Modification of the β2-Adrenergic Receptor to Engineer a Receptor-Effecter Complex for Gene Therapy*

Received for publication, March 27, 2001, and in revised form, June 6, 2001
Published, JBC Papers in Press, June 11, 2001, DOI 10.1074/jbc.M102734200

Kersten M. Small‡†, Kari M. Brown‡†, Susan L. Forbes‡†, and Stephen B. Liggett‡‡¶
From the Departments of §Medicine and ¶Pharmacology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

Depressed G-protein-coupled receptor (GPCR) signaling has been implicated as a component of the pathophysiology of a number of complex diseases including heart failure and asthma, and augmentation or restoration of signaling by various means has been shown to improve organ function. Because some properties of native GPCRs are disadvantageous for ectopic therapeutic expression, we utilized the β2-adrenergic receptor (β2AR) as a scaffold to construct a highly modified therapeutic receptor-effector complex (TREC) suitable for gene therapy. Altogether, 19 modifications were made to the receptor. The ligand-binding site was re-engineered in TM-3 so that a β-hydroxymethyl side chain acts as a proton donor for the binding of a novel ligand. In addition, sites critical for agonist-promoted down-regulation in the amino terminus and for phosphorylation by GPCR kinases, and protein kinases A and C, in the third intracellular loop and the carboxyl terminus of the receptor were altered. These modifications of the receptor resulted in depressed agonist-stimulated adenylyl cyclase activity (26.8 ± 2.1 versus 41.4 ± 8 pmol/min/mg for wild-type β2AR). This was fully restored by fusing the carboxyl terminus of the modified receptor to Gαs (43.3 ± 2.7 pmol/min/mg). The fully modified fused receptor was not activated by β-agonists but rather by a nonbiogenic amine agonist that itself failed to activate the wild-type β2AR. This two-way selectivity thus provides targeted activation based on physiologic status. Furthermore, the TREC did not display tachyphylaxis to prolonged agonist exposure (desensitization was 1 ± 5% versus 55 ± 4% for wild-type β2AR). Thus, despite extensive alterations in regions of conformational lability, the β2AR can be tailored to have optimal signaling characteristics for gene therapy. As a general paradigm, TRECs for enhancement of other G-protein signaling appear to be feasible for modification of other pathologic states.

Cell surface receptors that activate signaling via coupling to G proteins comprise a large superfamily consisting of several hundred distinct receptors. G-protein-coupled receptors are widely expressed and serve diverse functions within hormonal, neurotransmission, immune, and growth systems. Receptor signaling in this family is dynamically regulated such that the cell integrates a large number of incoming signals and ultimately adapts to short and long term events. Although such regulation can occur at the level of G proteins or subsequent downstream participants in the pathway, highly specific mechanisms acting at the receptor itself are the primary mechanisms by which the signal transduction of a given receptor is selectively modulated (1, 2).

Both the β1-adrenergic receptor and β2-adrenergic receptor (β2AR)1 subtypes couple to the stimulatory guanine nucleotide binding protein Gs, resulting in activation of adenylyl cyclase and increased intracellular cAMP. Rapid regulation of βAR coupling efficiency (1) is mediated by phosphorylation of the receptor by G protein-coupled receptor kinases (GRKs), as well as protein kinase A (PKA) and protein kinase C (PKC). GRK-mediated desensitization is evoked by agonist occupancy, whereas PKA- and PKC-mediated phosphorylation are mechanisms of heterologous desensitization. Receptor-mediated responsiveness is also dependent on cell surface βAR expression, which is regulated by complex sorting and trafficking mechanisms involving internalization, recycling, and degradation of receptors, as well as transcriptional mechanisms (3–5). Although regulation of βAR function serves important roles in maintenance of homeostasis under normal and compensatory states, in certain pathologic conditions depressed function appears to contribute to the disease state or may limit the therapeutic response to agonist (6). For example, in the lung airflow smooth muscle β2AR act to relax the muscle resulting in bronchodilation (7). β2AR on airway epithelial cells also contribute to the establishment of bronchomotor tone (7, 8). In asthma, a disease characterized by bronchial smooth muscle contraction, airway epithelial and smooth muscle β2AR function is depressed, thus contributing to airway obstruction (7, 9). Furthermore, regular use of β-agonists in the treatment of bronchospasm acts to desensitize β2AR (termed tachyphylaxis), thus potentially worsening obstruction and limiting the effectiveness of this therapy (10–12). In the heart, β1-adrenergic receptor and β2AR expressed on myocytes act to increase the rate and force of contraction (13, 14). In human heart failure as well as in many animal models of the syndrome, changes in βAR responsiveness are well documented (15, 16). Mechanisms of such dysfunction include down-regulation of receptor number, phosphorylation of receptors by the βAR kinase (βARK) and PKC, and changes in downstream elements of the signal transduction cascade such as G proteins and adenylyl cyclase (14–19).

