodes of acute bronchospasm. Consistent with these physiologic events, frequent β -agonist use is associated with increases in asthma exacerbations and other indices of morbidity and mortality (8–10). Indeed, some investigators have supported the notion of gradual withdrawal of β -agonists as a therapeutic approach in resistant asthma (11). Analogous to the use of β AR antagonists in the treatment of heart failure, the concept of “reverse pharmacology” has been raised as a potentially viable strategy for diseases such as asthma, where chronic agonists are used (12). Airway smooth muscle tone and reactivity is primarily affected by $G_{\alpha s}$ -coupled receptors (relaxation) and $G_{\alpha q}$ -coupled receptors (contraction) (13). The former include β ARs (primarily the β_2 AR subtype in humans), the PGE_2 receptor, and the vasoactive intestinal peptide receptor. Smooth muscle relaxation results from a decrease in intracellular Ca^{2+} , a consequence of cAMP-dependent protein kinase A phosphorylation of multiple proteins (14). The contractile $G_{\alpha q}$ -coupled receptors include muscarinic acetylcholine subtype 3 receptor (M_3R), thromboxane A_2 receptor (TXA_2R), a 5-hydroxytryptamine receptor (likely subtype 2; 5-HT $_2R$), cysteinyl leukotriene, histamine, platelet-activating factor, and tachykinin receptors. Smooth muscle contraction occurs by receptor-mediated activation of phospholipase C (PLC), leading to inositol 1,4,5 trisphosphate (IP $_3$) production and release of Ca^{2+} from the sarcoplasmic reticulum (14).

This incongruity in β -agonist therapy has been the source of considerable debate, in part because no cohesive mechanism accounts for the potential deleterious effects of chronic administration, the benefits of minimized usage, or the implied paradoxical regulation by β AR of airway sensitivity to contraction. Recent development of mice lacking β AR, as well as those overexpressing β AR in airway smooth muscle, provides a means to identify heretofore unrecognized β AR signaling events in airway smooth muscle that regulate in vivo bronchial tone and responsiveness. The overexpression of β_2 AR results in signaling even in the absence of agonist, since there is an increase in the proportion of receptors in the spontaneously active (termed R^*) state, thereby resulting in a continuous level of signaling. The knockout and overexpressing mice thus represent two conditions: a total

lack of β AR activity and persistent activity in the airway. In this study we use these mice to define this paradoxical regulation and have identified the molecular basis whereby airway β AR amplify or attenuate $G_{\alpha q}$ receptor signaling in direct proportion to β AR activity, which we refer to as antithetic regulation.

Methods

Transgenic and gene ablation mice. Mice with ablated β_1 AR and β_2 AR genes were generated as described elsewhere (15). (Mice lacking both receptor subtypes were used to ensure ablation of all β AR activity in the airway.) For the β_2 AR-overexpressing mice, the α -smooth muscle actin promoter was used to target expression of the human receptor to airway smooth muscle by standard transgenic techniques as described previously (16). For the current studies, a line of these transgenic mice (generations F_5 – F_8) was used that have low levels of β_2 AR overexpression (see Results). Of note, we have reported previously the effects of massive (approximately 75-fold) overexpression of β_2 AR on airway smooth muscle (16). These mice are markedly bronchodilated at rest, which limits the generation of airway tone in vivo, and thus were not amenable to the physiologic experiments in the current work where we sought to examine sensitivity to several constrictive agents, some of which are of low contractile efficacy (see Discussion). In all studies, mice were compared with the appropriate strain- and age-matched wild-type littermates, which were exposed to the identical housing conditions.

Noninvasive measurement of airway reactivity. Airway responsiveness to methacholine was measured noninvasively in conscious, unrestrained mice as described (16), using a whole-body plethysmograph (Buxco Electronics Inc., Troy, New York, USA). Using this system, the volume changes that occur during a normal respiratory cycle are recorded as the pressure difference between the animal-containing chamber and a reference chamber. The resulting signal is used to calculate respiratory frequency, minute volume, tidal volume, and enhanced pause (Penh). Penh is a unitless value that is a function of the maximum inspiratory and maximum expiratory pressures and the timing of expiration. For acute airway challenges, Penh has been shown to closely correlate with invasive measurements of airway resistance (17) and was used as one measure of airway responsiveness in intact mice in the current study. Mice were placed in the chamber and allowed to adjust to their surroundings for 10 minutes. They were then exposed to aerosolized PBS (to establish baseline), followed by increasing concentrations of aerosolized methacholine (2.5–80 mg/ml). Each dose of methacholine was delivered for 1 minute, and respiratory measurements were recorded and averaged for a 4-minute period after delivery.

