

Update on current concepts of the molecular basis of β_2 -adrenergic receptor signaling

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The proposed manner by which β_2 -adrenergic receptors signal has dramatically changed from earlier concepts that centered on a lock-and-key mechanism in which the receptor acts as a simple switch. We now know that β_2 -adrenergic receptors spontaneously toggle to an activated state (R^*) and that the equilibrium between R (the inactive state) and R^* can be altered by ligands. In addition, the R^* conformation is likely to consist of multiple subspecies that may favor certain signaling pathways or regulatory events. Changes in agonist structure alter the abundance of certain subspecies of R^* . Indeed, multi-functional coupling is common with many G-protein-coupled receptors and can be modulated pharmacologically to attain specific outcomes. In addition to providing the basis for development of new β -agonists for unique signaling, these properties can be extended such that β_2 -adrenergic receptors, or highly modified "designer" receptors, can be used for gene therapy with highly specific effects. (*J Allergy Clin Immunol* 2002;110:S223-8.)

Key words: Adenylyl cyclase, G protein, desensitization, gene therapy

The β_2 -adrenergic receptor (β_2 AR) is a member of the large superfamily of G-protein-coupled receptors (GPCRs). Recent studies with this receptor and other GPCRs have refined our understanding of how signaling occurs and is regulated, revealing means by which to modulate function for therapeutic benefit. This review discusses the current concepts of structure and function relationships, how agonists activate β_2 AR, and the mechanisms of coupling of this receptor to multiple effectors.

β_2 AR STRUCTURE AND FUNCTION RELATIONSHIPS

Like all GPCRs, the β_2 AR is a protein that has 7 transmembrane-spanning domains, an amino terminus that is extracellular, a carboxyl terminus that is intracellular, 3 interconnecting extracellular loops, and 3 intracellular loops (Fig 1). Primarily through site-directed mutagenesis and recombinant expression, a number of regions of

Abbreviations used

α -AR: α -Adrenergic receptor
 β -AR: β -Adrenergic receptor
cAMP: Cyclic AMP
GPCR: G-protein-coupled receptor
GRK: G-protein-coupled receptor kinase

the receptor critical to receptor function have been identified. Although depicted in Fig 1 in a flat plane, the transmembrane-spanning domains appear to form a barrel-like configuration, and agonist binding occurs within the pocket formed by these domains.¹

Several critical contact points have been identified as absolutely necessary for β -agonist activation of β_2 AR.²⁻⁴ Asp113, which is in the third transmembrane-spanning domain, forms an ion pair with the amine group of agonists and its carboxylate side chain.² The hydroxyl groups of the phenyl ring of catecholamines interact with Ser204 and Ser207 of the fifth transmembrane-spanning domain.³ The β -hydroxyl group of β -agonists binds to Asn293 of the sixth transmembrane-spanning domain. This particular interaction requires positioning of the hydroxyl group that is afforded only by the *l*-isomer of agonists such as isoproterenol, epinephrine, and albuterol.⁴ Although the described relationships appear to be critical, subtle interactions with other transmembrane-spanning domains occur, leading to high-affinity, full-agonist activation of the receptor.

Once bound by agonists, the receptor is maintained in a conformation favorable for coupling to heterotrimeric G proteins, particularly G_s . This results in activation of adenylyl cyclase and conversion of ATP to cyclic AMP (cAMP). The interactions required for binding and activation of $G_{\alpha s}$ appear to be complex and involve the second intracellular loop, the proximal and distal portions of the third intracellular loop, and the proximal portion of the cytoplasmic tail. The latter includes the region between the seventh transmembrane-spanning domain and the palmitoylated cysteine that anchors a portion of the tail to the membrane. Similar to the crystal structure of bovine rhodopsin,¹ this portion of the tail forms an α helix (Fig 1). Despite extensive mutagenesis studies, no specific residue, or even portions of these loops, has been identified as a singular contact point for G proteins. It appears that $G_{\alpha s}$ coupling occurs through multiple interactions at various points within the receptor. Interesting-

