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A small region of the β-adrenergic receptor is selectively involved in its rapid regulation

(sequestration/phosphorylation/desensitization)

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ABSTRACT **Plasma membrane receptors that couple to guanine nucleotide-binding regulatory proteins (G proteins) undergo a variety of rapid (minutes) and longer term (hours) regulatory processes induced by ligands. For the β_2-adrenergic receptor (β_2AR), the rapid processes include functional desensitization, mediated by phosphorylation of the receptor by the cAMP-dependent protein kinase and the β-adrenergic receptor kinase, as well as a loss of hydrophilic ligand binding proposed to represent sequestration of receptors into a cellular compartment distinct from the plasma membrane. The slower processes include β_2AR down-regulation (i.e., a decrease in the total cellular complement of receptors). It is not yet known whether β_2AR phosphorylation and/or sequestration are prerequisites for down-regulation of the receptor. Like other G protein-coupled receptors, the β_2AR molecule spans the plasma membrane seven times, and the cytoplasmic carboxyl-terminal domain has been proposed to contain molecular determinants for each of these regulatory processes. We replaced four serine and threonine residues located within a 10-amino acid segment of this domain of β_2AR and thereby prevented agonist-promoted phosphorylation, sequestration, and rapid desensitization of the adenylyl cyclase response. In contrast, these mutations did not affect functional coupling to the stimulatory G protein G_s or long-term down-regulation. These findings thus define a small, hitherto unappreciated region of the receptor molecule that may selectively subserve its rapid regulation. In addition, with the demonstration that β_2AR does not have to be phosphorylated or sequestered in order to enter the down-regulation pathway, the results suggest that the classical receptor endocytosis model may not be appropriate for β_2AR regulation.**

The binding of many hormones and neurotransmitters to their plasma membrane receptors triggers two distinct sets of biochemical reactions. First, a rapid conformational change occurs in receptors, which permits them to bind to and activate specific guanine nucleotide-binding proteins (1, 2) (G proteins); these, in turn, affect the functioning of various effector molecules such as adenylyl cyclase, ion channels, and phospholipases (3, 4). Activation of receptors also initiates, on a slightly slower time frame, a series of processes that cause them to become progressively *less* able to interact with G proteins. These processes underlie the frequently made observation that the cellular responses to agonists often wane, or desensitize (5), despite continuous exposure to the agonists.

The molecular mechanisms underlying functional desensitization of the adenylyl cyclase response stimulated by the β_2-adrenergic receptor (β_2AR) have been the object of a particularly intensive investigation (6–9). The most rapid events causally implicated in desensitization of β_2AR function include its phosphorylation by two kinases. The cAMP-dependent protein kinase (PKA) is activated upon exposure of cells to nanomolar or greater levels of the β-adrenergic agonist isoproterenol (Iso) (10–12) and is believed to phosphorylate β_2AR on residues adjacent to regions implicated in coupling of the receptor to the stimulatory G protein G_s. Such phosphorylation may then directly perturb functional activation of the G protein. Micromolar levels of Iso also lead to phosphorylation of β_2AR on its cytoplasmic tail (10, 13) by the β-adrenergic kinase (βARK) (14). βARK phosphorylation has been proposed to facilitate the binding of the recently identified cytosolic protein β-arrestin (15) to the receptor molecule, which further disrupts β_2AR–G_s coupling.

Another regulatory event triggered within minutes by agonist exposure is the sequestration of β_2AR. Sequestration is manifested as a rapid loss of hydrophilic (but not lipophilic) ligand binding, although the precise cellular location and nature of sequestered receptors remain controversial (8, 16–18). In addition, sequestration does not appear to play a substantive role in the rapid functional desensitization of β_2AR: prevention of sequestration by various pharmacological agents generally has little effect on the rapid desensitization process (8). Furthermore, β_2AR phosphorylation by PKA or βARK is not required for sequestration to occur (10, 11, 13).

Finally, exposure of cells to Iso for an hour or more results in a progressive loss of total cellular receptors as detected by lipophilic ligand binding (down-regulation). More prolonged exposure to Iso (>5 hr) is also associated with decreases in the levels of mRNA encoding β_2AR (19). In analogy with the regulation of the transferrin and growth factor receptors, it has been proposed that β_2AR sequestration represents an obligatory precursor stage for the ultimate down-regulation of the receptor (16).