Invasive measurement of airway reactivity. Invasive assessment of respiratory mechanics was carried out in intact, intubated, anesthetized mice using a

computer-controlled small-animal ventilator in a manner similar to that reported previously (18). Briefly, mice were anesthetized with pentobarbital and the trachea cannulated with an 18-gauge metal needle. Mechanical ventilation (FlexiVent; SCIREQ Inc., Montreal, Canada) was applied at a frequency of 150 breaths per minute, a tidal volume of 10 ml/kg (approximately 250 μ l/breath), and positive end-expiratory pressure of 2.5 cm H₂O. To deliver contractile agonists, the inspiratory limb of the ventilator was transiently diverted through the reservoir of an ultrasonic nebulizer. During the 30 seconds when aerosolized drugs were delivered, the ventilator frequency was decreased to 100 breaths per minute and the tidal volume increased to 20 ml/kg. To measure airway resistance (R_{aw}), the ventilator was set to apply a low-amplitude flow oscillation to the lung during a period of apnea. Using software provided by the manufacturer, airway resistance was determined from airway pressure and piston position data. The mechanical impedance of the ventilator tubing and tracheal cannula were accounted for in a calibration procedure performed prior to intubation of each mouse. Prior to challenge, stabilization of R_{aw} was attained, followed by administration of aerosolized PBS. The resistance after PBS was considered to be baseline R_{aw} . Contractile agonists (diluted to the indicated concentrations in PBS) were then administered by the nebulizer. R_{aw} was measured every 30 seconds for the first 5 minutes after delivery of each drug, with maximal response typically occurring 90–120 seconds after delivery. R_{aw} was allowed to return to baseline before proceeding with the next challenge.

Tracheal ring contractility. Studies of mouse tracheal contractility were performed as reported previously (16). Tracheas were excised and dissected free of surrounding tissues and cut into rings of 5 mm in length. The tracheal rings were mounted on stainless steel wires connected to isometric force transducers and immersed in a physiologic saline solution (in millimoles: 118 NaCl, 11 glucose, 4.73 KCl, 1.2 MgCl₂, 0.026 EDTA, 1.2 KH₂PO₄, 2.5 CaCl₂, 25 NaH₂CO₃) at 37°C and bubbled with 95% O₂/5% CO₂ to maintain a pH of 7.40. Each tracheal ring was stretched to a tension of 5 mN, an optimal passive tension for maximizing active force, equilibrated for 20 minutes, and then the indicated agents were added to the bath and the maximal response over the next 1 minute recorded. Cumulative concentration-isometric force curves were generated for the responses using standard curve-fitting techniques.

Airway smooth muscle cell cultures. Primary cultures of airway smooth muscle cells were derived from explants of excised tracheas from the mice as reported previously (16) and maintained in DMEM, 10% FCS, and 1 \times antibiotic-antimycotic solution (Invitrogen, Grand Island, New York, USA) at 37°C in a 95% air, 5% CO₂ incubator. As demonstrated previously (16), this isolation technique provides for

adherent cells with morphologic and immunohistochemical characteristics of smooth muscle cells for at least 12 passages. All experiments were performed on confluent cells at matched passage numbers four through nine.

Inositol phosphate accumulation. [³H]inositol phosphate formation was determined in intact airway smooth muscle cells using methods similar to those we have detailed elsewhere (19). Briefly, near-confluent airway smooth muscle cells in 12-well plates were incubated with inositol-free media containing [³H]myo-inositol (5 μ Ci/ml) for 24 hours at 37°C in 5% CO₂ atmosphere. Cells were then washed and incubated with PBS for 30 minutes followed by a 30-minute incubation with 20 mM LiCl in PBS. Cells were then exposed to PBS (basal) or carbachol, serotonin, or U46619 for 15 minutes, after which total inositol phosphates were extracted by column chromatography (AG1-X8 columns; Bio-Rad Laboratories Inc., Hercules, California, USA).

