Pleiotropic β -Agonist–Promoted Receptor Conformations and Signals Independent of Intrinsic Activity

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β -Agonists used for treatment of obstructive lung disease have a variety of different structures but are typically classified by their intrinsic activities for stimulation of cAMP, and predictions are made concerning other downstream signals based on such a classification. We generated modified β_2 -adrenergic receptors with insertions of energy donor and acceptor moieties to monitor agonist-promoted conformational changes of the receptor using intramolecular bioluminescence resonance energy transfer in live cells. These studies suggested unique conformations stabilized by various agonists that were not based on their classic intrinsic activities. To address the cellular consequences of these differences, G_s -coupling, G_i -coupling (p44/p42 activation), G protein–coupled receptor kinase–mediated receptor phosphorylation, internalization, and down-regulation were assessed in response to isoproterenol, albuterol, terbutaline, metaproterenol, salmeterol, formoterol, and fenoterol. In virtually every case, agonists did not maintain the classic rank order, indicating that distinct signaling is evoked by β -agonists of different structures, which is unrelated to intrinsic activity. The extensive pleiotropy of agonist responses shown here suggests that classification of agonists by cAMP-based intrinsic activity is inadequate as it pertains to other intracellular events and that it may be possible to engineer a β -agonist that stabilizes conformations that evoke an ideal portfolio of signals for therapeutic purposes.

Keywords: β -agonist; β -adrenergic receptor; adenylyl cyclase; G-protein; asthma

The traditional concept of G protein–coupled receptor (GPCR) activation, whereby agonist binds to receptor and imposes a conformational change in the receptor that initiates signaling, has evolved into a complex paradigm. It is now well established that many GPCRs, including the β_2 -adrenergic receptor (β_2 AR), oscillate into the active state in the absence of agonist with the equilibrium favoring the inactive (R) state (1, 2). Agonist occupancy acts to constrain, or stabilize, the active conformation (R*), leading to a shift in the equilibrium from R to R* and sustained intracellular signal amplification. In the case of the β_2 AR, coupling to $G_{\alpha s}$ is one consequence of agonist stabilization, resulting in activation of adenylyl cyclase, increased cAMP, and activation of protein kinase A (PKA), which acts to relax smooth muscle of the airway (3). The fact that GPCRs can signal in the absence of agonist has brought into question whether there is more than one “active state” and whether β -agonists can stabilize different active β_2 AR conformations, or sets of conformations, based on their chemical structures (2, 4). Classi-

CLINICAL RELEVANCE

We show that β -agonists commonly used to treat obstructive lung disease confer different conformations of the β_2 -adrenergic receptor, which results in different downstream signaling.

cally, β -agonists have been characterized by their efficacies (as well as potencies and subtype-specificities) in relation to a benchmark agonist such as isoproterenol. These efficacies, often denoted as their “intrinsic activities,” are based on maximal responses such as intracellular cAMP or tracheal relaxation. As such, β -agonists used for treating asthma and chronic obstructive pulmonary disease are often categorized as full or near-full agonists (e.g., isoproterenol, fenoterol, or formoterol) or partial agonists (e.g., albuterol, metaproterenol, terbutaline, or salmeterol). With the addition of the long-acting formoterol and salmeterol, the duration of action has been added as a descriptor of a given β -agonist. However, because there has been little differentiation of the intracellular effects of the various β -agonists available for clinical use, the molecular and physiologic actions of β -agonists are often considered as “class-effects.”

This broad classification is further confounded by the realization that β_2 AR activation results in a number of distinct “signals” aside from activation of adenylyl cyclase. As an example, some signals are evoked by receptor phosphorylation by G protein–coupled receptor kinases (GRKs), which provide a substrate for the binding of β -arrestins (5). The β -arrestins uncouple the receptor from G_s and scaffold other proteins to the complex, thereby evoking additional intracellular signaling/trafficking. Agonist occupancy of β_2 AR initiates receptor internalization, which serves as a mechanism for short-term desensitization, dephosphorylation, and recycling of receptor to the cell surface or to degradation pathways. β_2 AR can couple to the inhibitory G-protein G_i , potentially modulating adenylyl cyclase activity, but also activating p44/p42 mitogen-activated protein kinase (MAPK) (6, 7). A common initiating event of these signals is the stabilization of the receptor into one or more conformations by the bound agonist. However, little data have been generated as to how agonists of diverse structures, such as those used for treating obstructive lung disease, initiate these events.

