Molecular Mechanisms of β_2-Adrenergic Receptor Function and Regulation

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It is now clear that the β_2-adrenergic receptor continuously oscillates between various conformations in the basal state, and that agonists act to stabilize one or more conformations. It is conceivable that synthetic agonists might be engineered to preferentially confine the receptor to certain conformations deemed clinically important while having a less stabilizing effect on unwanted conformations. In addition, studies of genetically engineered mice have revealed previously unrecognized cross-talk between the β_2-receptor and phospholipase C, such that removal of the primary dilating pathway results in downregulation of constrictive pathways and overactivity of the dilating pathway increases the contractile response. These results indicate a dynamic interaction between β_2-receptor activity and G_q-coupled receptors that constrict the airway. Potentially, then, during chronic β-agonist therapy, expression of phospholipase C is increased, the functions of G_q-coupled constrictive receptors are enhanced, and there may be an increased tendency for clinical decompensation due to asthma and chronic obstructive pulmonary disease triggers. Antagonists to these receptors might be able to act synergistically with chronic β-agonists to block the effect of phospholipase C. Alternatively, perhaps novel phospholipase C antagonists would provide the most efficacious approach to blocking the physiologic sequelae of cross-talk between the β_2-receptor and phospholipase C.

Keywords: asthma; β-agonist; chronic obstructive pulmonary disease; G protein–coupled receptor; phospholipase C

G PROTEIN–COUPLED RECEPTOR SUPERFAMILY

G protein–coupled receptors (GPCRs) represent the largest signaling family in the human genome. They transduce signals from the cell exterior to the interior from a host of systems, including the sight, smell, hormonal, neurotransmitter, autocrine, and paracrine systems. The β_2-adrenergic receptor (β_2AR) was one of the first receptors to be identified by radioligand binding, which solidified the notion that a receptor was in fact a *bona fide* entity rather than a theoretical concept that was useful for describing physiologic processes. Subsequent studies revealed that agonists for β_2AR led to activation of adenylyl cyclase, and thus an increase in cyclic adenosine monophosphate (cAMP), via receptor interaction with a third protein, now termed the stimulatory guanine nucleotide–binding protein, G_s. G_s is a heterotrimer, consisting of an α subunit (the dissociated form stimulates adenylyl cyclase) and βγ subunits (which also transduce signals). Each of the aforementioned components of the β_2AR pathway has been cloned and its amino acid sequences delineated. On the basis of structure–function studies and the ability to express these components in various cell types and genetically altered mice, concepts about β_2AR signaling have changed fundamentally.

STRUCTURE OF THE β_2AR

Like all GPCRs, the β_2AR has seven transmembrane-spanning segments, which are α helices. As a consequence, there are three extracellular and three intracellular loops. The amino terminus is extracellular and the carboxy terminus is intracellular. Several posttranslational modifications are noteworthy. The human β_2AR is N-glycosylated at amino acids 6, 15, and 187; these are important for proper insertion of the receptor into the membrane as well as for agonist trafficking (1, 2). Notably, the glycosylation site at amino acid 187 is restricted to higher order primates (1). At amino acid position 341, the cysteine of the human β_2AR is palmitoylated (3). This acts to anchor this part of the carboxy terminus to the cell membrane and imparts several functional features (3). The region between the seventh transmembrane-spanning domain and the palmitoylated cysteine has also been shown to be an α helix in the homologous protein rhodopsin. Therefore, this region is sometimes denoted as the fourth intracellular loop or the eighth α helix. Agonist-promoted phosphorylation of the β_2AR occurs via protein kinase A at serines in the third intracellular loop and the proximal cytoplasmic tail. Such phosphorylation decreases the coupling of the receptor to the G protein G_s and is one mechanism of agonist-promoted desensitization (4). A family of kinases termed G protein–coupled receptor kinases (GRKs) phosphorylates the β_2AR at multiple serines and threonines in the cytoplasmic tail (4). Subsequent binding of β-arrestins to the phosphorylated receptor serves to (*1*) uncouple the receptor from G_s and thus desensitize receptor function (4), (*2*) promote receptor internalization, and (*3*) by virtue of the scaffolding action of β-arrestins, bring other proteins into the receptor's microdomain (5). An example of the latter is phosphodiesterase-4, which is recruited by β-arrestin and acts to metabolize the locally generated cAMP (6). Another posttranslational modification of the β_2AR is ubiquitination, which occurs via an E3 ligase and is a prerequisite for receptor degradation (7).