Other studies. For Western blot analysis, cells were lysed and solubilized in 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors (10 μ g/ml benzamidine, 10 μ g/ml soybean trypsin inhibitor, 10 mg/ml aprotinin, and 5 μ g/ml leupeptin) in PBS. After centrifugation at 10,000 g, the proteins in the supernatant were fractionated on polyacrylamide gels (6–10%), transferred to PVDF membranes, and incubated overnight with primary Ab's. The anti-PLC- β 1 (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) Ab was diluted 1:500. Anti-G_{s α} (Perkin Elmer, Boston, Massachusetts, USA) and anti-G_{q α /11} (Calbiochem, San Diego, California, USA) Ab's were each diluted 1:1,000. After further washing and hybridization with a goat anti-rabbit secondary Ab (diluted 1:6,500), bands were visualized by enhanced chemiluminescence and quantitated with Scan Analysis software (Biosoft, Cambridge, United Kingdom), with the data reported in relative units (RUs) of pixel density. Radioligand binding with ¹²⁵I-cyanopindolol (¹²⁵I-CYP) was carried out as described (20) on membrane preparations from cultured airway smooth muscle cells. Response curves were analyzed using Prism (GraphPad Software for Science Inc., San Diego, California, USA). Comparisons were by ANOVA or *t* tests as indicated, with *P* values less than 0.05 considered significant. Results are reported as means plus or minus standard errors.

Results

To determine whether ablation of β AR signal transduction altered intrinsic airway reactivity or constrictive receptor signaling, we initially measured the bronchoconstrictor response to the M₃R agonist methacholine in β AR^{-/-} mice with whole-body plethysmography (Figure 1, a and b). Strain-matched wild-type mice showed a robust dose-dependent bronchoconstrictor response, with a maximum Penh of 4.9 ± 0.24 . In contrast, methacholine-induced bron-

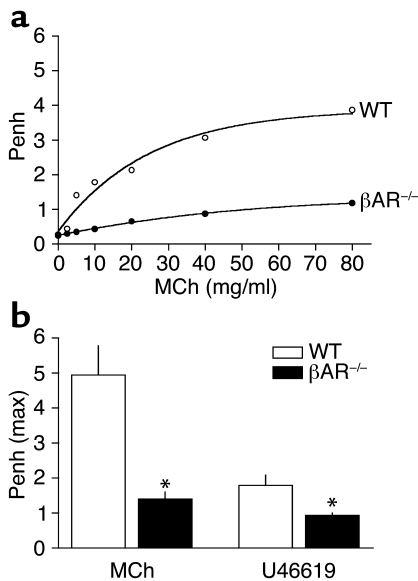


Figure 1

βAR^{-/-} mice display paradoxical contractile responses as assessed by whole-body plethysmography. Mice inhaled the indicated agents in an unrestrained state in a small-animal body box, and Penh was measured as described in Methods. (a) A representative dose-response study with methacholine (MCh) is shown. (b) Mean results from eight to twelve mice in each group in response to methacholine and the thromboxane analogue U46619 are shown. **P* < 0.001 versus wild type.

choconstriction in the βAR^{-/-} mice was only 1.40 ± 0.07 (*P* < 0.001). In addition, the βAR^{-/-} mouse response was less sensitive than that of wild-type mice, with an ED₂₀₀ for methacholine of approximately 20 mg/ml compared with approximately 5 mg/ml for wild-type mice. This result was somewhat unexpected, because we had anticipated that the loss of a potent bronchoprotective pathway would serve to augment bronchoconstrictor responses. To determine whether this response was specific for methacholine, we exposed mice to the bronchoconstrictor U46619, which is a thromboxane mimetic acting at the TXA₂R. Once again, we found that bronchoconstriction was substantially diminished in the βAR^{-/-} mice (Figure 1b).