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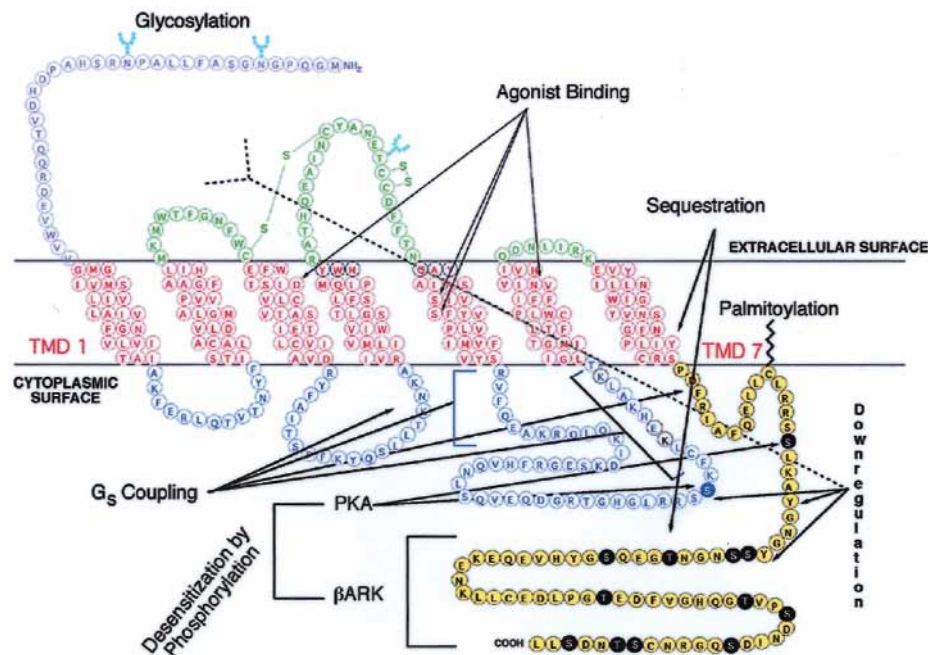


FIG 1. Amino acid sequence and proposed membrane topology of the human β_2 AR. Regions or specific domains with structural significance are labeled. TMD 1 and TMD 7 indicate the first and seventh transmembrane-spanning domains, respectively. β ARK, β -AR kinase.

ly, an amino acid alignment of G_s -coupled receptors within the GPCR superfamily fails to identify a G_s consensus sequence, which suggests either that $G_{\alpha s}$ activation is achieved by complex interactions at the tertiary structure level that we cannot model or that activation can occur by many different types of interaction.

Once $G_{\alpha s}$ is activated by agonists, mechanisms to limit responsiveness at the receptor level are put into play. The earliest response is phosphorylation by one or more members of the class of serine-threonine kinases designated GPCR kinases (GRKs). The prototypic GRK, originally designated the β AR kinase (now also known as GRK2), phosphorylates β_2 AR at serine and threonine in the carboxyl-terminal portion of the receptor (Fig 1). GRK phosphorylation alone, however, is not sufficient to uncouple the receptor from $G_{\alpha s}$. Binding of a member of the arrestin family of proteins, in this case β -arrestin, is necessary for this rapid form of desensitization.^{5,6} On the basis of the composition of intracellular domains and of findings from specific structure and function studies, it appears that about two thirds of nonodorant receptor GPCRs undergo rapid agonist-promoted desensitization by this GRK-arrestin mechanism. For the β_2 AR, a second rapid phosphorylation then occurs through the cAMP-dependent protein kinase. This occurs at sites at the third intracellular loop and the carboxyl terminus. GRK phosphorylation and β -arrestin binding also act to internalize the β_2 AR and direct it to specific endosomes, where it can be dephosphorylated and either recycled to the cell surface or degraded. In addition, the β -arrestin binding may in fact serve as a signal itself, because it acts as an adapter to trigger coupling to other pathways, such

as c-Jun-N-terminal kinase and extracellular-signal regulated kinase.⁷ After more prolonged agonist activation, the net receptor complement is decreased, a process termed down-regulation. Detectable down-regulation typically requires several hours of agonist exposure, and the mechanisms leading to the process appear to be highly cell-type dependent. These mechanisms include a decrease in gene transcription, destabilization of mRNA, and degradation of receptor protein.