In an attempt to design a mutant receptor with specific regulatory deficits, we altered the codons encoding four serine and threonine residues in the proximal portion of the carboxyl tail of the human β_2AR. Studies with this mutated receptor, described here, provide strong evidence that causally dissociates the rapid regulatory processes of phosphorylation and sequestration from long-term regulation of the receptor. In addition, these mutations highlight the existence of a small region of β_2AR whose conformation appears to be crucial for its rapid regulation.

EXPERIMENTAL PROCEDURES

Mutagenesis and Stable Expression. Site-directed mutagenesis using oligonucleotide primers was performed with cDNA

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Abbreviations: β_2AR, wild-type human β_2-adrenergic receptor; G protein, guanine nucleotide-binding regulatory protein; G_s, stimulatory G protein; Iso, isoproterenol; βARK, β-adrenergic receptor kinase; PKA, cAMP-dependent protein kinase.

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encoding the human β_2AR according to the Amersham protocol and was confirmed by sequencing (20). The mutant cDNA and that encoding wild-type human β_2AR were cloned into the eukaryotic expression vector PBC12BI and cotransfected with pSVNeo (Pharmacia) into Chinese hamster fibroblasts and Chinese hamster ovary cells by calcium phosphate precipitation, as described (20). The recipient cell type did not qualitatively influence the parameters of receptor functioning (adenylyl cyclase activities and receptor sequestration) assessed for both receptors expressed in both cell types. Furthermore, wild-type β_2AR expressed in either Chinese hamster fibroblasts (10, 13, 21) or Chinese hamster ovary cells showed similar capacities to stimulate adenylyl cyclase, to become desensitized upon exposure to agonist, and to undergo receptor sequestration and down-regulation.

Whole-Cell Phosphorylation. Cells [1.2×10^8; two 245 × 245 × 20 mm dishes (Nunclon Delta) per condition] were detached by collagenase pretreatment and incubated for 90 min in phosphate-free medium supplemented with 0.1 mM ascorbic acid, 0.1 mM sodium phosphate, and ≈3 mCi (1 Ci = 37 GBq) of carrier-free $^{32}P_i$ (DuPont/NEN). Cells were then incubated with or without Iso for 10 min; receptors were solubilized and purified by affinity chromatography and examined by polyacrylamide gel electrophoresis and autoradiography, as described (10). Laser densitometric scanning of the autoradiograms, performed under linear detection conditions, was used to assess the incorporation of $^{32}P_i$ into receptors, with correction for random differences between gel lanes in background intensity. In each experiment, the difference between the basal and agonist-induced phosphorylation of β_2AR was first set equal to 100%, and the corresponding difference for the mutant receptor (termed S355-364) was normalized to that value; alternatively, the agonist-induced phosphorylation for β_2AR and S355-364 were expressed as the fold increase over the respective basal phosphorylation in the same experiment.

G Protein-Coupling Assessments. Following preincubation with or without Iso, cells were washed with cold Dulbecco's phosphate-buffered saline (PBS; GIBCO) and scraped into 10 ml of 5 mM Tris (pH 7.4), 2 mM EDTA, and 1 mM sodium phosphate, rendering >90% of the cells permeable to trypan blue. Lysed cells were centrifuged at 40,000 × *g* for 30 min at 4°C, and the particulate fractions were assayed for adenylyl cyclase activities (free $[Mg^{2+}]$ = 1 mM) as described (20). In equilibrium binding assays, membranes were incubated with 25–50 pM ^{125}I-labeled cyanopindolol and various concentrations of Iso in a buffer containing 75 mM Tris (pH 7.4), 12.5 mM $MgCl_2$, and 2 mM EDTA for 90 min at 25°C. Binding competition curves were analyzed by computer as described (20).