Although Penh has been reported to correlate with other measurements of airway resistance (17), some have questioned whether Penh accurately reflects the extent of acute bronchoconstriction under certain circumstances (21). Our results described above therefore prompted additional, confirming studies using an anesthetized, intubated mouse model, which provides a direct measure of airway responses in absolute resistance units in the lung (Figure 2). As shown, initial studies showed that the contractile response to methacholine in wild-type mice was ablated by coinhalation of the βAR agonist isoproterenol, confirming that the model detects the expected constrictive as well as dilatory responses in normal mice and that the βAR^{-/-} mice are indeed physiologic knockouts of βAR responses in the airway. Basal (“resting”) airway resistance, measured

after aerosolization of PBS alone, was not found to be different between βAR^{-/-} and wild-type mice (1.04 ± 0.08 versus 1.36 ± 0.11 cm H₂O/ml/s; *P* = 0.07). Remarkably, the maximal increase in resistance to methacholine was severely blunted in the βAR^{-/-} mice (3.0 ± 0.9 versus 24 ± 6.2 cm H₂O/ml/s for wild type), and the response to serotonin was essentially absent (Figure 2).

We next measured contractile activity in excised tracheal rings so that we could specifically isolate the physiologic response of airway smooth muscle (Figure 3). In wild-type mice, acetylcholine-mediated maximal active isometric force was 13.3 ± 2.1 mN/mm². In contrast, active force attained with βAR^{-/-} rings was only 5.8 ± 0.6 mN/mm². In addition, the contractile response to U46619 was approximately sevenfold lower in the βAR^{-/-} mice compared with wild type. Importantly, though, the response following permeabilization with β-escin, which evokes contraction by an influx of extracellular Ca²⁺, was retained in βAR^{-/-} rings and indeed was slightly greater than wild type (6.6 ± 0.6 versus 4.5 ± 0.6 mN/mm²; *P* = 0.04). These latter results indicated that the intrinsic ability of airway smooth muscle to generate force (i.e., contract) is not altered by βAR gene ablation.

The results from these three types of physiologic studies were in complete agreement and indicated that βAR gene ablation decreased responsiveness of airway smooth muscle to exogenous contractile agents. Because the agonists used in the above studies each contract airway smooth muscle through activation of receptors that couple to G_{αq} (M₃R, 5HT₂R, and TXA₂R), we considered that an active cross talk between these signaling pathways and the G_{αs}-coupled βAR was influenced by the complete loss of βAR signaling in the βAR^{-/-} mice. To test this hypothesis, we measured agonist stimulation of inositol phosphate production, a downstream consequence of

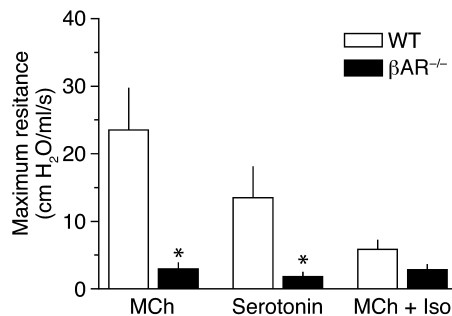


Figure 2

In vivo airway resistance responses to contractile ligands are decreased in βAR^{-/-} mice. Intubated mice were administered aerosolized solutions of PBS, methacholine (MCh) (80 mg/ml), serotonin (20 mg/ml), and methacholine with 1.0 mg/ml isoproterenol (MCh + Iso) in PBS, and then airway resistance was measured after each administration. Baseline resistance was not different between wild-type and βAR^{-/-} mice (see text), but the latter had virtually no contractile responses to methacholine or serotonin (*P* < 0.001 by ANOVA). Results are from four to six mice of each genotype. *Not different (*P* > 0.05) from baseline resistance.

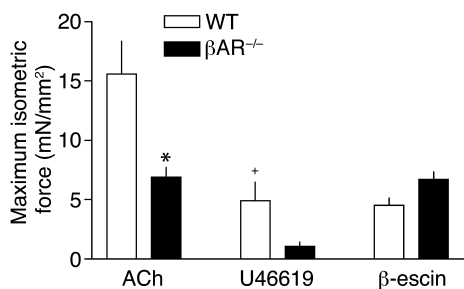


Figure 3

Airway smooth muscle contractions to G_{α_q} -coupled receptor agonists are impaired in $\beta AR^{-/-}$ tracheal rings. The contractile responses to the M_3R agonist acetylcholine (ACh) (10 nM–1.0 mM) and the TXA_2R agonist U46619 (1.0 nM–0.1 mM) were ascertained in tracheal rings from $\beta AR^{-/-}$ mice and wild-type littermates. Shown is the maximal absolute isometric force evoked by the indicated agents. Maximal responses to acetylcholine and U46619 were depressed in the $\beta AR^{-/-}$ mice, while the contractile responses to the cell-permeabilizing agent β -escin (500 μM) were preserved. Results are from five to seven experiments. * $P = 0.005$; * $P = 0.024$.