THE "ACTIVATED" β_2 AR

Concepts concerning how agonists trigger GPCR signaling have changed markedly during the past few years. Early models were typified by the concept of a lock and key mechanism whereby agonists fit into the receptor, which induced a conformational change in the receptor protein, leading to G-protein coupling. Thus the receptor was considered to act as a simple switch. Multiple studies have now shown that this is not the case with β_2 AR and many other GPCRs.

The current concepts involve the notion of spontaneous receptor activation (Fig 2). Thus it appears that the β_2 AR toggles between the active conformation (R^*) and the inactive conformation (R) in the absence of any agonist binding. The equilibrium favors the inactive conformation in the absence of agonist (Fig 2, A, large arrow). This spontaneous activation is most easily observed in transfected cells, in which multiple levels of expression in different clonal cell lines can be obtained. When basal (non-agonist-stimulated) adenylyl cyclase activities are quantified in membrane preparations from such cells, a

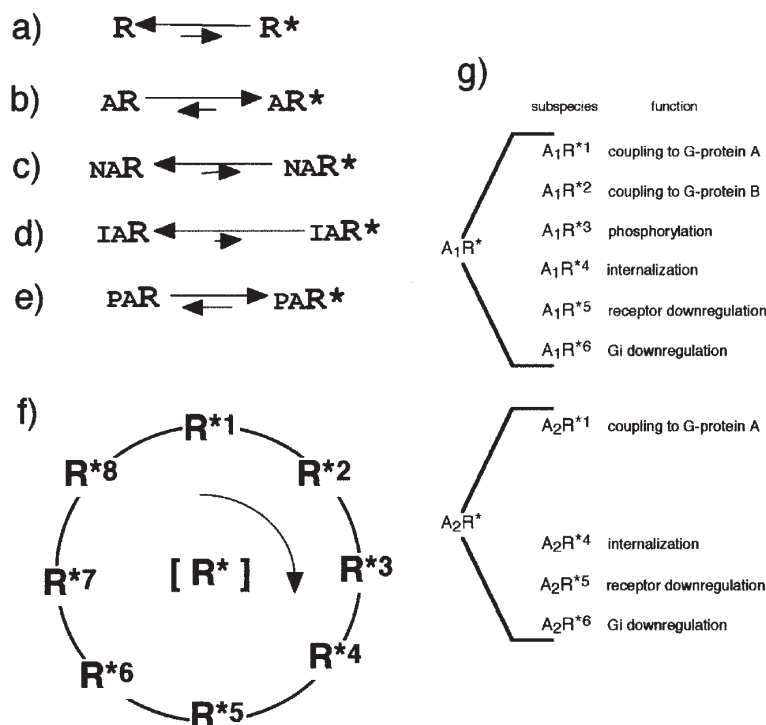


FIG 2. Characteristics of spontaneously active GPCRs. A, Agonist; NA, neutral antagonist; IA, inverse agonist; PA, partial agonist; A₁ and A₂, agonist 1 and agonist 2; R^{*1}, R^{*2}, etc, various conformations of an activated receptor. (G from Jewell-Motz EA, Small KM, Theiss CT, Liggett SB. α_2A/α_2C -adrenergic receptor third loop chimera show that agonist interaction with receptor-subtype backbone establishes G protein-coupled receptor kinase phosphorylation. J Biol Chem 2000;275:28989-93. With permission.)

linear relationship between receptor expression and basal adenylyl cyclase activities is observed. With the higher levels of expression, enough receptors toggle to the activated state and adenylyl cyclase activity is measurably increased. In this model, agonist preferentially binds with high affinity to the spontaneously activated state, stabilizing this conformation and shifting the equilibrium to the right (Fig 2, B, large arrow). Neutral antagonists show no preference for R or R^{*} and thus do not alter basal activity (Fig 2, C). Of course, in the presence of tonic stimulation by agonist, β AR-neutral antagonists lower adenylyl cyclase activities by displacing the agonist and therefore evoke their physiologic responses. The binding of certain antagonists (referred to as reverse antagonists or inverse agonists, with the latter termed now being preferred) stabilizes the inactive conformation, shifting the equilibrium to the left (Fig 2, D). Partial agonists can act in several ways. They may simply have less capacity to bind or to stabilize the active conformation. More than likely, however, partial agonists stabilize a slightly different conformation of the receptor than does a full agonist. Regardless, less-stabilized, functional receptors (at least as defined by a single pathway) are present at equilibrium with partial agonists (Fig 2, E).