In the current work, we established a method to assess the conformational change in the β_2 AR stabilized by various β -agonists using what we term “intramolecular” bioluminescence resonance energy transfer (BRET). In addition, a number of important downstream events from receptor activation by various β -agonists, including G_s coupling, GRK-mediated receptor phosphorylation, G_i coupling (p44/p42 MAPK activation), receptor internalization, and receptor downregulation, were determined. We found extensive agonist pleiotropy based on structure that could not be reconciled based on full or partial agonist

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activity. Thus, it seems that each β -agonist has properties that are dependent on specific conformations of the agonist-stabilized β_2 AR, resulting in unique cellular responses.

MATERIALS AND METHODS

Constructs, Transfections, and Cell Culture

The cDNA for the FLAG- β_2 AR was constructed by standard techniques and consisted of the FLAG epitope (DYKDDDDA) fused in-frame to the amino terminus of the human β_2 AR (with the polymorphic positions at amino acids 16 and 27 being Gly and Glu, respectively) in pcDNA3. For intramolecular BRET studies, the human β_2 AR cDNA was mutated to have in-frame insertions of yellow fluorescent protein (YFP) cDNA in the second intracellular loop or the third intracellular loop and the Renilla luciferase (Rluc) cDNA substituted in frame 3' of the full-length carboxy-terminus or 3' of a truncated carboxy-terminus (see Figure 1 for locations of the insertions). Human airway smooth muscle (HASM) cells were obtained from Clonetics (Walkersville, MD) and grown in the supplied medium. Genotyping (8) of the genomic DNA from these cells at the β_2 AR positions 16 and 27 loci showed heterozygosity at both sites, which is the most common genotype in the population (9). All studies with HASM cells were performed from passages 3–8. HEK-293 cells were transfected with the indicated cDNAs using lipofectamine (Invitrogen, Carlsbad, CA) and were used for studies 2 d later. These cells were maintained in monolayers in Dulbecco's modified Eagle's medium with 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a 5% CO₂ atmosphere. Chinese hamster fibroblasts (CHW-1102 cells) stably expressing the human β_2 AR at $\sim$ 200 fmol/mg were derived from transfections as previously described (10) and maintained as monolayers in the same medium as previously described supplemented with 80 μ g/ml G418.

Agonists

To facilitate the large number of assays with the seven β -agonists, single concentrations representing the maximal effect were used. The concentrations chosen were based on the maximal response attained in agonist-stimulated adenylyl cyclase activity assays that we have previously published (11) and were 2 orders of magnitude greater than the EC₅₀ for the response. These concentrations were isoproterenol (Iso) (5 μ M), albuterol (Alb) (50 μ M), terbutaline (Terb) (175 μ M), metaproterenol (Met) (375 μ M), salmeterol (Salm) (1 μ M), formoterol (Form) (1 μ M), and fenoterol (Fen) (15 μ M).

Intramolecular BRET

BRET-1 was performed on live cells using methods essentially as previously described (12, 13). Briefly, HEK-293 cells were transfected with the indicated constructs and 2-d later were plated in 96-well dishes in PBS with 0.1% glucose at a density of $\sim$ 80,000 cells/well. Agonist or PBS, in 100 μ M ascorbic acid, were added at 37°C; 15 min later, coelenterazine (5 μ M) was added, and the dishes were incubated for 7 min at 25°C. Light emission and acquisition were performed using a VICTOR³ multi-label counter (Perkin-Elmer, Boston, MA). The Rluc signal was obtained at 460 nm and the YFP signal at 535 nm. The BRET ratio was calculated as described (12, 13). Expression levels of the transfected receptors were routinely monitored by the Rluc signal, which correlated with radioligand binding and typically ranged from 500 to 1,000 fmol/mg.