G_{α_q} -coupled receptor activation, in primary cultures of airway smooth muscle cells derived from the two mouse lines. Compared with cells from wild-type littermates, inositol phosphate accumulation was substantially lower in cells from the $\beta AR^{-/-}$ mice in response to agonists acting at all three receptors (Figure 4).

Although β -agonists are potent acute bronchodilators, numerous studies have suggested that chronic use of these agents promotes increased airway hyper-reactivity or loss of protective effect in asthmatics (see Introduction). This observation, taken together with our finding that βAR signaling modulates the responsiveness of airway smooth muscle to contractile agonists by a mechanism other than direct opposition, suggested the possibility that chronic βAR stimulation could have the opposite physiologic and biochemical effects to those found in the mice without βAR activity. To address this, we used transgenic mice with targeted overexpression of $\beta_2 AR$ ($\beta_2 AR-OE$) in airway smooth muscle. $\beta_2 AR$ expression in cultured airway smooth muscle cells from these mice was 580 ± 140 fmol/mg (as determined by ^{125}I -CYP radioligand binding), which represents approximately tenfold overexpression compared with wild-type cells. This expression provides for persistent activation of this signaling pathway specifically in smooth muscle cells of the airway (16). In cultured airway smooth muscle cells we found that the inositol phosphate responses to carbachol, serotonin, and U46619 were indeed increased in cells from $\beta_2 AR-OE$ mice compared with cells from wild-type littermates (Figure 4). We expected, then, that the physiologic response to contractile agents would be enhanced in the $\beta_2 AR-OE$ mice, as well. To test this, we used the isolated tracheal ring preparations since in this assay the $\beta_2 AR-OE$ mice are not influenced by circulating epinephrine (as would be the case in the intact animal), thereby

maximizing sensitivity for detecting potential subtle differences. Using this approach (Figure 5), we found that the concentration-response curves between the tracheal rings from the wild-type and $\beta_2 AR-OE$ mice were clearly different ($P < 0.001$), and maximal active force generated by $\beta_2 AR-OE$ rings in response to acetylcholine was modestly, but significantly, greater (approximately 33%) than the force generated by the wild-type mice (Figure 5).

Taken collectively, these data in mice that lacked and overexpressed βAR indicated that G_{α_q} -coupled receptor signal transduction was proportionately regulated by βAR activity in airway smooth muscle cells. Since the response to multiple contractile agonists was similarly attenuated, we considered that a signaling component common to these three contractile receptors was a likely nodal point for the tight control by way of such cross talk. We directed our investigation to signaling components distal to the receptor but upstream of Ca^{2+} -mediated contraction, particularly the relevant G protein (G_q) and the effector phospholipase $C\beta$. Using Western blot analysis, we found that G_{α_q} content in $\beta AR^{-/-}$ and $\beta_2 AR-OE$ airway smooth muscle cells was not different compared with their respective control (wild-type) cells (Figure 6). PLC- $\beta 1$ content in the $\beta AR^{-/-}$ cells, however, was decreased by over 60% compared with wild-type-derived cells ($23,350 \pm 3,536$ versus $69,640 \pm 2,442$ RU, respectively; Figure 6a). This decrease is consistent with the decreased agonist-promoted inositol phosphate production and the decreased contractile responses observed in the $\beta AR^{-/-}$ mice. Since the signaling and contractile functions