Overexpression of β2AR or inhibition of βARK has been shown to markedly improve organ function in animal models of asthma and/or heart failure. For example, we have recently

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* This work was supported by a grant from the Caroline Halfter Spahn Trust Genetic Research Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: 231 Albert Sabin Way, Rm. G062, Cincinnati, OH 45267-0564. Tel.: 513-558-4831; Fax: 513-558-0835; E-mail: stephen.liggett@uc.edu.

1 The abbreviations used are: βAR, β2 adrenergic receptor; TREC, therapeutic receptor-effector complex; PKA, protein kinase A; PKC, protein kinase C; GRK, G protein receptor-coupled kinase; HA, hemagglutinin; βARK, βAR kinase.
shown that transgenic overexpression of β2AR in airway smooth muscle results in mice that are resistant to methacholine-induced bronchial constriction (20). In another set of studies, we showed that β2AR overexpression (of as little as ~2-fold over background) in airway epithelial cells of transgenic mice decreased ozone-induced bronchial hyperreactivity and the constrictive response to methacholine (8). We have also found that overexpression of β2AR in the hearts of transgenic mice increases resting and agonist-stimulated ventricular function (21, 22). Lefkowitz and co-workers (23, 24) have demonstrated that transgenic expression and ex vivo and in vivo delivery of viral constructs to express β2AR or a βARK inhibitor can significantly enhance ventricular function. In several animal models with impaired cardiac contractility (28–31), such intervention results in improved, often fully restored, cardiac function.

The above studies indicate the potential for gene therapy via expression of the β2AR in the lung and heart. The wild-type receptor, however, has a number of properties that may limit the effectiveness of this approach, particularly with lower levels of expression, as is likely in human therapy. Thus, under pathologic conditions where βARK activity is increased, phosphorilation and desensitization of the genetically expressed β2AR may ultimately limit therapeutic efficacy. Similarly, PKA or PKC activation in the cell, because of homologous or heterologous mechanisms, could alter receptor function. Also, agonist-promoted receptor down-regulation, which is partially due to degradation of receptor protein (5), could decrease the available number of cell surface receptors after initial successful expression. In addition, certain conformational and spatial properties of the receptor-Gi interface limit the efficiency of functional coupling of the wild-type β2AR (32). Finally, a therapeutically expressed wild-type β2AR is activated by the same endogenous catecholamines and synthetic agonists as is the endogenously expressed β2AR. Thus, under these circumstances there is no mechanism to selectively activate or deactivate the genetically expressed receptor as may be dictated by the clinical status.

We have considered, however, that the β2AR can be utilized as a scaffold upon which to construct a synthetic G-protein-coupled receptor with specific properties to optimize gene therapy. It is not clear, however, whether such a highly modified receptor can maintain functional integrity, because multiple mutations throughout the protein would need to be introduced in known areas of conformational lability. In the current work, we utilize the human β2AR in the above fashion to create a protein with markedly different properties from an adrenergic receptor and with characteristics suitable for gene therapy. As a general paradigm the creation of such designer receptors is likely to be amenable for enhancement of other G-protein signaling for the modification of other pathologic states.

**EXPERIMENTAL PROCEDURES**

** Constructs—**The human wild-type β2AR cDNA cloned into the expression vector pBCBI were used as a template for mutagenesis. From this, an 1862-base pair SacI/SalI fragment encompassing the entire wild-type β2AR coding region was isolated and subcloned into M13mp19 for oligonucleotide-directed mutagenesis (33). Sequence encoding the HA epitope tag (MYGPDVDYDPSA) was incorporated into the amino terminus of the receptor for all subsequent constructs. Amino acid 27 was exchanged from Gln to Ghu, and amino acid 113 was changed from Asp to Ser, to make the receptor refractory to agonist-promoted down-regulation and to alter the ligand binding specificity, respectively. In addition, potential PKA/PKC phosphorylation sites at amino acids 272, 273, 356, and 357 were changed to Ala. Similarly, potential GRK phosphorylation sites at amino acids 366, 367, 371, 375, 395, 404, 407, 412, 418, 419, and 422 were changed to Ala. Another modification included the mutation of the stop codon to alanine and an in-frame ligation at the NcoI site immediately preceding the initiator methionine codon of the entire open reading frame of the rat Go, cDNA (long form).