Studies with related receptors,⁷⁻⁹ such as α_1 AR and α_2 AR, suggest that GPCRs oscillate between many different R^{*} conformations (Fig 2, F). These have been

designated as R^{*1}, R^{*2}, R^{*3}, and so on. We originally referred to these states as R^{*}, R^Δ, and so on,⁹ but the numeric nomenclature seems more appropriate because there may be an infinite number of activated states. Structural differences between different agonists have the potential to direct stabilization of one particular R^{*} subset, and thus a range of activities can be achieved. This property has implications beyond simply the degree of activation, appearing to be relevant to signaling specificity as well.

MULTIEFFECTOR COUPLING

Fig 3 shows some of the mechanisms that lead to multifunctional coupling of GPCRs. As Fig 3, A, indicates, two receptors that are highly homologous (subtypes) can couple to two different G proteins, and if a nonselective agonist is used, two different signals can be observed. An example is the stimulation of extracellular signal-regulated kinase 1 and 2 by β_2 AR but not β_1 AR. This scenario is a special case, and the rest of the examples represent a single receptor that results in activation of different pathways.

Fig 3, B, shows a receptor coupling to two different G α subunits, resulting in activation of two effectors and production of two second messengers. Fig 3, C, depicts a similar scenario, but the effects of the two G α subunits have opposing actions on one effector. So here there is

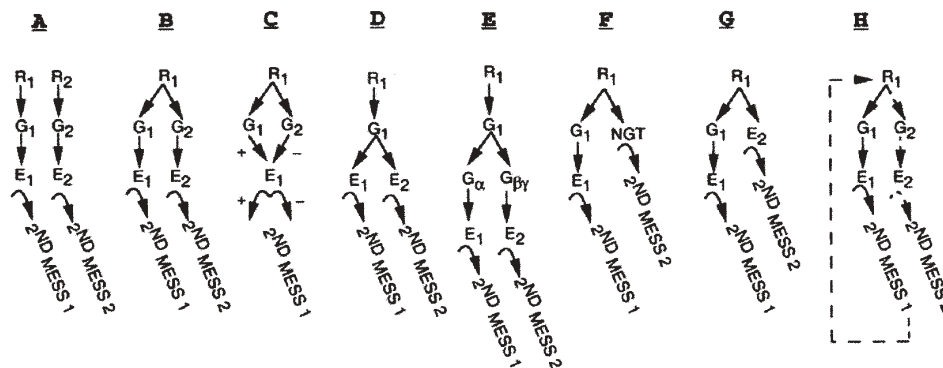


FIG 3. General mechanisms of multifunctional coupling of GPCRs. *R*, Receptor; *G*, G-protein α subunit; *E*, effector; *NGT*, non-G-protein transducer; *2nd MESS*, second messenger.

one signal, but it is modulated by one receptor coupling to two G proteins. An example of this is the α_{2A} AR, which couples to $G_{\alpha i}$ and $G_{\alpha s}$, resulting in dual modulation of adenylyl cyclase.¹⁰

In Fig 3, *D*, one receptor couples to one G protein, which activates two effectors. An example is coupling of β AR to L-type calcium channels and adenylyl cyclase through $G_{\alpha s}$. Similarly, the $\beta\gamma$ subunits of a heterotrimeric G protein may act to signal to a second effector that is completely unrelated to the G_{α} pathway (Fig. 3, *E*). The coupling of α_{2A} AR to G_i results in inhibition of adenylyl cyclase through $G_{\alpha i}$ and stimulation of phospholipase C through $G_{\beta\gamma}$.¹¹