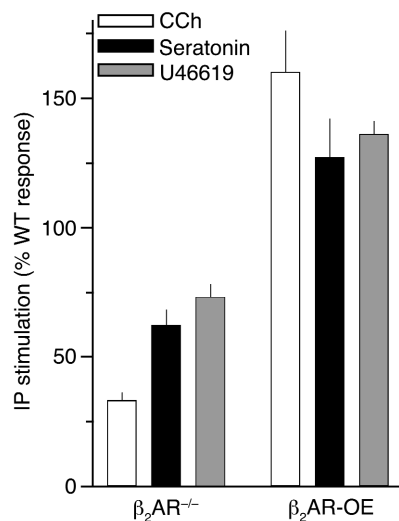


Figure 4

Antithetic regulation of G_{α_q} receptor signaling by βAR activity in isolated airway smooth muscle cells. Primary airway smooth muscle cell cultures were established from tracheas from the $\beta AR^{-/-}$, the $\beta_2 AR-OE$, and their wild-type (WT) littermates. Maximal whole-cell inositol phosphate accumulation in response to carbachol (CCh), serotonin, and U46619 were reduced in $\beta AR^{-/-}$ cells and increased in the $\beta_2 AR-OE$ cells ($P < 0.01$ for all responses versus wild-type littermates). Results are from five experiments performed in triplicate.

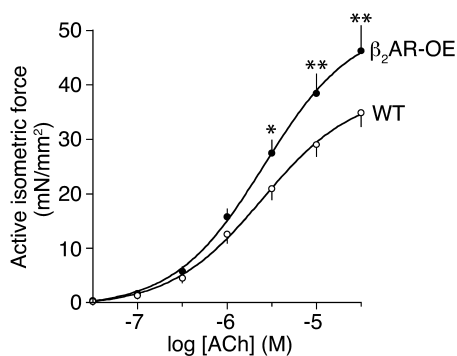


Figure 5
 β_2 AR-OE mice have increased contractile responses in airway smooth muscle. Tracheal rings from the β_2 AR-OE mice and wild-type littermates were contracted by the indicated concentrations of acetylcholine (ACh). The overall responses differed ($P < 0.001$ by ANOVA), and the maximal response was approximately 33% greater for the β_2 AR-OE rings ($P < 0.001$). Results are from six experiments. *P* values of individual post hoc comparisons: * $P = 0.02$; ** $P < 0.001$.

were increased in the β_2 AR-OE, we expected that PLC- β 1 expression would be increased as well, if the evolving paradigm was correct. Indeed, PLC- β 1 expression in cells from β_2 AR-OE mice was increased twofold over those from wild-type littermates ($76,700 \pm 8,226$ versus $38,280 \pm 3,279$ RU, respectively; Figure 6b). Neither mouse line had altered levels of $G_{\alpha s}$, the cognate G protein for β AR.

Discussion

The mechanistic basis of enhanced airway responsiveness to constrictive stimuli during chronic β -agonist treatment of asthma has been elusive. This treatment-related phenomena has been considered a physiologically relevant adverse effect, particularly since the increased use of β -agonists has been associated with increased asthma morbidity and mortality (8–10). The enhanced sensitivity to contractile stimuli has been thought to be consistent with a greater propensity for acute flares of bronchospasm, also termed loss of “asthma control,” during chronic β -agonist therapy (11). While β -agonists administered on a chronic basis are efficacious, their use in asthma has been called into question because of the aforementioned deleterious physiologic effects on airway reactivity and these epidemiologic associations. One hypothesized mechanism of this altered hyperresponsiveness, or loss of bronchoprotective effect, has been that desensitization of the β_2 AR by chronic agonists lessens the opposition to contractile events (7, 22). We show here, however, that cross talk between β AR and $G_{\alpha q}$ receptor signaling results in enhanced signaling of the contractile pathway. So, rather than simply a loss of relaxation, the program evoked by chronic β -agonists includes a gain of contractile signaling.

In the current work, we approached this issue using mice with an absolute lack of β AR activity or persistent β AR activation in airway smooth muscle. We

assumed that these extreme conditions would provide the greatest opportunity to delineate the interplay between β AR and constrictive receptors. The initial findings with the β AR $^{-/-}$ mice using whole-body plethysmography were somewhat surprising. These mice lacked the bronchodilatory β AR, so the expectation was that they would have enhanced responsiveness to contractile agents. This potentially “unopposed” state paradoxically resulted in a marked decrease in airway responsiveness to the inhaled M_3 R agonist methacholine, however. Subsequent studies in an intubated mouse model confirmed that this response is indeed occurring in the airway and also revealed that serotonin-promoted contraction was also markedly reduced in the β AR $^{-/-}$ mice. Tracheal ring preparations, which provided the most direct approach to isolate airway smooth muscle responses, again confirmed hyporesponsiveness to M_3 R activation, as well as to the thromboxane analogue U46619. The contractile responses to cell permeabilization by β -escin, which is due to an influx of Ca^{2+} , were equivalent between wild-type and β AR $^{-/-}$ mice, indicating that the underlying contractile mechanisms are intact in the knockout mice.