cAMP Determinations

HASM cells were plated in 24-well dishes and grown to 95% confluence. The medium was replaced with serum-free medium (SFM) containing 2.0 μ Ci [³H]adenine and incubated at 37°C for 2 h. The medium was removed, and cells were incubated with fresh SFM containing the indicated agonists for 10 min. Reactions were stopped by the addition of 0.2N HCl. To control for individual column recovery, 5,000 DPM [¹⁴C]cAMP was added to the samples. [³H]cAMP and [¹⁴C]cAMP were separated by alumina column chromatography, and the eluent was counted in a dual channel liquid scintillation counter.

p44/p42 MAPK Assays

These activities were determined by Western blots using phospho-specific antibodies essentially as previously described (12, 14). HASM cells were plated in 12-well dishes, grown to 95% confluence, and incubated in serum free media at 37°C for 4 h. The indicated agonists were added, and incubations continued for 5 min. Reactions were stopped by the addition of a solubilization buffer (1% IGEPAL, 0.5% Na deoxycholate, 0.1% SDS, in PBS) with the protease inhibitors benzamide, soybean trypsin inhibitor, aprotinin, and leupeptin (5 μ g/ml each). The wells were scraped, and the contents were sheared with a 21-g needle, rotated in a microfuge tube for 1 h at 4°C, and centrifuged for 5 min to pellet unsolubilized material. An aliquot of the supernatant was quantitated for protein, and equal amounts of protein (8 μ g) were loaded onto 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes, and Western blots were performed with an antibody to the phosphorylated

Figure 1. Modification of the β_2 AR for intramolecular BRET. Shown are the locations where YFP or Rluc were inserted into the β_2 AR.

form of p44/p42 MAPK (1:2,000 titer; Cell Signaling, Beverly, MA) and reagents from a chemiluminescence system (Amersham, Piscataway, NJ). Images were acquired directly from the membranes using a Fuji LAS-3000 charged-coupled device camera (Fuji, Stamford, CT), and bands were quantitated using the included software. To control for potential differences in protein transfer, the membranes were stripped and probed for GAPDH using an antibody titer of 1:1,000 (Santa Cruz Biotechnology, Santa Cruz, CA) and processed as described previously.

Receptor Phosphorylation

HEK-293 cells in 100-mm dishes were transfected with 20 μ g FLAG- β_2 AR-pcDNA3, and whole cell receptor phosphorylation studies were performed as described (13). Briefly, 2 d after transfection, the medium was replaced with serum- and phosphate-free medium containing [32 P] orthophosphate (500 μ Ci/ml) and incubated for 2 h at 37°C. Cells were exposed to the indicated agonists for 10 min and washed five times with 4°C PBS. Scraped cells were homogenized by repeated pipetting and solubilized by rotation in a microfuge tube for 2 h at 4°C in solubilization buffer consisting of 1% Triton X-100, 0.05% SDS, 0.5 mM EGTA, 1 mM EDTA (pH 7.40), 10 mM NaF, and 10 mM sodium pyrophosphate in PBS. The protease inhibitors were included in this and all subsequent steps. Unsolubilized material was removed by centrifugation at 20,000 $\times g$ at 4°C for 10 min. FLAG- β_2 AR were immunoprecipitated by incubating the supernatant with an anti-FLAG antibody preincubated to agarose beads (Sigma, St. Louis, MO) for 18 h at 4°C. After immunoprecipitation, the beads were washed four times by centrifugation and resuspended in cold solubilization buffer, and the FLAG- β_2 AR was eluted from the beads by incubation with 3x FLAG peptide (Sigma). The eluates were fractionated on 10% SDS-polyacrylamide gels. Signals were acquired from the dried gels with a phosphorimager (Molecular Dynamics, Piscataway, NJ) and analyzed with the accompanying software.