AGONIST INTERACTION WITH THE β_2AR: WHAT IS THE "ACTIVE" STATE?

The traditional concept of agonist activation of the β_2AR was akin to a lock and key: the agonist fit into the receptor and caused it to adopt a conformation that was favorable for coupling to G_s. However, it is now clear that the receptor is continuously "toggling" between various conformations in the absence of agonist. This is readily appreciated by measuring basal adenylyl cyclase activity in membranes from transfected cells expressing various levels of the β_2AR (8) (Figure 1A). As expression increases, so does basal (nonagonist) adenylyl cyclase activity. In this model, agonist occupancy increases the probability that the receptor will be in the active state (Figure 1B). Neutral antagonists do not affect the equilibrium and thus adenylyl cyclase activities are not altered. (Of course, in a clinical setting in which the levels of synthetic or endogenous agonist are elevated,

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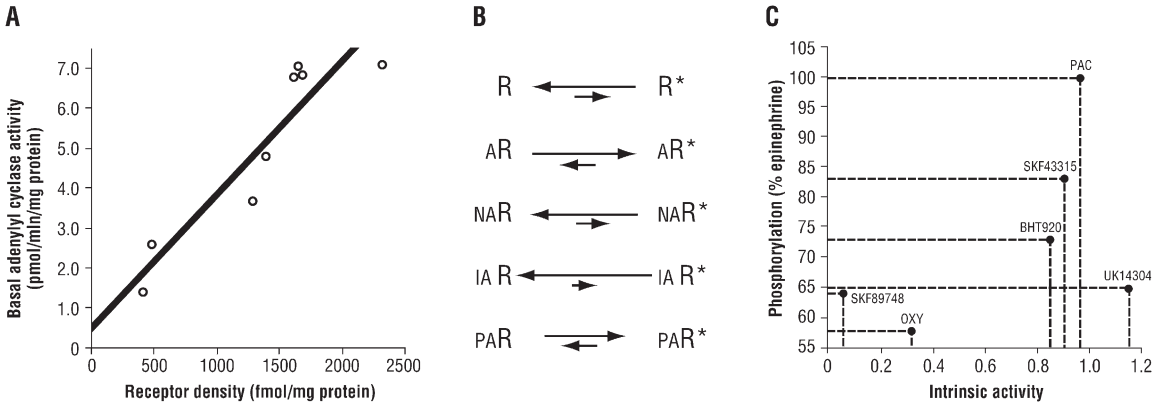

Figure 1. Features of the activation states of G protein–coupled receptors (GPCRs). (*A*) Spontaneous activation of the β_2-adrenergic receptor (β_2AR) in the absence of agonist is shown by the increase in basal adenylyl cyclase activity as β_2AR expression increases (8). (*B*) Actions of various ligands on the equilibrium between inactive receptor (R) and active receptor (R*). A = agonist; NA = neutral antagonist; IA = inverse agonist; PA = partial agonist. (*C*) Lack of correlation between the intrinsic activity of an agonist and agonist-promoted receptor phosphorylation by GRK2 of the α_{2A}AR. PAC = *p*-aminoclonidine; OXY = oxymetazoline.

neutral antagonists nevertheless have a physiologic effect, because they block agonist access to the receptor.) Inverse agonists actually lower basal adenylyl cyclase activities because they preferentially bind to receptor in the inactive state. Data have suggested that most antagonists probably have the properties of an inverse agonist, but their efficacy in decreasing adenylyl cyclase is low and is probably clinically insignificant. Partial agonists either stabilize a different conformation of the receptor compared with full agonists or stabilize the same active conformation as a full agonist but do so less frequently.