Importantly, the three contractile agonists used in these studies act through receptors (M_3 R, TXA_2 R, and 5-HT $_2$ R) that couple to $G_{\alpha q}$, suggesting a common mechanism. We thus concentrated on shared elements of these signaling pathways, since it seemed unlikely that expression of all three of these receptors would be reduced. Contraction of smooth muscle by $G_{\alpha q}$ -coupled receptors is due to IP $_3$ receptor-mediated Ca^{2+} release from the sarcoplasmic reticulum. Since β -escin responses were equivalent, it seemed reasonable that the adaptive event(s) were proximal to the Ca^{2+} -dependent step, particularly at the level of $G_{\alpha q}$, PLC isoforms, inositol phosphates (or their precursors), or

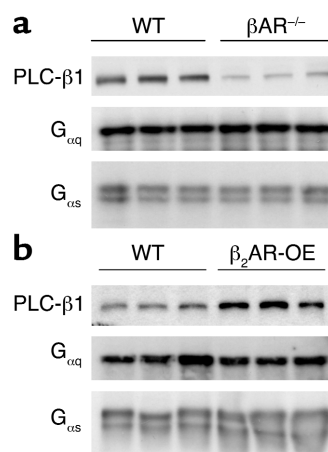


Figure 6
 Antithetic regulation of PLC- β 1 expression by β AR activity in airway smooth muscle cells. (a) PLC- β 1 was decreased approximately 60% in β AR $^{-/-}$ cells ($P < 0.001$) and (b) increased twofold in β_2 AR-OE cells ($P = 0.01$), compared with their wild-type littermates. In contrast, expression levels of $G_{\alpha q}$ and $G_{\alpha s}$ were not different.

the IP₃ receptor. In cultured airway smooth muscle cells inositol phosphate production in response to all three agonists was significantly reduced in β AR^{-/-} cells compared with those from wild-type mice, further narrowing the sites of potential interaction. Subsequent studies revealed that expression of PLC- β 1 was markedly reduced in the β AR^{-/-} cells, while levels of G_{αq} and G_{αs} were not appreciably changed.

This cross talk between bronchodilating and bronchoprotective pathways suggests that an adaptive program that promotes a defined equilibrium is in play so as to maintain bronchomotor tone or reactivity within a specific range. If this is so, we hypothesized that an antithetic response would be evident with the two extremes of β AR activation, manifested as opposite physiologic, signaling, and protein-expression events. This was indeed found, as indicated by our results from the overexpressing mice. In these β ₂AR-OE mice, the sensitivity to methacholine and the maximal contractile response were increased. In addition, inositol phosphate production from activation of M₃R, TXA₂R, and 5-HT₂R were all increased in airway smooth muscle cells from β ₂AR-OE mice compared with wild type. Finally, consistent with the physiologic and cell-based signaling and the cross-talk paradigm, expression of PLC- β 1 was found to be increased in these mice with persistent β ₂AR activation.

The current results with β AR knockout mice and low-level β ₂AR-overexpressing mice need to be reconciled with our earlier report with markedly overexpressed β ₂AR (approximately 75-fold over endogenous levels) in airway smooth muscle (16). In that study, it was found that the bronchodilating effect of such overexpression resulted in resistance to bronchoconstriction. This scenario is consistent with the notion that airway tone and responsiveness is ultimately set by the net effect of G_q and G_s signaling events. While we have shown in the current report that persistent β ₂AR signaling can augment G_q signaling, it is clear from the high overexpressor data that this can be overcome, at some point, by increasing G_s signaling to extraordinary levels. In essence, overexpression to this extent obscures the physiologic sequelae of altered G_q signaling due to such marked, virtually “fixed,” bronchodilatation. Further confirmation of the β AR-G_q receptor cross talk comes from the β AR knockout mice, where overexpression issues are not a concern. In these mice the alterations in G_q signaling at the physiologic and biochemical level and PLC- β 1 regulation were the opposite of the β ₂AR overexpressors in each case, which indicates a dynamic and directional regulation. In some ways the biology of extreme overexpression has also been experienced by us (23, 24) and others (25) with overexpressing β ₂AR in the heart. With very high expression levels, physiologic function (rate and force of contraction) was maximal in the absence of agonist (25). Only with mice expressing β ₂AR at a lower level could meaningful physiologic and biochemical correlates be established (23, 24).