A corollary to this is the direct coupling through a non-G-protein transducer. As shown in Fig 3, *F*, this is the case with the β_2 AR, in which coupling to $G_{\alpha s}$ results in activation of adenylyl cyclase and the binding of β -arrestin, which is an independent trigger of various events such as extracellular signal-regulated kinase 1 and 2 and Jun-N-terminal kinase activation.⁷

Similarly, the β_2 AR can couple directly to the sodium-hydrogen ionic exchanger regulatory factor,¹² which regulates the activity of sodium-hydrogen ionic exchanger type 3 (Fig 3, *G*). This interaction occurs through a direct coupling with the last amino acid of the carboxyl terminus of the receptor. This does not require an intermediate G-protein transducer for the effect, as is shown. Activation of two G proteins by a single effector may require an initial signal that subsequently modifies the receptor such that coupling to another G_{α} protein occurs. This is the apparent case with β_2 AR, in that $G_{\alpha s}$ coupling increases cAMP and activates cAMP-dependent protein kinase, which in turn phosphorylates the receptor and results in β_2 AR coupling to $G_{\alpha i}$ (Fig 3, *H*).^{13,14}

Taken together, these general mechanisms (and probably many more) must be considered each time a signal event, whether biochemical or physiologic, is detected when an agonist is presented to a receptor. This should be done to assign which mechanisms and signals result in the desired outcome. In addition, there may be unexpected activation of a pathway, which could be deleterious when a drug is used long term, so exploring multiple signals may be useful for screening for adverse cellular events.

Some of the scenarios depicted in Fig 3 can be considered in the multiactivation state model (Fig 2, *G*). Certain agonists could preferentially stabilize one particular conformation of R^* that is specific for one G protein versus another. One wonders whether potential agonists that have been screened for activity by measurement of one signaling outcome (such as increased cAMP) or one physiologic outcome (such as bronchodilation) may have been discarded because of such limited studies. This concept can be extended further by considering other agonist-triggered events. For example, is the conformation that maximally activates G_s necessarily the same one that is preferred for GRK2 phosphorylation? Is it possible to achieve high or moderate degrees of G_s activation and little or no desensitization by GRK2? Studies with the α_{2A} AR indeed suggest that there is no correlation between agonist efficacy (intrinsic activity compared with that of a prototypic full agonist) and agonist-promoted receptor phosphorylation by GRK2.⁸ Of particular importance for β -agonists, it may be possible for certain agonists to fully activate G_s but undergo little phosphorylation or down-regulation.

DESIGNER β_2 AR-LIKE RECEPTORS FOR GENE THERAPY

This concept suggests that agonists could be designed to provide highly specialized signaling through the β_2 AR. We therefore considered that perhaps an engineered receptor could be used for gene therapy in the lung. As discussed elsewhere in this supplement (see article by McGraw on page S236), we have previously shown with targeted transgenic mice that overexpression of β_2 AR in certain cell types of the lung confers therapeutic responses. When β_2 AR was overexpressed in Clara cells of the upper airway (CC10 promoter), mice displayed a decreased bronchial hyperresponsiveness to methacholine as well as to ozone.¹⁵ When β_2 AR was overexpressed in airway smooth muscle cells (SMP8 promoter), transgenic mice displayed a marked resistance to bronchoconstriction by methacholine in the absence of β -agonists.¹⁶ Indeed, this protection was greater than that provided by inhaled albuterol. This