Sequestration and Down-Regulation. Following exposure to Iso and 0.1 mM ascorbic acid for the indicated times, cells were washed with cold PBS and incubated for 15 min with cold PBS containing 5 mM EDTA, and the cells were detached by vigorous shaking of the flasks. Greater than 90% of the cells remained impermeable to trypan blue following this treatment. Cells (5×10^5 per ml) were incubated at 13°C for 3 hr with 200 pM [^{125}I]iodopindolol either alone (to define total binding), in the presence of 1.0 μM (−)-propranolol (to define nonspecific binding), or in the presence of 0.1 μM CGP 12177 [a hydrophilic ligand commonly used to assess cell surface receptor numbers (8)]. There was no difference between the apparent binding affinities of CGP 12177 for intact, untreated cells expressing either β_2AR or S355-364 ($IC_{50} \approx 3$ nM), and 0.1 μM CGP 12177 displaced ≈90% of the radioligand binding to untreated cells in both cases. Receptor sequestration was defined as the percentage of specific radioligand binding not displaced by CGP 12177. Down-regulation was defined as the loss in specific [^{125}I]iodopindolol binding.

RESULTS

The carboxyl-terminal domains of G protein-coupled receptors have been implicated in the regulation of receptor function by biochemical and mutagenesis studies (10, 13, 22–24). The proposed topographical arrangement of the human β_2AR is schematically depicted in Fig. 1 *Left*. High-

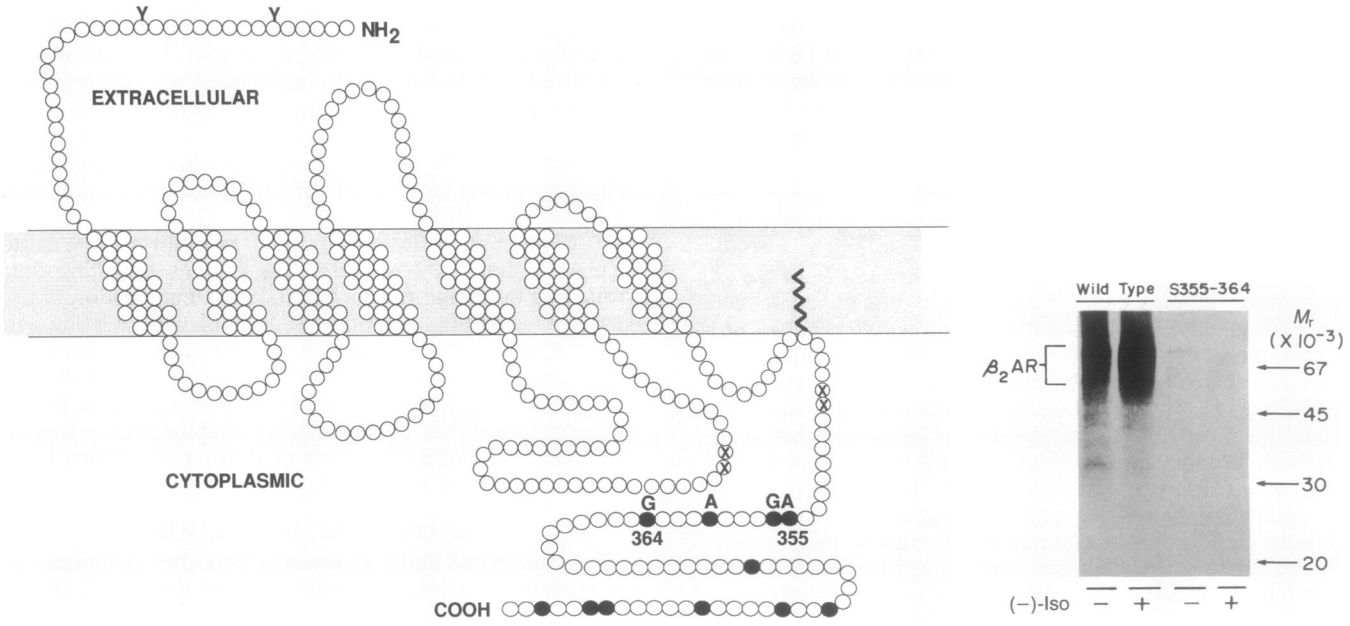

FIG. 1. (*Left*) Schematic depiction of the topographical arrangement of wild-type and mutant human β_2AR. Filled circles and circles containing an X represent potential βARK or PKA phosphorylation sites, respectively. In S355-364, the following amino acid substitutions were made: Ser-355 → Ala (S355A), S356G, T360A, and S364G. (*Right*) Iso-induced phosphorylation of wild-type and S355-364 β_2AR in intact cells. An autoradiogram of purified receptors from cells prelabeled with $^{32}P_i$ and incubated for 10 min in the presence or absence of 10 μM Iso is shown. Each lane was loaded with ≈0.2 pmol of purified receptor (quantitated by ^{125}I-labeled cyanopindolol binding). Iso-induced phosphorylation of S355-364 was 11% ± 7% of that seen in parallel incubations with β_2AR (n = 3).

