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Mutations of the Human β_2-Adrenergic Receptor That Impair Coupling to G_s Interfere with Receptor Down-Regulation but Not Sequestration

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SUMMARY

The integrity of coupling of the β_2-adrenergic receptor (β_2AR) to its guanine nucleotide-binding protein, G_s, and phosphorylation events on the receptor molecule have been proposed to be important determinants in the processes of receptor sequestration and down-regulation. However, little is known about the molecular mechanisms underlying these processes, and the regions of the receptor molecule that specifically subserve sequestration and down-regulation have yet to be delineated. To address these questions, we stably transfected eight mutant β_2AR genes into Chinese hamster fibroblasts and evaluated the coupling, sequestration, and down-regulation properties of the mutated receptors. These mutant receptors have been previously demonstrated either to exhibit abnormal coupling to G_s or to lack functionally important phosphorylation sites for either the cAMP-dependent protein kinase or the agonist-dependent β-adrenergic receptor kinase. All eight mutants exhibited receptor sequestration equivalent in extent to that of the β_2AR, regardless of their coupling or phosphorylation status. However, four mutants that exhibited various degrees of impairment in coupling to G_s showed blunted receptor down-regulation patterns. Simultaneous treatment with isoproterenol and dibutyryl-cAMP did not improve the abilities of the mutant receptors to undergo down-regulation. These findings demonstrate that a variety of mutant β_2AR with impaired coupling to G_s are, nevertheless, able to be sequestered normally. In contrast, agonist-induced down-regulation appears to require coupling of the β_2AR to G_s but is largely independent of the generation of cAMP. Our results also suggest that molecular determinants of the β_2AR involved in receptor sequestration are distinct from those participating in the down-regulation process.

The β_2AR-G_s-adenylyl cyclase transmembrane signal transduction complex is one of the most thoroughly studied systems that exemplifies the phenomenon of desensitization, a process whereby a physiological response wanes over time despite the presence of a stimulus of constant intensity (1). Three major processes have been postulated to contribute to agonist-induced desensitization. First, a rapid (seconds to minutes) uncoupling event renders the receptors less able to functionally activate G_s. The major mediator of this process appears to be receptor phosphorylation by distinct protein kinases, PKA and βARK (2–6). Two consensus sequences exist on β_2AR for phosphorylation by PKA, one in the third intracellular loop and a second in the proximal part of the carboxyl terminal tail (4), whereas putative phosphorylation sites for βARK reside in a serine- and threonine-rich segment of the distal portion of the carboxyl terminal tail (2, 4, 7).

A second proposed mechanism of desensitization is receptor sequestration. This process is generally envisioned as a rapid (minutes) agonist-promoted internalization of receptors to a cellular compartment distinct from the plasma membrane, but wherein the receptors are still detectable using hydrophobic radioligands. Upon removal of agonist, these receptors are thought to be recycled to the cell surface in a fully functional state (8, 9). An alternative hypothesis postulates, however, that the receptors are not physically translocated within the cell but, rather, are sequestered in the plasma membrane in a conformation that does not support the binding of hydrophilic ligands (10).

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ABBREVIATIONS: AR, adrenergic receptor; CHW, Chinese hamster fibroblast (CHW-1102); βARK, β-adrenergic receptor kinase; ISO, (−)-isoproterenol; PKA, cAMP-dependent protein kinase; G_s, stimulatory guanine nucleotide-binding regulatory protein; ^{125}I-CYP, [^{125}I]iodocyanopindolol; ^{125}I-PIN, [^{125}I]iodopindolol; IBMX, isobutylmethylxanthine; G418, Geneticin; CGP 12177, 4-(3-*t*-butylamino-2-hydroxypropoxy)-benzimidazole-2-one HCl; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; G protein, guanine nucleotide-binding protein; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Finally, prolonged treatment (hours) of cells with agonist results in receptor down-regulation, a process whereby the total cellular β_2AR binding is decreased. Under certain experimental conditions, the reappearance of receptor binding following removal of agonist requires *de novo* protein synthesis (11–14). There appear to be both cAMP-dependent and -independent mechanisms that lead to receptor down-regulation; they may include alterations in mRNA stability as well as in the rates of gene transcription, protein synthesis, and receptor degradation (15–19).

Although regions, and in some cases individual amino acid residues, of the β_2AR that are likely involved in ligand binding (20–27), G_s coupling (25, 28–30), and phosphorylation (2, 4, 5) have been identified, the determinants of the molecule responsible for mediating receptor sequestration and down-regulation are unknown. Strader and co-workers (31, 32) have reported that large deletions in the third intracellular loop of β_2AR impair the abilities of the mutant receptors to couple to adenylyl cyclase and to undergo receptor sequestration, and these investigators have proposed a role for G_s coupling in the process of receptor sequestration. However, this notion is difficult to reconcile with results from earlier studies showing that S49 lymphoma cells lacking either the α subunit of G_s (cyc^-) or PKA activity (kin^-) or possessing a defective G_s (UNC) (33, 34) sequester their βAR normally. In contrast, there is more persuasive evidence for a role for G_s coupling in agonist-induced receptor down-regulation. Both cyc^- and UNC cells show little agonist-induced down-regulation of β_2AR (33–36) and β_2AR mRNA (37). Moreover, a recent study by Valiquette *et al.* (38) has shown that a mutant β_2AR bearing substitutions of tyrosines 350 and 354 in the proximal portion of the cytoplasmic tail is markedly impaired in its ability both to couple to G_s and to undergo agonist-induced down-regulation. However, because receptor-mediated activation of adenylyl cyclase does not appear to be required for down-regulation (33, 37), the precise role of G_s in the latter process is unclear.