Oscillation of the β_2AR between various conformations brings into question whether there is a single "active" conformation. Traditionally, the active state was defined by a single function, which was coupling to G_s, with the "readout" being cAMP production or adenylyl cyclase activities. However, it is now clear that multiple signals are promoted by β_2AR, and that the "ideal" conformation for one effector signal may not be the same as that for another. For example (9), the β_2AR activates the sodium–hydrogen exchanger regulatory factor by direct interaction with the carboxy-terminal domain of the receptor (no G protein is required). Conceivably, an agonist might preferentially activate the G_s pathway compared with the sodium–hydrogen exchanger regulatory factor pathway. Other conformations, such as those that favor GRK-mediated phosphorylation, β-arrestin binding, mitogen-activated protein kinase activation, or G_i coupling, might also be selectively activated, or not activated, by synthetic agonists. Indeed, for the closely related α_2ARs, it has been shown that there is a poor correlation between agonist-promoted phosphorylation of α_2AR by GRKs and the intrinsic activity (compared with the prototypic full agonist epinephrine) of the given synthetic agonist (10) (Figure 1C). Similarly, some agonists show a relative preference for α_2AR–G_s coupling over α_2AR–G_i coupling (11). Taken together, the data suggest that GPCRs, including the β_2AR, oscillate between many conformations in the basal state, and that agonists act to stabilize one or more conformations. Thus, it is conceivable that synthetic agonists might be engineered to preferentially confine the receptor to certain conformations deemed clinically important (e.g., coupling to G_s) while having a less stabilizing effect on potentially unwanted conformations such as phosphorylation by GRKs.

AFTER RECEPTOR ACTIVATION: DEEP PATHWAY EFFECTS

With prolonged agonist activation, β_2ARs undergo processes that limit function, generically termed desensitization. These events are critical for the cell to integrate the myriad signals being received, and for adaptation to physiologic and pathologic states. Desensitization may also limit the therapeutic effectiveness of prolonged agonist exposure (tachyphylaxis), although this may be highly dependent on the structure of the agonist, the cell type, the disease being treated, and the outcome measures deemed to be relevant. Long-term desensitization of the β_2AR is the result of the net effect of several processes, including the short-term events of protein kinase A and GRK phosphorylation, G_i coupling, and the long-term events leading to a decrease in receptor expression (termed downregulation), which is due to transcriptional as well as protein degradation mechanisms.

Several studies (12, 13) have begun to investigate the effects of long-term β_2AR activation on signaling elements far removed from the receptor, G protein, and effector. The bases for such investigations with β_2AR in asthma and chronic obstructive pulmonary disease (COPD) were intriguing clinical observations made during chronic β-agonist therapy that were not readily reconciled by the traditional desensitization paradigm. For example, some, but certainly not all, studies have shown that β-agonists administered on a regular schedule result in a decrease in bronchodilator function. β_2AR desensitization presumably causes this decrease in bronchodilator function. In addition, chronic use of β-agonists causes an increase in sensitivity (i.e., a decrease in the provocative concentration of an agonist causing a 20% fall in FEV_1) to the bronchoconstrictive effects of agents such as methacholine and histamine. This phenomenon appears to be observed more consistently than tachyphylaxis of bronchodilatation from chronic β-agonists. It has also been ascribed to β_2AR desensitization, supposedly because of less opposed bronchoconstriction, and thus increased hyperreactivity.

We hypothesized that these events might be explained by regulation of other signaling elements apart from the receptor (12). To explore this possibility, we studied genetically altered mice in which the β_2AR was overexpressed in airway smooth muscle (mimicking constant β_2AR activation) and mice in which the β_1- and β_2AR genes were ablated (equivalent to an absolute lack of airway βAR activity). In the βAR knockout ($\beta AR^{-/-}$)