lighted are serine and threonine residues representing the putative phosphorylation sites for PKA and for βARK (14). A cluster of four of these residues located in the proximal end (positions 355–364) of the carboxyl tail (potential βARK sites) was replaced by alanine and glycine residues (Fig. 1 *Left*), and the cDNAs encoding this or the wild-type human β_2AR were stably expressed in mammalian cells. As depicted in Table 1, the mutant receptor (termed S355-364) exhibited wild-type parameters of G_s coupling, whether assessed in a functional assay (Iso stimulation of adenylyl cyclase activity) or as the ability of the receptor to exhibit a high-affinity binding state for Iso in an equilibrium binding assay. Formation of such a state is believed to occur only when receptors physically interact with G proteins (3, 4).

To examine the ability of S355-364 to undergo agonist-induced phosphorylation, cells were preincubated with $^{32}PO_4$ and then treated with 10 μM Iso for 10 min; next the receptors were purified and examined by gel electrophoresis and autoradiography. Wild-type β_2AR migrates as a relatively broad band (sometimes apparent as discrete, multiple bands) centered at ≈67 kDa (Fig. 1 *Right*); the broadness of the band has been attributed to a high level of glycosylation (13). Both β_2AR and S355-364 purified from untreated cells exhibited basal phosphorylation, and pretreatment of cells expressing β_2AR with agonist resulted in a 3.7 ± 0.2-fold increase (n = 3) in incorporation of $^{32}PO_4$ into the receptor relative to this basal phosphorylation (Fig. 1 *Right*). In parallel experiments, in contrast, there was no consistent agonist-induced phosphorylation of S355-364 (1.3 ± 0.3-fold increase). These results were surprising, since other residues believed to be phosphorylated in wild-type β_2AR under these conditions remain intact in this mutant (10, 13), including both consensus sites for PKA, as well as proposed phosphorylation site(s) for βARK among the four serine and threonine residues located at the distal tip of the tail (W.P.H. and R.J.L., unpublished data).

In parallel experiments, cells expressing β_2AR and S355-364 were exposed to Iso (10 μM for 10 min), and membrane fractions were tested for their respective abilities to mediate stimulation of the adenylyl cyclase response upon rechallenge with Iso. Pretreatment of cells expressing β_2AR in this manner diminished the maximal adenylyl cyclase response attainable with Iso to a level that was 67% ± 13% of that seen in membranes from untreated cells (Fig. 2 *Upper*). This pretreatment not only reduced the maximal response but also the sensitivity of the functional response to the agonist, such that the response of the desensitized membranes to restimulation by a submaximal concentration of Iso (10 nM) was only 37% ± 6% of that obtained with membranes from untreated cells (Fig. 2 *Upper*). In contrast, membranes prepared from pretreated cells expressing S355-364 exhibited maximal and submaximal adenylyl cyclase responses that were 100% ± 15% and 97% + 24%, respectively, of the corresponding responses in untreated membranes (Fig. 2 *Lower*). Cells expressing S355-364 also failed to undergo desensitization following pretreatment with a lower Iso concentration (10 nM; data not shown), a dose at which phosphorylation (10) and desensitization (10–12) of β_2AR are mainly due to PKA.