**Tissue Culture—**Chinese hamster fibroblasts (CHW-1102) were grown in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were transiently transfected with a concomitant infection of a “helper” adenovirus using methods as described (34). Briefly, 7 × 10⁶ cells were plated in a 7.5 cm flask ~16 h prior to transfection. The cells were washed twice with Hank’s balanced salt solution and incubated in 0.5 ml of a transfection/infection mixture containing 1 × 10⁹ plaque-forming unit purified E1A-deleted adenovirus, 2% fetal calf serum, 80 μg/ml DEAE-dextran, and 15 μg of plasmid DNA in the aforementioned expression vectors, at 37 °C and 5% CO₂ with gentle rocking. After 2 h, the solution was aspirated, and the cells were incubated with 10% Me₂SO in Dulbecco’s modified Eagle’s medium for 2 min. After a final wash, the cells were preincubated with 1 mg/ml of rat anti-HA antibody (Babco) at a dilution of 1:1000 for 30 minutes. The cells were then incubated with 2% type IV collagenase and 0.5 mg/ml of soybean trypsin inhibitor, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 5 μg/ml benzamidine, 10 °C for 15 min. The cells were then plated at 0.1 mg/ml into each well of a 96-well plate and incubated at 37 °C for 48 h before proceeding with experiments.

**Radioisotopic Binding—**The cells were washed twice with phosphate-buffered saline, detached with a rubber policeman in 5 mM Tris, pH 7.4, 2 mM EDTA buffer, and centrifuged at 30,000 × g for 10 min. To determine receptor density, membranes pellets were resuspended in 75 mM Tris, pH 7.4, 12.5 mM MgCl₂, 2 mM EDTA, and radioligand binding with ³²P-cyano were performed for cells expressing the wild-type β2AR as described previously (35). To determine adenylyl cyclase activities, the membranes were incubated with 30 mM Tris, pH 7.4, 2 mM MgCl₂, 0.8 mM EDTA, 120 μM ATP, 60 μM GTP, 2.8 mM phosphoenolpyruvate, 50 μg/ml myokinase, 100 μM cAMP, and 1 μCi of [α-³²P]ATP for 30 min as described (35). The reactions were carried out with water, 100 μM forskolin, 10 μM epinephrine, 100 μM norepinephrine, and varying concentrations of alfubutol or L-158,870 (Merck).

**Therapeutic Receptor Effector Complex (TREC)**

**FIG. 1. Schematic of the modifications imposed on the β2AR protein.** The shaded areas represent transmembrane domains I–VII. The dark blocks represent fusions to the HA epitope tag and GαS. The tick marks indicate individual mutations (see text for details). The brackets indicate which alterations are included in the modified receptor and the TREC.
RESULTS AND DISCUSSION

Shown in Fig. 1 are the modifications imposed on the β2AR. To facilitate identification by immunoblotting, all receptors were fused at the amino terminus with the hemagglutinin (HA) epitope tag. The combined effect of mutations that remove sites required for down-regulation and phosphorylation was considered to be unlikely to alter ligand binding, so these were imposed collectively in the initial round of mutagenesis. Ultimately, two modified receptors and the wild-type β2AR were studied. One receptor contained the single mutations introduced to change amino acids at the indicated positions to alter the ligand-binding site, receptor down-regulation and receptor phosphorylation as further described below. This receptor is denoted as the “modified receptor.” A second receptor contained these mutations in addition to being fused to Gαs and is denoted as the therapeutic receptor-effector complex (TREC). Receptors were transiently expressed in CHW-1102 cells, and expression was verified by 125I-cyanopindolol binding (∼400 fmol/mg) or Western blots.