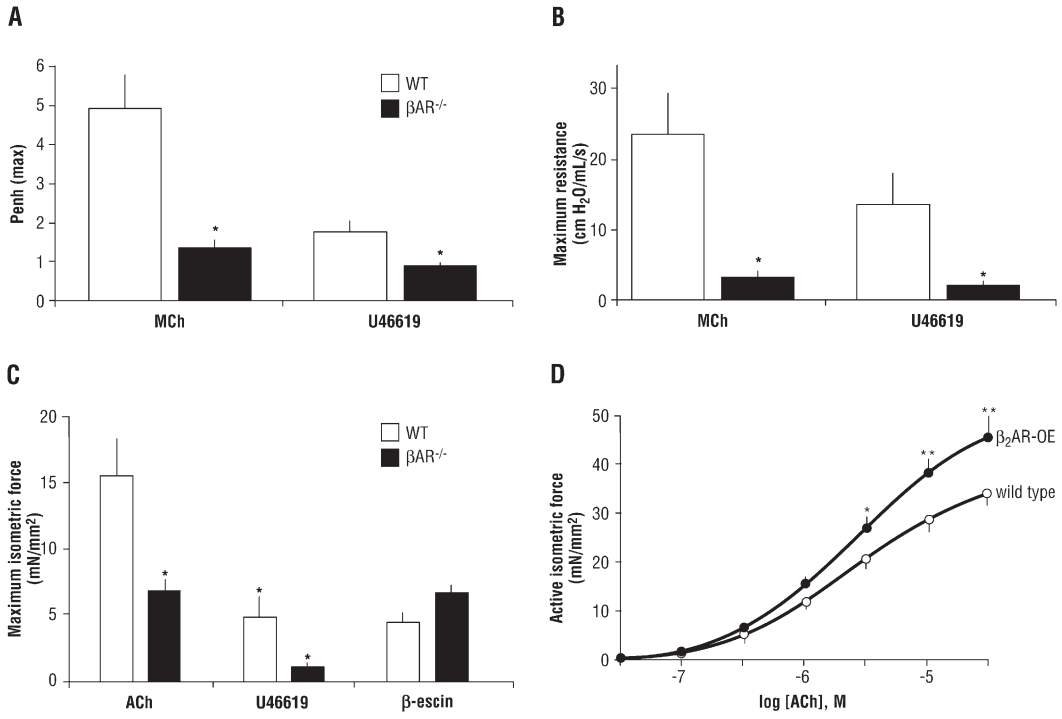

Figure 2. Functional consequences of altered βAR activity in the airways of genetically modified mice. (*A*) $\beta AR^{-/-}$ mice display a paradoxical decrease in the bronchoconstricting effect of methacholine (MCh) and the thromboxane mimetic U46619, as assessed by whole-body plethysmography. P_{enh} indicates enhanced pause, a function of the maximum inspiratory and maximum expiratory pressures and the timing of expiration. * $p < 0.001$ compared with wild type (WT). (*B*) *In vivo* airway resistance responses to contractile ligands are also decreased in $\beta AR^{-/-}$ mice. * Not different ($p > 0.05$) from baseline resistance. (*C*) Airway smooth muscle contractions in response to G_q-coupled receptor agonists are impaired in $\beta AR^{-/-}$ tracheal rings. ACh = acetylcholine. * $p = 0.005$. (*D*) β_2AR-overexpressing (β_2AR-OE) mice have increased contractile responses in airway smooth muscle. * $p = 0.02$; ** $p < 0.001$ (12).

mice we were surprised to find that removal of this bronchodilating pathway resulted in a paradoxical decrease in the bronchoconstricting effect of methacholine and the thromboxane mimetic U46619, as studied by whole-animal plethysmography (Figure 2A). This finding was confirmed by measuring airway resistance in an intubated mouse model, in which the contractile responses to methacholine and serotonin were also found to be decreased (Figure 2B). Finally, tracheal rings were studied *ex vivo*, and the phenomenon was again observed (Figure 2C). Here we were able to expose the rings to β-escin, an agent that makes the cell permeable, allowing an influx of extracellular calcium and subsequent contraction via non-GPCR mechanisms. We found that contraction was the same for $\beta AR^{-/-}$ rings compared with rings from wild-type mice, which indicated that the basic contractile apparatus was intact in $\beta AR^{-/-}$ mice.