Receptor Internalization

Loss of cell-surface β_2 AR after brief exposure to β -agonist was quantitated using the hydrophilic radioligand [3 H]CGP-12177 using methods previously described (10). CHW-1102 cells were plated in monolayers in 24-well dishes and the next day were incubated with the indicated agonists for 30 min at 37°C in SFM. Cells were washed four times with cold PBS and incubated for 3 h at 4°C with 10 nM [3 H]CGP-12177 in the absence or presence of 10 μ M propranolol used to define nonspecific binding. Cells were washed three times with cold PBS to remove unbound radioligand and lysed by the addition of 500 μ l 1.0% SDS in PBS, which was aspirated and counted in a liquid scintillation counter.

β_2 AR Down-Regulation

Initial studies revealed that, after ≥ 2 h of incubation of cells with formoterol and salmeterol, these agonists could not be readily removed from cells by washing with PBS, thus giving a falsely low expression of density as assessed by [125 I]-CYP radioligand binding to membrane preparations. To avoid this problem, quantitative immunoblots were performed using a human β_2 AR-specific antibody. CHW-1102 cells stably expressing β_2 AR at 90% confluence were treated with 100 μ M ascorbic acid (vehicle) or ascorbic acid with the indicated agonists in SFM for 12 h at 37°C. Cells were washed four times with 5 ml PBS, scraped in 5 mM Tris and 2 mM EDTA (pH 7.4) at 4°C, and pelleted by centrifugation at 19,000 $\times g$ for 10 min. The pellet was resuspended in the same solubilization buffer plus protease inhibitors used in the MAPK assays. After rotation in a microfuge tube for 1 h, the unsolubilized material was pelleted by centrifugation, and the supernatant was quantitated for protein concentration. Thirty micrograms of solubilized membranes were subjected to 10% SDS-PAGE. Immunoblots were performed with a β_2 AR antibody at a titer of 1:200 (H20; Santa Cruz); the polyvinylidene difluoride membranes were processed with an enhanced chemiluminescence system (Amersham) and directly visualized using a Fuji LAS-3000 charged-coupled device camera with bands quantitated using the included software.

Radioligand binding with [125 I]-CYP was performed as described previously (15) using 1 μ M propranolol to define nonspecific binding. Protein concentrations were determined by the copper bicinchoninic acid method (16). Comparisons of agonist effects were by one-way ANOVA followed by the Newman-Keuls test with correction for multi-

ple comparisons. For BRET, one-way ANOVA without post-hoc *t* tests was used to test for trend. *P* values < 0.05 were considered significant. Data are presented as mean $\pm$ SE of *n* independent experiments.

RESULTS

The cAMP Response

The β_2 AR-G α_s -cAMP response was assessed in HASM cells in monolayers exposed to the indicated agonists for 10 min. The results reveal the intrinsic activities as follows: Iso $>$ Fen = Form $>$ Alb = Terb = Met $>$ Salm (Figure 2). These data are consistent with the known properties of these agonists as assessed by a variety of *in vitro* and *in vivo* methods (reviewed in [17–19]).

Receptor Conformation Responses as Assessed by Intramolecular BRET

For these studies, β_2 AR constructs were generated to express modified receptors (Figure 1) in-frame with YFP in the second intracellular loop and Rluc at the full-length or at a truncated carboxy-terminus (β_2 AR:YFPi2-RlucCT and β_2 AR:YFPi2-RlucCT, respectively) or YFP in the third intracellular loop and Rluc at the full length or a truncated carboxy-terminus (β_2 AR:YFPi3-RlucCT and β_2 AR:YFPi3-RlucCT, respectively). When YFP and Rluc are in close proximity (10–80 Angstroms) (20), the energy from Rluc is sufficient to excite YFP, leading to the BRET signal. As has been shown for the β_2 AR (21) and other 7-TM receptors (22), an agonist-promoted change in the conformation of the receptor alters the distance between these intracellular domains, providing a measurement of a conformational change (Figure 1). The agonist-promoted change in BRET signal (compared with PBS alone) is dependent on where the donor and acceptor moieties have been placed within the receptor. We used the second and third intracellular loops because they are contiguous with the third and fifth transmembrane spanning domain, respectively, which are attachment sites for β -agonists (23). Based on mutagenesis studies, it is evident that these loops, when in the activated conformations, bind G-proteins (23). The goal of these studies was to assess the possibility of pleiotropic agonist responses at the level of conformational change. Thus, in experiments with a given construct, we hypothesized that the conformations stabilized by agonist would not necessarily be directly related to classical agonist intrinsic activity, such that the magnitude or direction of the BRET change would not mimic a full versus partial agonist rank-order. We also recognized that the results from one modified β_2 AR would

Figure 2. Agonist-promoted cAMP response in HASM cells. Cells were incubated with the indicated agonists for 10 min at 37°C. Results are from five experiments. **P* < 0.01 versus Iso. #*P* < 0.005 versus any other agonists.