Reported here, our results in airway smooth muscle delineate one mechanism by which β -agonists act to increase the potential for airway hyperresponsiveness and support a clinical benefit for minimizing β -agonist use when possible. They also identify a potential asthma-modifier gene, PLC- β , which may also represent a novel target for therapy. Inhibitors of one or more of the PLC isoforms would be predicted to decrease G_{αq} receptor-mediated bronchoconstriction and minimize the deleterious enhancement of contractile sensitivity induced by β -agonists. Virtually all the endogenous constrictive substances implicated in the bronchospastic component of asthma, including acetylcholine, thromboxane, histamine, leukotrienes, and prostaglandins, act through receptors that couple to G_{αq}. These are released in neuronal, autocrine, paracrine, or humoral programs instigated by the inflammatory state of the disease. Interestingly, agents that are antagonists at individual G_{αq} receptors (such as montelukast, ipratropium, and seratrodoast) have limited efficacy in treating acute bronchospasm in asthma, which may be due to the large number of constrictive receptors that are activated in the disease. Thus, agents that decrease the activity of a common distal element in the pathway may be more useful. In combination with a β -agonist, both interventions could lead to persistent bronchodilatation without increased airway hyperresponsiveness.

This current study also appears to distinguish between desensitization of airway β AR and airway hyperresponsiveness, both of which occur with chronic β -agonists. While β AR desensitization may contribute to less-effective dilatation, the current data do not support this as the major mechanism of β -agonist-induced airway hyperreactivity for two reasons. First, the ultimate desensitization of β AR function occurs when expression is absent, as is the case with the β AR^{-/-} mice. These mice, however, do not have enhanced constrictive responses, but indeed have a marked reduction in this parameter. Second, the transgenic expression of β ₂AR on airway smooth muscle mimics a persistently activated state due to the receptor overexpression. We have no evidence for desensitization of receptors in such mice (16). Taken together, the results indicate that β ₂AR activation initiates and maintains events that lead to enhanced constrictive responses. Of note, some studies suggest that partial β -agonists evoke fewer adverse effects than full agonists (reviewed in ref. 10) and that certain β ₂AR polymorphisms with altered downregulation phenotypes influence hyperresponsiveness (26, 27). Based on the current work, these observations reflect how the trigger for the events leading to enhanced contractile responsiveness by way of β ₂AR activation can be modified. Furthermore, with the identification of this cross talk event, the genes encoding the PLC isoforms are candidates that should be interrogated for polymorphisms (28), given that there is interindividual variability in β -agonist-promoted bronchial

hyperreactivity and bronchoprotection in humans. Concerning regulatory regions within the PLC gene that may be influenced by β AR activity, we are unaware of any reports of potential control motifs or transcription-binding sites. Indeed, the genomic organization of the PLC- β 1 gene has been discerned only recently (29).

In conclusion, we have identified a mechanism by which chronic airway β AR activation leads to an increase in airway hyperresponsiveness over time during chronic activation. In contrast to the notion that this pathophysiologic outcome is due to β AR desensitization, we find that β AR activation initiates cross talk with the constrictive G_{α_q} -coupled receptor pathway, leading to enhanced signaling and airway hyperresponsiveness. Such regulation is due to modulation of PLC- β 1 expression. Furthermore, this process is antithetic in nature; cessation of β AR activation decreases G_{α_q} receptor signaling and constrictive airway responsiveness. This provides a mechanistic basis for the beneficial clinical effect of limiting chronic β -agonist administration in the management of bronchospastic diseases such as asthma. Whether there is a role for “reverse” or “paradoxic” pharmacology (12) in the treatment of asthma, analogous to the apparent effects of β AR antagonists in heart failure, remains speculative. Since the deleterious cross talk between β ARs and G_{α_s} receptors appears to be by altered expression of PLC, however, novel therapeutics targeting this enzyme may have substantial benefit, particularly when chronic β -agonists are required.

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