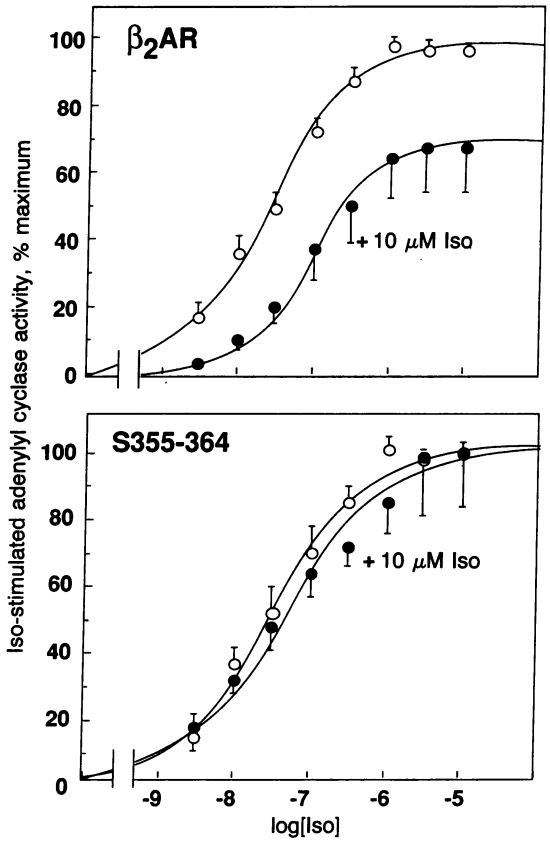

FIG. 2. Iso-induced desensitization of adenylyl cyclase responses to Iso in β_2AR and S355-364. Cells expressing wild-type (*Upper*) and S355-364 (*Lower*) receptors were incubated with (●) or without (○) 10 μM Iso for 10 min, and adenylyl cyclase activities were measured in particulate fractions in response to increasing concentrations of Iso. In each experiment, the maximum Iso stimulation in control membranes expressing β_2AR and S355-364 was first set equal to 100%, and all other adenylyl cyclase values for the respective cell type were expressed as percentage of that maximal response. Each data point is the mean ± SEM (n = 5–10). Absolute adenylyl cyclase values for control membranes are presented in Table 1.

The ability of S355-364 to undergo sequestration and down-regulation was assessed by measuring the changes in the radioligand binding properties of intact cells after pretreatment with 10 μM Iso for various times. β_2AR underwent sequestration with a $t_{1/2}$ of ≈5 min; >30% of the total receptors in the cell ultimately remained sequestered. In

Table 1. G_s coupling properties of β_2AR and S355-364

	Adenylyl cyclase activity				Iso binding constants		
Receptor	Basal	Iso_{max}	EC_{50} for Iso, nM	NaF	K_H, nM	K_L, nM	$\%R_H$
β_2AR	9 ±1	85 ± 17	23 ± 3	61 ± 14	0.59 ± 0.29	58 ± 22	42 ± 4
S355-364	10 ± 1	80 ± 16	27 ± 7	69 ± 13	0.77 ± 0.28	119 ± 56	50 ± 2

Membranes were assayed for adenylyl cyclase activity in response to various concentrations of (−)-Iso or to 10 mM NaF as described in *Experimental Procedures*. Basal, NaF, and maximally stimulatory (Iso_{max}) activities were expressed as pmol of cAMP per min per mg of membrane protein (means ± SEM from 11 to 15 experiments). Binding parameters were estimated by nonlinear least-squares analyses, as described (20), and are the means ± SEM from three experiments. K_d values (equilibrium dissociation constants) for binding of ^{125}I-labeled cyanopindolol were ≈30 pM for both receptor types. K_H and K_L are the high- and low-affinity equilibrium dissociation constants for Iso, respectively. $\%R_H$ is the percentage of receptors in the high-affinity agonist binding form. Receptor expression levels (B_{max}) for β_2AR and S355-364 were 700 ± 150 and 700 ± 190 fmol/mg, respectively.

contrast, under the same conditions <5% of S355-364 was sequestered (Fig. 3). However, β_2AR and S355-364 exhibited patterns of down-regulation similar in both rate and extent. For both receptors, exposure of cells to Iso for 90 min was sufficient to evoke some down-regulation, with at least 50% of the receptors undetectable by radioligand binding at 24 hr (Fig. 3 *Lower*).