Figure 3. Agonist-promoted intramolecular BRET. Shown are single experiments performed in quadruplicate with the indicated three modified β_2 ARs. Results are representative of three to five experiments. Results from all three modified receptors revealed a relationship between the array of agonists and the BRET signal ($P < 0.01$ by ANOVA).

be unlikely to be the same as another, given the different placements of YFP and Rluc within the receptor, but that pleiotropy would nevertheless be identified. As shown in Figure 3A with β_2 AR:YFPi2-RlucCT, Form increased, whereas Iso decreased, the BRET signal compared with control (PBS). Minor decreases in BRET were observed with Met and Salm, but not Alb, Terb, or the full agonist Fen. With β_2 AR:YFPi3-RlucCT, Form and Iso displayed decreases in BRET, but the other agonists imposed no changes. In studies with β_2 AR:YFPi2-RlucCT (Figure 3C), Fen displayed the greatest decrease in BRET, with smaller decreases with Form and Salm, but not with the other agonists, including Iso. Receptors expressing YFP in the third intracellular loop with Rluc at the end of the full-length carboxy tail showed no agonist-promoted changes in BRET. Taken together, these studies suggested pleiotropic agonist-promoted conformations that were not related to the classic intrinsic activity of these agents and prompted additional targeted studies of various aspects of receptor signaling to ascertain the effects of such differences.

The p44/p42 MAPK Responses

For these studies, HASM cells in monolayers were treated with agonists for 5 min, which provided the maximal activation (phosphorylation) response of the kinase over the basal (vehicle-treated) signal. From each gel, the response from that experiment for each agonist was compared with that of Iso. Iso exposure resulted in a ~ 1.5 -fold increase in phosphorylated p44/p42 over basal (Figure 4A). All the other agonists used had equivalent p44/p42 MAPK activation compared with Iso, including partial agonists such as albuterol and salmeterol (Figure 4B). Therefore, for this response, which is considered to be G_i mediated, there was no relationship between G_s -intrinsic activity and p44/p42 MAPK activation.

The Receptor Phosphorylation Response

For these studies, HEK-293 cells were transfected to express the FLAG epitope-tagged human β_2 AR at ~ 8 pmol/mg. Cells were metabolically labeled with 32 P-orthophosphate and exposed to agonist for 10 min. Receptor was purified by immunoprecipitation with Flag antibody followed by SDS-PAGE. A typical autoradiogram is shown in Figure 5A; mean results are provided in

Figure 5B. Iso, Form, and Fen had equivalent agonist-promoted β_2 AR phosphorylation. The Alb response was also equivalent to Iso. Terb and Met evoked receptor phosphorylation at $\sim 60\%$ that of Iso, and Salm evoked the least phosphorylation.

The Receptor Internalization Response

CHW cells expressing ~ 150 fmol/mg were grown in monolayers, and the exposure to agonist and radioligand binding was performed in 24-well dishes. After agonist exposure at 37°C , cells were washed with cold PBS to remove the agonist, and [^3H]CGP-12177 radioligand binding was performed at 4°C . This hydrophilic agent identifies only cell surface receptors; thus, the extent of internalization is determined by comparing binding to agonist-treated versus vehicle-treated cells. In preliminary studies, we

Figure 4. Agonist-promoted p44/p42 response in HASM cells. Cells were incubated with the indicated agonists for 5 min at 37°C . (A) A typical Western blot of phosphorylated p44/p42. (B) Mean data from five experiments normalized to the Iso response. All agonists stimulated p44/p42 to the same extent.