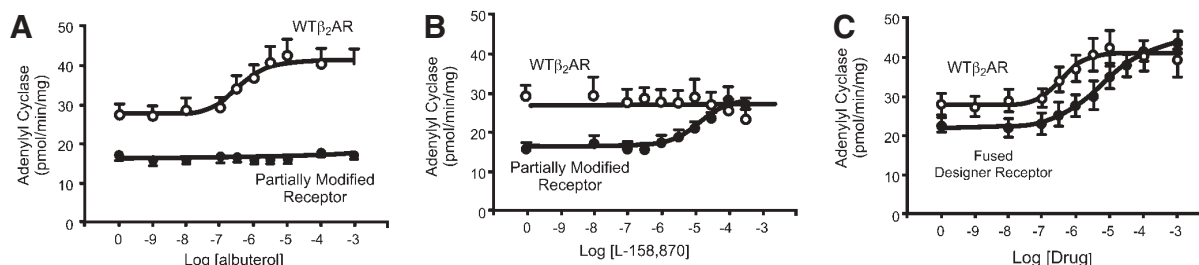


FIG 4. Properties of a designer β_2 -adrenergic-like receptor for gene therapy. **A** and **B**, “Two-way” selectivity. The mutated receptor fails to respond to albuterol but does respond to the modified, nonbiogenic amine-activated agonist denoted L-158,870. In contrast, whereas the wild-type β_2 -AR (WT β_2 AR) responds to albuterol, it does not respond to L-158,870. **C**, Results of fusing the modified receptor to G_{α_s} . The designer receptor achieves basal and agonist-stimulated adenylyl cyclase activation similar to that of the wild-type β_2 -AR. (**A** and **B** from Small KM, Brown KM, Forbes SL, Liggett SB. Modification of the β_2 -adrenergic receptor to engineer a receptor-effector complex for gene therapy. *J Biol Chem* 2001;276:31596-601. With permission.)

response to β_2 AR overexpression in airway smooth muscle was found to be due to spontaneous activation of the receptor.¹⁶ In transgenic mice, overexpression of β_2 AR in type II cells (surfactant protein C promoter) resulted in enhanced alveolar fluid clearance,¹⁷ which was probably caused by activation of receptor by endogenous epinephrine.

We considered that for human gene therapy for diseases such as asthma, chronic obstructive pulmonary disease, or pulmonary edema perhaps a modified receptor with unique properties would provide the greatest clinical efficacy. With the β_2 AR as a scaffold, we mutated the receptor at 19 positions to achieve highly specific properties.¹⁸ Of particular interest was the potential to re-engineer the agonist binding site such that the receptor was activated not by endogenous epinephrine or typical β -agonists but by a synthetic noncatecholamine agonist that in turn did not activate endogenous β_2 AR or other GPCRs. Thus the signaling would be initiated only by administration of a specific drug to a patient who had received gene therapy.

To accomplish this, Asp113 of the human β_2 AR was mutated to serine. This resulted in the loss of the native receptor's ability to form the ion pair with the amine head group of catecholaminelike β -agonists. The synthetic agonist 1-(3',4'-dihydroxyphenyl)-3-methyl-1-butanone (provided by Merck and designated L-158,870) has spatial characteristics similar to those of catecholamines, but it has the capacity to accept (rather than donate) a hydrogen bond in the vicinity of residue 113. The substituted serine has a β -hydroxymethyl group that provides this interaction. As shown in Fig 4, **A** and **B**, the modified receptor indeed displayed this “two-way” selectivity. It was responsive to L-158,870 and unresponsive to albuterol, whereas L-158,870 failed to activate wild-type β_2 AR.¹⁸ At this juncture the receptor was no longer a biogenic amine-activated receptor. As can be seen, basal and maximal agonist-promoted adenylyl cyclase activities were depressed as a consequence of this mutation. To improve coupling efficiency and to direct coupling specifically to G_s , the carboxyl terminus of the modified receptor was fused to the α subunit of G_s .^{18,19} This not

only directed coupling specifically to this pathway but also maintained a 1:1 stoichiometric relationship between receptor and G protein. The result was a receptor with nearly the same signaling efficiency as wild-type β_2 AR in stimulation of adenylyl cyclase (Fig 4, **C**). To eliminate short-term agonist-promoted desensitization, all potential serine-threonine pairs of cAMP-dependent protein kinase and GRK phosphorylation sites were mutated to alanine. Long-term agonist-promoted down-regulation was eliminated by mutating Gln27 of the amino terminus to glutamic acid. After 24 hours of exposure to the agonist albuterol, wild-type β_2 AR underwent $55\% \pm 4\%$ desensitization. In contrast, exposure of the fully modified designer receptor to L-158,870 for the same period failed to promote desensitization ($1\% \pm 5\%$). From such studies it appears that GPCRs can be engineered to have specific properties by mutating a parent receptor in multiple regions of potential structural lability. Such designer receptors may be useful for gene therapy, although efficient delivery to the lung for gene therapy has yet to be optimized.