The agonist-binding site was re-engineered to satisfy several criteria. First, we wanted to render the receptor unresponsive to endogenous catecholamines and other synthetic β-agonists. In addition, we aimed to have the mutated receptor respond to a unique agonist. Finally, we preferred that this unique agonist not activate wild-type β2AR. The key interactions (36–38) of the β2AR with catecholamines occur in transmembrane domain III at Asp113, transmembrane domain VI at Asn293, and transmembrane domain V at Ser204 and Ser207. At Asp113, it has been proposed that the binding of agonists (and antagonists) involves the formation of an ion pair between the amine group of the ligand and the Asp carboxylate side chain. This interaction can apparently be brought about in mutant forms of the receptor by using different classes of ligands (39). We thus mutated Asp113 to Ser, which substitutes the β-carboxymethyl side chain with a β-hydroxymethyl group. This renders the receptor incapable of activation by biogenic amines. However, compounds that otherwise satisfy requirements for β2AR binding and are capable of accepting hydrogen bonds from the engineered Ser in transmembrane domain III, would activate the receptor. Based on work by Strader et al. (39), we chose 1-(3′,4′-dihydroxyphenyl)-3-methyl-1-butanone as the agonist specific for the modified β2AR. This compound, also known as L-158,870, retains the catechol ring but instead of the amine containing alkyl-side chain has a four carbon length moiety, keto-substituted at the α-carbon. The functional results of the Asp to Ser substitution are shown in Fig. 2. Because the therapeutic β-agonists commonly utilized clinically are partial agonists, we used albuterol as the benchmark agonist. As shown in Fig. 2A, albuterol stimulated adenylyl cyclase by the Asp113 wild-type (the wild-type allele) receptor ∼50% over basal levels, whereas there was no stimulation by albuterol of the modified mutant (including Ser113) receptor. In contrast, L-158,870 stimulated adenylyl cyclase by the modified receptor ∼70% above basal but did not activate wild-type β2AR (Table I). In addition, studies with the modified receptor, neither norepinephrine nor epinephrine nor ephedrine nor norepinephrine stimulated adenylyl cyclase activities over basal levels (5 ± 10% and 10 ± 10%, respectively; n = 6; p > 0.05 versus basal levels).

One consequence that we observed with the Ser substitution at position 113 of the third transmembrane domain was a decrease in basal adenylyl cyclase activities compared with the wild-type β2AR (16.6 ± 1.3 versus 27.8 ± 2.6 pmol/min/mg). So, whereas the two agonists stimulated their respective receptors to the same fold extent over basal, the absolute levels of maximal stimulation of the Ser113-modified β2AR was nonetheless depressed (Fig. 2B). This effect on basal coupling is likely due to conformational effects on the intracellular coupling domains that are imposed by this substitution in transmembrane domain III. Of note, Asp in this position is invariant in all biogenic amine-ligated G-protein-coupled receptors. To attempt to overcome this therapeutically disadvantageous characteristic, the intracellular carboxyl terminus of the modified receptor was fused to the amino terminus of the α-subunit of Gαs. At least with wild-type β2AR, such a fusion has been reported to increase basal (non-agonist-stimulated) levels of adenyl cyclase activity (35). Because the Ser substitution at residue 113 ablated 125I-cyanopindolol binding, immunoblotting using HA antibody was used for verification of the expression of the fused receptor. As shown in Fig. 3, the wild-type β2AR migrated at a molecular mass of ∼60 kDa, with an additional band representing more highly glycosylated states observed between 80 and 100 kDa. The TREC migrated at molecular masses consistent with the addition of the 42 kDa Gαs protein, with a band at ∼100 kDa and another broad band representing the highly glycosylated states at ≥120 kDa.

The results from adenylyl cyclase studies of the TREC are shown in Fig. 4. The fusion with Gαs indeed increased basal levels from 16.2 ± 1.3 to 23.1 ± 1.9 pmol/min/mg. Agonist stimulation was preserved and in fact increased from ∼70% over basal in the absence of Gαs to ∼90% over basal with the fusion. With these changes, maximal adenylyl cyclase activities were equivalent between wild-type β2AR and the fully modified fused receptor in the basal state and with the respective agonist activation (Table I).