To isolate the mechanism of regulation of these constrictive G_q-coupled receptors by the absence of βAR, we generated primary airway smooth muscle cells from each line. As shown in Figure 3, inositol 1,4,5-trisphosphate (IP_3) production was decreased in $\beta AR^{-/-}$ mice. In addition, consistent with what we term antithetic regulation, IP_3 production was increased in β_2AR-overexpressing (β_2AR-OE) mice. This, along with the β-escin data, indicated that the mechanism of this regulation was likely to be at the level of a receptor, G_q, or the effector phospholipase C (PLC), rather than IP_3 precursors, the endoplasmic reticulum IP_3 receptor, or altered intracellular Ca^{2+} stores. We believed it unlikely that the G_q-coupled receptors that we activated (M_3-muscarinic, $5HT_2$, and thromboxane) were all downregulated in $\beta AR^{-/-}$ mice and upregulated in βAR-OE mice. We thus concentrated on elements shared by these receptors. Immunoblotting of airway smooth muscle cell lysates revealed no change in $G_{\alpha q}$ content or in the levels of the cognate G protein for the β_2AR, $G_{\alpha s}$. However, in $\beta AR^{-/-}$ mice, the PLC-β_1 content of $\beta AR^{-/-}$ airway smooth muscle cells was markedly decreased compared with cells from wild-type mice (Figure 3). And, in β_2AR-OE cells, PLC-β_1 was increased about threefold over wild type. The decrease in PLC-β_1 in $\beta AR^{-/-}$ mice was entirely consistent with the decreases in bronchoconstriction and IP_3 production observed in response to G_q-coupled receptor agonists. Tracheal ring studies from β_2AR-OE mice revealed what might be predicted from the IP_3 results and the immunoblotting: acetylcholine evoked greater contraction and had greater potency in these mice compared with wild type (Figure 2D). These data revealed a previously unrecognized interaction between β_2AR and PLC. It appears that there is a mechanism in place to cross-regulate bronchodilating and bronchoconstricting responses. Removal of the primary dilating pathway resulted in a downregulation of constrictive pathways, and overactivity of the dilating pathway had the effect of increasing the contractile response. Data from Callaerts-Vegh have shown in a mouse model of asthma that chronic treatment (28 days) with β-blockers decreases contractile responses (13), analogous to the results we found in $\beta AR^{-/-}$ mice (12).

These results indicate a dynamic interaction between β_2AR activity and receptors that act to constrict the airway. The local concentrations of agonists for these receptors, such as acetylcholine, serotonin, thromboxane, histamine, certain prostaglandins, and certain leukotrienes (acting at the cysteinyl leukotriene-1 receptor) are increased in asthma, and potentially in chronic bronchitis. Thus, during chronic β-agonist therapy, when PLC expression is increased and the functions of these G_q-coupled constrictive receptors are enhanced, there may be an increased tendency for clinical decompensation due to infection, allergy, or other asthma/COPD triggers. As such, antagonists to these receptors may be particularly useful under these circumstances. Interestingly, as monotherapy in asthma, these antagonists (such as ipratropium, montelukast, and seratrodast) have limited efficacy.