Figure 5. Phosphorylation of β_2 AR by various β -agonists. HEK-293 cells were transfected to express FLAG- β_2 AR and incubated with 32 P-ortho-phosphate as described in MATERIALS AND METHODS. Cells were treated with the indicated agonists for 10 min at 37°C and purified by immunoprecipitation. (A) Representative autoradiogram. (B) Mean results from five experiments. * $P < 0.05$ versus Iso. # $P < 0.05$ versus any other agonist.

found that Salm could not be washed off the receptor using these methods. This is consistent with our previous report where extensive continuous subfusion of monolayers was required to remove Salm binding (15). Thus, Salm was not included in these studies. Iso evoked $\sim 40\%$ β_2 AR internalization (Figure 6A). This was equivalent to that found with Form and Fen. Alb, Terb, and Met evoked substantially less internalization than the other agonists, amounting to $\sim 18\%$.

The Receptor Down-Regulation Response

We used quantitative immunoblots to avoid the artifactual β_2 AR down-regulation that is apparent when using radioligand binding because of the difficulty in washing off the lipophilic agonists Salm and Form after their incubation with cells. (In preliminary studies with several other agonists, we found a high correlation between down-regulation as measured by radioligand binding versus immunoblotting.) The results are shown in Figure 6B and reveal that Iso evoked a $\sim 70\%$ loss of β_2 AR after 12 h of exposure. This same extent of down-regulation was observed with Form and Fen. Alb, Terb, Met, and Salm had less down-regulation ($\sim 40\%$) and were not different from each other.

DISCUSSION

We show that β -agonists with different structures have the potential to stabilize the β_2 AR in different conformations and that this has consequences on intracellular signaling. Early studies with overexpression of the β_2 AR showed that basal levels of membrane adenylyl cyclase activities increased with expression (24), which is consistent with the receptor toggling to the “active” state, and thus capable of coupling to G_s in the absence of agonist. In the presence of agonist, the active conformation is stabilized, resulting in measurable signal transduction/amplification. Subsequent studies (reviewed in [1]) have refined the model and indicate that there are multiple different spontaneous conformations that can be attained by GPCRs, although the equilibrium in the

Figure 6. Agonist-promoted internalization and down-regulation of β_2 AR. (A) Cells were treated with the indicated agonists for 30 min at 37°C, and cell surface receptor was quantitated by hydrophilic radioligand binding with intact cells. * $P < 0.01$ versus Iso. (B) Cells were treated with the indicated agonists for 12 h at 37°C and solubilized, and proteins were subjected to 10% SDS-PAGE. Western blots were performed on the transferred proteins using a β_2 AR antibody, and enhanced chemiluminescence and the immunoreactive bands were quantitated by a charged coupled device camera (see MATERIALS AND METHODS). * $P < 0.01$ versus untreated but not different from each other. # $P < 0.01$ versus untreated and Iso, Form, and Fen but not different from each other.

absence of agonist highly favors the inactive conformation. Such multiple conformations may have fully overlapping functions such that there is efficient transduction of several signals, or certain conformations (R^{*1} , R^{*2} , R^{*3} , etc.) may be more favorable for a given downstream event; this provides the potential for an agonist to have some degree of selectivity for one or more pathways (Figure 7). The model, therefore, indicates that a rank-order of β -agonists based on one signal (such as G_s -coupling) might not be true for another signal.

The notion that a β -agonist, regardless of structure, can be classified as a full or partial agonist, and thereby the biochemical and physiologic effects of signal transduction implied based on such classification, is an important issue in asthma where safety issues of chronic use of β -agonists has been called into question (25–27). To pursue the issue of compound-based unique signaling, we used intramolecular BRET as a screen looking for trends in agonist-promoted changes in the BRET signal. We found that the apparent major conformation attained by the β_2 AR by the binding of some β -agonists used for asthma treatment seem to

Figure 7. Mechanism of pleiotropic signaling by structurally diverse β -agonists. In this depiction, each agonist is denoted by a subscript (i.e., A_1 , A_2 , etc.), and each unique conformation of the receptor is denoted by a superscript (i.e., R^{*1} , R^{*2} , etc.). Each conformation represents a distinct signaling pathway. A given agonist can stabilize one or more of these active conformations, based on the structure of the compound. This leads to a portfolio of signals that is evoked by each agonist.