We next considered the effects of modifications imposed to limit receptor desensitization. These mutations included substitution of all Ser and Thr in the cytoplasmic tail of the receptor with Ala. A similar mutant β2AR, which consisted of
Gly and Ala substitutions for Ser and Thr, has been shown to lack sites for GRK (βARK)-mediated phosphorylation (40). There are two potential PKA/PKC sites (Arg-Arg-Ser-Ser*) at amino acid positions 273 and 357 that were changed to Ala. Because the Ser immediately adjacent to the second Arg could also serve as a phosphoacceptor, these were mutated to Ala as well. Because long term agonist exposure evokes desensitization via these phosphorylation events as well as down-regulation of receptor number, we also sought to render the receptor resistant to down-regulation. We thus substituted Glu for Gln at amino acid position 27 of the amino terminus (which is a naturally occurring polymorphism in the human gene), which we have previously shown to result in a functional receptor that fails to undergo agonist-promoted destabilization and subsequent degradation (41). Thus, to test whether the above modifications in fact limit desensitization, cells in culture expressing the wild-type β2AR or the TREC were incubated with equipotent concentrations of albuterol (10 μM) or L-158,870 (100 μM) for 24 h. This exposure provides the ultimate verification of a functional knockout of desensitization from all the above mechanisms and also mimics the in vivo setting of repetitive dosing over prolonged periods of time. The cells were then placed on ice and washed, membranes were prepared, and agonist-stimulated adenylyl cyclase activities were determined. The results of such studies are shown in Fig. 5. Wild-type β2AR underwent extensive functional desensitization under these conditions that amounted to a loss of 55 ± 4% of the maximal response observed in control cells (Fig. 5A). In marked contrast, the fully modified receptor failed to exhibit any loss of function with long term agonist treatment. As shown in Fig. 5B, the responses are superimposable, with mean desensitization being quantitated at 1 ± 5%. Also, the EC50 saw did not significantly change with the TREC following agonist exposure (6.8 ± 1.2 versus 12 ± 3.9 μM). Forskolin-stimulated activities were not altered by agonist treatments (untreated versus treated: 93 ± 7 versus 86 ± 6 pmol/min/mg for the wild-type β2AR and 94 ± 6 versus 110 ± 7 pmol/min/mg for the TREC), indicating that these responses are receptor-specific.

The above results indicate that constructing synthetic G-protein-coupled receptors with distinct properties for targeted modification of specific signaling pathways is feasible. Here we utilized the β2AR as a backbone for constructing such a highly modified receptor. In this case, the receptor created is no longer an adrenergic receptor, because it is not activated by catecholamines. This is a critical feature of the approach, because it provides for selective activation of the therapeutically expressed receptor via an agonist that does not activate endogenously expressed βAR. The need to activate (and to deactivate by withdrawal of agonist) the therapeutically expressed receptor is a mechanism by which the response can be modulated in accordance with physiologic need. We have shown, for example, that high overexpression of wild-type β2AR in the hearts of Gαs overexpressing transgenic mice worsens cardiac hypertrophy and dysfunction and increases mortality, whereas low levels of β2AR overexpression in the Gαs mouse are beneficial (28). So, by selectively activating the therapeutically expressed receptor with appropriate dosing of the agonist, a certain level of enhanced signaling could be obtained for effectiveness without adverse events. There may also be times, however, when no signal enhancement is desirable. Withdrawal of the specifically designed agonist would thus result in cessation of signaling by the genetically expressed receptor despite elevated catecholamines. It should be noted, however, that the ability of the therapeutically expressed receptor to spontaneously activate could still result in some degree of enhanced signaling even in the absence of agonist. As discussed above, our initial mutations resulted in a receptor with significantly depressed basal and agonist-stimulated adenylyl cyclase activities, which was considered therapeutically disadvantageous, so the fusion with Gαs was undertaken. Basal adenylyl cyclase activities were

### Table I

| Receptor          | Basal | Albuterol | L-158,870 |
|-------------------|-------|-----------|-----------|
|                   | pmol/min/mg | pmol/min/mg | %          | pmol/min/mg | %          | μM         |
| Wild-type β2AR    | 27.8 ± 2.60 | 41.4 ± 7.28 | 50 ± 2.5  | 18.7 ± 2.53 | 70 ± 10    | 0.39 ± 0.30 (albuterol) |
| Modified receptor | 16.6 ± 1.55 | 17.8 ± 1.32 |           | 26.8 ± 2.06 | 70 ± 10    | 11 ± 3.7 (L-158,870)    |
| TREC              | 23.2 ± 1.71 | 29.3 ± 2.47 |           | 43.3 ± 2.71 | 90 ± 20    | 7.4 ± 2.9 (L-158,870)   |

![Western analysis of the expression of β2AR and the TREC](image-url)
increased with the fused receptor but were no more than basal levels of the wild-type β2AR. Of note, we have not tested L-158,870 against other G-protein-coupled receptors, but based on its structure, it is unlikely to activate any other natively expressed receptors.