DISCUSSION

Over the past several years, a number of biochemical and mutagenesis techniques have been used to determine which regions of the β_2AR are involved in its various functions. Such studies have highlighted the importance of the transmembrane spanning domains in ligand binding and the second and third cytosolic loops in G-protein coupling (1, 2). In contrast, much less is known about the structural bases of receptor regulation, except that elements in the carboxyl-terminal tail are probably involved. In the present study we mutated a relatively small region (10 amino acids) of the proximal portion of the carboxyl tail of β_2AR and abolished all rapid regulatory processes of the receptor. In theory, the lack of rapid agonist regulation of S355-364 function shown here could be due to the absence of specific phosphate acceptor serine and/or threonine residues, which normally govern rapid regulation; alternatively, the effects may arise from conformational changes in this region of the receptor. With regard to the first possibility, there is substantial evidence from mutagenesis studies (10, 13) and with kinase inhibitors (11) that phosphorylation of the wild-type receptor is *not* a prerequisite for at least one aspect of rapid regulation, receptor sequestration. We therefore favor the possibility that the mutations introduced into S355-364 alter the conformation of this small region in such a way as to both block sequestration and to prevent phosphorylation of other, non-mutated residues of the receptor.

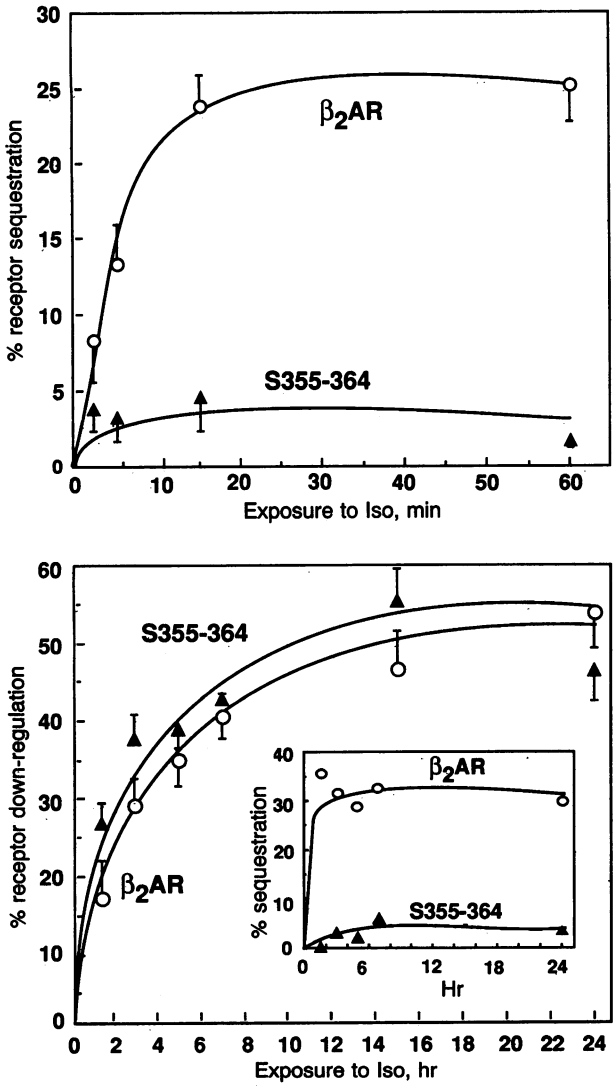

FIG. 3. Receptor sequestration and down-regulation properties of β_2AR and S355-364. Cells were exposed to 10 μM Iso for 2–60 min (*Upper*) or 1.5–24 hr (*Lower*), and receptor sequestration (*Upper* and *Inset*) and down-regulation (*Lower*) were assessed in a whole-cell radioligand binding assay. Data are means ± SEM (n = 4–8).

In this regard, we recently characterized the regulatory properties of a mutated receptor identical to S355-364 except that the remaining seven serine and threonine residues in the carboxyl terminus of β_2AR were also replaced by alanine and glycine (mutant B in ref. 10). This more extensive mutant, however, showed only partial impairments in agonist-induced phosphorylation and desensitization following pretreatment with micromolar levels of Iso and exhibited a wild-type pattern of receptor sequestration (10, 13). Thus, the additional mutations in the distal portion of the carboxyl terminus of mutant B substantially suppressed the effects of the mutations in the proximal portion of the tail. The mechanistic basis for this suppression is unknown at present. One possibility is that the additional mutations in the distal portion of the carboxyl terminal in mutant B somehow counteract the inhibitory effects of the conformational change in the proximal portion of the tail. A second possibility is that the additional mutations simply prevent the conformational change from occurring in the first place. If the latter possibility were true, differences should exist in the conformation of the region between amino acids 355 and 364 among S355-364, mutant B, and β_2AR. In fact, differences in the secondary structures of this domain are predicted to exist between all three receptors by computer-assisted structure analyses (25, 26). No single amino acid change appears to be solely responsible for the functional effects, since mutant receptors comprised of single point mutations in which only one of the four residues mutated in S355-364 is replaced by alanine or glycine fail to fully reproduce the regulatory phenotype of S355-364 (and are markedly different from S355-364 in predicted secondary structure; data not shown).