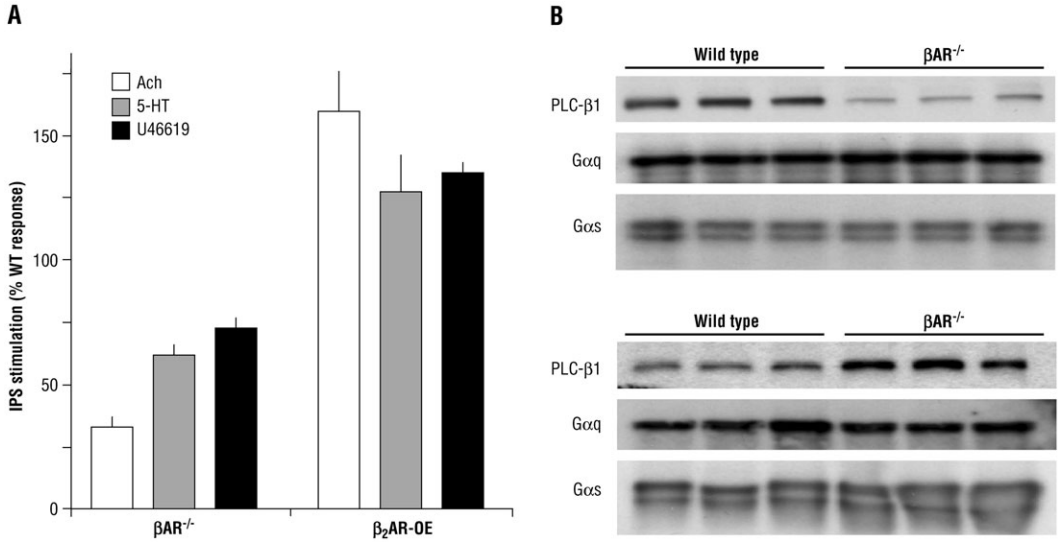

Figure 3. Signaling consequences of altered βAR activity in airway smooth muscle cells derived from genetically modified mice. (*A*) IP_3 responses to the indicated G_q-coupled receptor agonists. ACh = acetylcholine; β_2AR-OE = β_2AR-OE cells; 5-HT = serotonin. (*B*) Immunoblots indicating regulation of phospholipase C (PLC)-β_1 by β_2AR activity (12).

However, they could act synergistically with chronic β-agonists to block the PLC effect and thus achieve a more satisfactory clinical response. Still, because there are so many G_q-coupled constrictive receptors, it will be difficult to fully block this pathway with receptor antagonists. Perhaps new pharmacologic agents that antagonize PLC-β_1 would provide the most efficacious approach to blocking the physiologic sequelae of the chronic β-agonist cross-talk. The events discussed above are also expected in patients with COPD who are treated long term with β-agonists. However, we know less about which endogenous G_q-coupled receptors are activated in this disease. Certainly the favorable response to ipratropium in COPD and the synergy of this anticholinergic with β-agonists are consistent with the cholinergic pathway as an active participant. The potential clinical relevance of β_2AR–PLC cross-talk is illustrated in Figure 4.

It is intriguing to consider this paradigm in the light of several studies that show associations between β_2AR polymorphisms (14, 15) and worsening asthma control (16–18). Because the trigger for this PLC-based constrictive pathway cross-talk is the β_2AR, any genetic variation that alters expression, function, or regulation of the β_2AR will alter the degree of augmentation of G_q receptor signaling. As such, a β_2AR polymorphism might also influence the response to a G_q receptor antagonist such as ipratropium. This indeed appears to be the case in the *b*eta-*a*drenergic *r*esponse by *g*enotype (BARGE) trial (18). Patients with asthma who were homozygous for Arg-16, when withdrawn from β-agonist therapy and provided with ipratropium for rescue purposes, had a significant increase in morning peak expiratory flow. Gly-16 homozygotes showed no such improvement under the same conditions. Although the trial was not specifically designed to address ipratropium responsiveness, the results are nevertheless consistent with the concept of chronic β_2AR cross-talk with M_3-muscarinic receptor signaling of the airway.

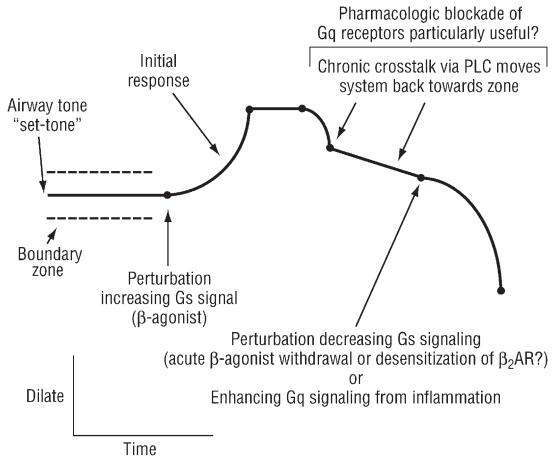

Figure 4. Potential clinical relevance of β_2AR–phospholipase C (PLC) cross-talk. β-Agonists initially dilate the airway but also evoke cross-talk with constrictive receptors, essentially moving airway tone back toward the "set point" after prolonged treatment. A perturbation of contraction–relaxation signaling under these conditions could lead to a substantial decrease in airway caliber. Such a perturbation might be abrupt withdrawal of β-agonist (unopposed contraction) or further activation of G_q signaling from constrictive ligands released during inflammation.

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