The TREC also lacks the ability to undergo agonist-promoted phosphorylation and down-regulation by degradation. These modifications were based on multiple reports that β2AR function is depressed in asthma and heart failure via these and other mechanisms (see the Introduction). It seemed appropriate, then, to render the modified receptor incapable of such function with agonist exposure. The results are from four independent experiments.

Fig. 5. The TREC fails to undergo agonist-promoted desensitization. CHW-1102 cells in monolayers transiently transfected with each receptor were incubated with (filled symbols) and without (open symbols) 10 μM albuterol or 100 μM L-158,870 for 24 h and washed. The membranes were prepared, and the adenylyl cyclase activities were determined as described under “Experimental Procedures.” The results are presented as percentages of the maximum activity for the untreated control. Although the wild-type β2AR underwent ~55% desensitization, the TREC failed to undergo any decrease in function with agonist exposure. The results are from four independent experiments.

In the heart, β2AR overexpression in transgenic mice increases inotropy and chronotropy, as has been shown in multiple studies (21–23). Cardiomyopathic changes are noted, however, with high levels of expression or with moderate levels over a long period of time (22). This emphasizes the need for careful control of receptor signaling, a major feature of our receptor-effector complex. In double transgenic mice, we have recently shown that the hypertrophy and resting cardiac contractile dysfunction due to Goq overexpression is partially rescued by β2AR overexpression (28). Another method of improving βAR function in the heart has been with an inhibitor of the βAR kinase. Transgenic overexpression of a peptide inhibitor (ARKCT) rescues ventricular function, and in some cases hypertrophy and other pathologic features, of several mouse models of heart failure. This includes the muscle-specific LIM protein knockout mouse (29), the modified myosin heavy chain overexpressing mouse (31) and a mouse overexpressing calcineurin (30). The use of a βARK inhibitor may have particular advantages in pathologic states where overstimulation is deleterious, because the inhibitor would act to restore receptor function, avoiding excessive activation of the pathway. However, βARK inhibitors are likely to be most effective when βARK activity is enhanced in the disease. And, such inhibition lacks specificity because many G-protein-coupled receptors are phosphorylated by GRKs. Nevertheless, in the setting of the compromised heart, the in vivo consequences of βARK inhibition are substantial, and appropriately, the approach continues to be pursued.

Our modified receptor effector complex differs by a number of features from a mutated receptor reported by Coward et al. (44). This receptor consisted of a κ opioid receptor that was mutated such that the affinity for an endogenous peptide agonist (dynorphin) was decreased 200–2000-fold; yet affinity for some small synthetic molecules was relatively unchanged. Interestingly, the maximal response (inhibition of adenylyl cyclase) by dynorphin was not changed by the mutations. So, with high concentrations as might be seen in certain microenvironments, this receptor could still be activated by endogenous agonist. Furthermore, the small molecule agonists activated wild-type κ opioid receptors, so “two-way” selectivity was not attained. No modifications were made to alter coupling or regulation.

In summary, the framework of the β2AR was utilized to create a G-protein-coupled receptor suitable for therapeutic
modulation of signaling in pathologic states. Extensive modifications were undertaken to re-engineer the ligand-binding site, to remove sites for phosphorylation by GRKs and PKA/PKC, to depress agonist-promoted down-regulation, and to increase Gs coupling. The resulting receptor-effector complex was unresponsive to catecholamines and the prototypic therapeutic agonist albuterol but was activated by a synthetic agonist that is responsive to catecholamines and the prototypic therapeutic agonist-promoted down-regulation, and to increase Gs to remove sites for phosphorylation by GRKs and PKA/PKC, to activate adenyl cyclase activities. The receptor-effector complex showed no desensitization during agonist occupancy despite high agonist concentrations and prolonged exposures. Concomitant modifications of all these domains in a single receptor has not been previously reported. The fact that a stable, functional receptor, with such specific therapeutic properties can be made, is promising as a modality for enhancing βAR-like signaling in diseases where function is impaired. As a general paradigm, based on these results, it is likely that other “designer” G-protein-coupled receptor-effector complexes can be constructed to therapeutically modulate other signaling pathways.

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J. Biol. Chem. 2001, 276:31596-31601.
doi: 10.1074/jbc.M102734200 originally published online June 11, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102734200

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