be different and are not related to the classic intrinsic activity (i.e., full versus partial agonism of the G_s signal) of the drug. The approach used the placement of the Rluc energy donor at one of two sites in the carboxy-terminal tail of the receptor and the YFP energy acceptor in the second or third intracellular loop. Conformational changes were inferred when an altered BRET signal (indicative of the distance between the two moieties) by agonist was observed. Because the resonance energy transfer signal is proportional to the distance between the energy donor and acceptor by a factor of 10^6 (28), it can be a sensitive method for detecting changes in receptor conformation. Lohse and colleagues have recently used an approach similar to that of the current work with another GPCR, the α_{2A} AR (22). Distinct molecular conformations were noted for ligands with varying degrees of efficacies. This group has also used placement of one energy transfer moiety on a receptor and the other on the G-protein (29). Again, distinct “switches” were detected for different agonists at the α_{2A} AR, resulting in differential rates and extents of G-protein signaling. In the current work, we found changes in BRET due to agonist binding, and there was no relationship between these signals and classic intrinsic activity as defined by the G_s -coupling signal. This was so for the direction and magnitude of the change in BRET. Had the movement of

the Rluc-YFP moieties (i.e., conformational change) been strictly based on the agonist being partial versus full, we would have expected a graded change in the same direction, from the weakest to the most efficacious (full) agonists. By using receptors with the donor acceptor in different places, we show that the BRET changes are not mirrored from one modified receptor to another. This indicates that the changes in conformation stabilized by agonist cannot be fully assessed by this method using a single axis for detection of loop movement, consistent with complex three-dimensional alterations in the relationship between intracellular domains of the β_2 AR during agonist occupancy. Other approaches, which are more quantitative, have been used to explore this same issue. Kobilka and colleagues (30) have used fluorescence lifetime spectroscopy with detergent solubilized purified β_2 AR containing a fluorescein maleimide label to Cys 265, which is within the third intracellular loop of the receptor. Distinct conformations of the receptor were identified when bound by Iso, Alb, or dobutamine. Further studies using a number of approaches indicated that agonist binding involves conformational intermediates (reviewed in [31]), each of which could potentially direct a distinct signal. Studies have suggested that the noncatechol ring of albuterol binds to different residues of the β_2 AR compared with the aromatic ring of catecholamines (32), which is likely the basis for a different agonist-bound conformation.

From our screening results with intramolecular BRET, we assessed the propensity for agonists of different structures to evoke various events relevant to asthma. Initial studies of the G_s (cAMP) response revealed the expected classic intrinsic activity relationships, with Form and Fen being near full agonists ($\sim 80\%$ compared with Iso); Alb, Terb, and Met being partial agonists; and Salm being the weakest partial agonist. In contrast to G_s coupling, agonist-promoted stimulation of p44/p42 MAPK (a G_i -mediated event [6] that affects cell proliferation and hypertrophy [33]), was equivalent for all agonists, including the weakest partial G_s agonist Salm. The effects of this β_2 AR-mediated signal on airway smooth muscle proliferation, however, are complex. Agents that provide for robust and sustained activation of p44/p42 promote airway smooth muscle proliferation (34). Under such circumstances, β_2 AR agonists inhibit growth via transcriptional inhibition of promitogenic factors (reviewed in [3]). Regardless of the net effect of β_2 AR-promoted activation of p44/p42 on smooth muscle cell proliferation in intact lung within the asthmatic milieu, based on the current results there may be no advantage of one agonist over another.