The extreme sensitivity of this region to mutational perturbation may help to explain previous results with mutant receptors lacking large portions of the carboxyl tail. For example, Cheung *et al.* (22) observed that large truncations of the carboxyl tail of the hamster β_2AR proximal, but not distal, to this region impaired receptor sequestration. In a different study, truncation of the human β_2AR immediately distal to Ser-364 inhibited rapid phosphorylation and desensitization but not sequestration (13).

From a mechanistic standpoint, the apparent lack of requirement for receptor phosphorylation in down-regulation of β_2AR is consistent with the conclusions of other studies with a mutated receptor lacking all PKA and βARK sites (21) or with PKA-deficient cells (27). In contrast, the precise relationship between agonist-induced sequestration and down-regulation of β_2AR has been the subject of controversy. Perkins and colleagues (8, 16) have proposed on the basis of pharmacological and biochemical evidence that sequestered receptors are internalized in a subcellular compartment distinct from the plasma membrane and ultimately down-regulate in a manner similar to that described for the epidermal growth factor and transferrin receptors. The latter receptors, like many growth factor receptors, are believed to accumulate in clathrin-coated pits, which are internalized in an endocytotic pathway. This pathway culminates in the fusion of the endosomes with lysosomes, which leads to

degradation of the receptor (28). However, this model is not easily reconciled with several experimental observations regarding β_2AR sequestration and down-regulation. For example, certain mutations in β_2AR (21, 29) or in G_s (27) that impair receptor–G protein coupling inhibit down-regulation but not the more rapid sequestration process. (G_s has not been implicated in the endocytosis of growth factor receptors.) Conversely, nanomolar levels of Iso (W.P.H., P.T.C., and R.J.L., unpublished data) and so-called atypical agonists (30) do not elicit β_2AR sequestration but do induce partial, albeit delayed, down-regulation.

Further evidence that challenges the relevance of the endocytosis model to β_2AR regulation comes from studies in which receptor internalization was assessed either by the relative sensitivity of radioligand binding to cellular treatment with low pH (17) or by antibodies directed against β_2AR (18, 31, 32). From such studies two groups (17, 18), but not others (31, 32), proposed that standard measurements of sequestration (rapid and selective decreases in hydrophilic ligand binding) and down-regulation (slower losses of overall ligand binding) detect agonist-induced changes in receptor binding properties unrelated to any physical translocation or degradation of the receptor molecule.

In this context, the identification here of a small region of β_2AR selectively involved in rapid regulation of the receptor appears to permit a mechanistic dissociation of rapid receptor sequestration from the more slowly evolving down-regulation process, regardless of their precise biochemical natures. These results do not appear to be consistent with the predictions of the endocytosis model. Nevertheless, we cannot exclude the possibility that only a very small degree of receptor sequestration (such as that apparent with S355-364) is required to saturate the down-regulation pathway. However, such a mechanism would require that, while only a small fraction of sequestered, wild-type β_2AR receptors normally become down-regulated (and the vast majority recycle back to the plasma membrane), almost all sequestered S355-364 receptors are absorbed into the down-regulation pathway. Alternatively, it is also possible that β_2AR does undergo some form of endocytosis but that β_2AR sequestration is simply not part of this process.

Coupled with previous evidence that β_2AR sequestration does not substantially contribute to its rapid desensitization (cf. ref. 10), these data further suggest that attenuation of receptor functioning is not the principal biological consequence of sequestration (33, 34). Finally, several other G protein-coupled receptors that are highly homologous in primary structure (1, 2) also exhibit similar short- and long-term regulatory properties (5, 24, 35). Thus, these results may have mechanistic and structural implications for other receptors, especially those activated by small, nonpeptide ligands.

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