CONCLUSIONS

Recent studies have elucidated new details about the mechanism by which β_2 AR and other GPCRs carry out signal transduction. These mechanisms can potentially be exploited to attain highly specific properties in new agonists, pharmacologic or genetic agents that are distal to the receptor, or modified receptors used for gene therapy.

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DISCUSSION SESSION

Question: There are multiple potential conformations to the receptor-agonist complex. What happens when the receptor toggles spontaneously? Does it have the ability on its own to reach the conformations seen when it is bound to the different agonists?

Dr Liggett: I think that the receptor toggles to all of the conformations, but at a low level that we may not be able to detect.

Question: Use of a therapeutic receptor-effector complex is a great idea. When a therapeutic receptor-effector complex is used in gene therapy efforts, what happens to the endogenous receptor?

Dr Liggett: There is a cAMP-mediated pathway for down-regulation of endogenous β_2 receptor. So with a therapeutic receptor-effector complex we would probably lose the endogenous receptor, which may not be advantageous.

Question: How does changing the receptor, as with therapeutic receptor-effector complex, affect desensitization?

Dr Liggett: The receptor is less capable of desensitization.

Question: In the experiments performed with therapeutic receptor-effector complex, were there endogenous ligands present that might interact with the binding site?

Dr Liggett: I do not think that there was anything in the culture media that produced endogenous activation through a ligand-mediated event by way of the β -receptors.

Comment: If the therapeutic receptor-effector complex is good enough, it may supplant the endogenous β_2 -receptor, which may be an advantageous effect.

Dr Liggett: One of the reasons we were worried about the therapeutic receptor-effector complex was that the overall affinity of that synthetic agonist looked low. We were worried that we were not going to be able to give it in an appropriate concentration to elicit an effect in the model system that we used. Anything we do regarding gene therapy is probably going to have an effect on many different things, and we need to determine the net effect.

Question: Do different conformations affect the movement of receptors in the membrane? Does movement laterally in the membrane affect signaling?

Dr Liggett: As seen with cardiomyocytes, the receptors can colocalize into microdomains in which the effectors are highly concentrated. This compartmentalization is important. One can fail to detect an entire signal that is highly colocalized with a special effector. This generic statement would probably hold true for all GPCRs.

Question: The complexity of the ligand-receptor interaction is quite impressive. How does the autocrine secretion of other factors fit into the complexity of this paradigm?

Dr Liggett: We are just beginning to explore the physiologic relevance of the multifunctional coupling. Nothing is done in isolation. There is a huge morass of signaling occurring, and we may not always be interpreting the correct readout.

Question: Do all partial agonists interact with the receptor in the same way?

Dr Liggett: I think we have been naive to think that all partial β -agonists interact with the receptor in the same way. The structures of these agonists are drastically different. Agonists may preferentially affect one signal and not another completely. We have been remiss in not examining the spectrum of β -agonists and their actions in nontraditional signaling pathways.

Question: What effects do polymorphisms or mutations have on receptor conformation?

Dr Liggett: Some mutations can alter a receptor dramatically. With Ile164, for example, the receptor remains in the R state most of the time and rarely toggles to the R* conformation. The basal levels of Ile164 receptor cycles are lower than wild-type so that without ever even adding an agonist the complex does not oscillate to R* quite frequently. So all uncoupled receptors, by forced mutagenesis or natural polymorphisms, should have lower basal activity in these types of assays.

Question: What external factors alter the toggling of the receptor?

Dr Liggett: Membrane composition is a major factor that has not received as much attention as other factors. Alteration of the lipid content of the membrane and similar modifications have a dramatic effect on signaling. The differences in toggling attributed to differences in cell types might involve membrane composition, the variety of adenylyl cyclase isoforms, and multiple combinations of $\beta\gamma$ subunits.

Question: What is the effect of dimerization on coupling of the

receptor?

Dr Liggett: Receptors form dimers, possibly even with other GPCRs. Coupling may or may not be changed by the formation of dimers. Dimerization seems to be enhanced by agonists.

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