An early event after agonist binding to the β_2 AR is the attainment of an intracellular conformation that favors phosphorylation by GRKs. Such phosphorylation is independent of G_s coupling, and we considered that β -agonists of different structures could lead to varying degrees of receptor phosphorylation unrelated to intrinsic activity. From these studies, we found that the weak partial agonist Salm evoked the least phosphorylation, whereas the full agonists Iso, Fen, and Form were equivalent and promoted the greatest degree of phosphorylation. However, Alb, which is a partial agonist for the G_s signal (and equivalent to Terb and Met in this regard), evoked the same degree of receptor phosphorylation as was observed with the full agonist Iso, whereas Terb and Met evoked intermediate levels of phosphorylation. GRK-phosphorylated β_2 AR is a substrate for β -arrestin binding, which acts to partially uncouple receptor from G-protein but has recently been recognized as a signaling event itself, due to its scaffolding properties. The ultimate components of such translocation of proteins to microdomains is highly cell-type dependent. Examples of such β -arrestin-dependent events include recruitment of phosphodiesterases (35), elements required for p44/p42 MAPK activation (18), and

receptor ubiquitination (36) and dephosphorylation (37). However, we show here that there is a “disconnect” between the extent of p44/p42 MAPK activation and GRK-mediated receptor phosphorylation. These results and results from other studies (38, 39) suggest additional components that are necessary for the signaling of β_2 AR to these complex events.

Shortly after agonist exposure (and partially dependent on β -arrestin), β_2 ARs undergo endocytosis, which leads to a rapid loss of cell surface receptors and an increase in intracellular receptors in clathrin-coated vesicles (23). In the current studies, receptor internalization was quantitated using a hydrophilic, non-cell-permeable, radioligand with intact cells. The extent of internalization correlated well with the G_s -intrinsic activity (Figure 6A). Once internalized, β_2 AR are recycled to the cell surface or trafficked to a degradation pathway, leading to a net loss of cellular receptor (down-regulation). Like the internalization response, Iso, Fen, and Form evoked maximal down-regulation, amounting to an $\sim 80\%$ loss of receptors (Figure 6B). The G_s -partial agonists had less down-regulation ($\sim 40\%$). Salm resulted in the same extent of down-regulation compared with Alb, Terb, and Met but evoked less receptor cAMP accumulation and phosphorylation (Figure 5). Thus, except for the enhanced Salm down-regulation, the internalization and down-regulation responses followed the rank-order of intrinsic activity as defined by the G_s response. This does not imply that both processes are cAMP-dependent. Although down-regulation can be evoked by cAMP analogs, internalization requires receptor occupancy by agonist. In terms of the working paradigm, the conformation of the β_2 AR necessary for G_s coupling seems to be the same or very similar to that for internalization. Nevertheless, because the change in BRET signals differ between the two full agonists Iso and Form, other conformation-dependent events may differ between these two agents that were not recognized in the current study. Consistent with the notion that the conformations for G_s -coupling and internalization are similar, we have previously noted that the Ile164 polymorphic form of the β_2 AR has decreased G_s coupling and internalization to the same extent compared with the wild-type Thr164 receptor (24). The down-regulation response is a long-term consequence of receptor internalization but also involves cAMP-dependent and somewhat less-defined cAMP-independent events (23). Given that two pro-down-regulation functions have the same rank-order for agonists (G_s -cAMP and internalization), it is not unexpected that this response is similar to the classic intrinsic activity. Although Salm is the weakest of the partial agonists in terms of G_s coupling, because it evokes a similar degree of down-regulation, we conclude that there is a threshold effect in terms of cAMP and other factors that is achieved by Salm to promote receptor loss similar to that of other more efficacious partial agonists.

In conclusion, we have shown that the β -agonists used for the treatment of obstructive lung diseases differ in their signaling in a manner that is not predicted by classic G_s -based intrinsic activities and that this pleiotropy is based on different conformations that are stabilized by a given agonist. There is the potential for an agonist to have primarily, or exclusively, beneficial effects with little or no disadvantageous properties (Figure 7). What is not clear, though, is what constitutes the “ideal” profile. The compendium of signals evoked by activated β_2 AR is not well defined, particularly regarding complex crosstalk with other signal transduction pathways (13, 40). Nevertheless, these data support the notion that certain β -agonists dictate a specific set of endpoints in the treatment of obstructive disease. We show that it is potentially erroneous to assume that the group of downstream signal transduction events from one β -agonist is equivalent to that of a structurally different β -agonist.

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