To assess the structural basis of receptor sequestration and down-regulation, as well as the relationship between receptor-G_s coupling and these processes, we examined the sequestration and down-regulation characteristics of mutant forms of the β_2AR that have alterations in regions proposed to be involved in receptor-G_s coupling (28)[4] and in receptor phosphorylation (2, 4).

Experimental Procedures

Materials. ^{125}I-CYP, ^{125}I-PIN, [α-^{32}P]ATP, and [^{3}H]cAMP were obtained from New England Nuclear. (−)-Alprenolol, (−)-propranolol, forskolin, ISO, dibutyryl-cAMP, IBMX, ATP, GTP, cAMP, phosphoenolpyruvate, and myokinase were purchased from Sigma. Pyruvate kinase was from Calbiochem. G418, DMEM, fetal bovine serum, fungizone, glutamine, trypsin, penicillin, PBS, streptomycin, and HEPES were obtained from GIBCO. CGP 12177 was a gift from Ciba Geigy Co.

Cells. Wild-type or mutant human β_2AR were expressed in CHW cells, as previously described (39). Cells were maintained as monolayers in 75-cm^2 flasks in DMEM, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, in an atmosphere of 95% air/5% CO_2 at 37°.

Site-directed mutagenesis. Mutant β_2AR cDNA was constructed and cloned into eukaryotic expression vectors as described (40). Plasmids were cotransfected with pSV2-neo (41) into CHW cells by calcium phosphate precipitation as described (40), and clonal cells were selected for neomycin resistance as described (39).

Eight mutant β_2AR species were constructed (see Fig. 1); four that were substituted with corresponding sequences from the human α_{2A}AR (S216–237 = I L V Y V R I Y Q I A K R R T R V P P S R R, S263–274 = G G Q N R E K R F T F V, S327–339 = T I F N H D F R R A F K K I, S-III = S216–237 + S263–274 + S327–339); two in which the serine and threonine residues of the putative PKA and βARK phosphorylation sites were substituted with glycine or alanine [S-PKA ("Mutant A" in Ref. 4) = Ala^{261} + Ala^{262} + Ala^{345} + Ala^{346}; S-βARK ("Mutant B" in Ref. 4) = Ala^{355} + Gly^{356} + Ala^{360} + Gly^{364} + Ala^{384} + Ala^{393} + Gly^{396} + Ala^{401} + Gly^{407} + Ala^{408} + Ala^{411}]; one with Cys^{341} substituted with glycine (S341); and one with residues 267–273 deleted (D267–273).

Whole-cell radioligand binding assays. Nearly confluent cells were incubated at 37° for various periods of time in DMEM containing ISO (2 μM) plus ascorbic acid (0.1 mM) and/or dibutyryl-cAMP (1 mM) plus IBMX (0.1 mM). The flasks were immediately placed on ice, and the cells were washed four times with ice-cold PBS. Radioligand binding was performed as previously described (4), with down-regulation being defined as the decrease in specific ^{125}I-PIN binding due to exposure of cells to the desensitizing agent(s). The decrease in specific binding was expressed as a percentage of the specific binding of ^{125}I-PIN to untreated cells, in order to facilitate comparisons of down-regulation abilities between mutant clones expressing different receptor numbers. Expressed as a percentage, the ability of a given receptor species, including wild-type receptor, to undergo down-regulation was independent of its expression level (data not shown). For similar reasons, receptor sequestration was defined as the percentage of ^{125}I-PIN binding not displaced by CGP 12177.

Membrane radioligand binding assays. Cells were washed three times with ice-cold PBS, scraped into 10 ml of ice-cold 5 mM Tris·HCl (pH 7.4), 2 mM EDTA, and lysed by repeated suction through polyethylene pipets. The lysate was centrifuged at 45,000 × *g* for 20 min at 4°. The pelleted membranes were resuspended in ice-cold 75 mM Tris·HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM EDTA. For the routine assessment of receptor expression levels, ~10-μg membrane protein aliquots were incubated with 300 pM ^{125}I-CYP, in a buffer containing 75 mM Tris·HCl (pH 7.4), 12.5 mM $MgCl_2$, and 2 mM EDTA, at 25° for 90 min. Specific binding was defined as the amount of ^{125}I-CYP binding inhibited by 10 μM (−)-alprenolol. For agonist competition and ^{125}I-CYP saturation binding experiments, ~1–3-μg membrane protein aliquots were incubated in 75 mM Tris·HCl, 5 mM $MgCl_2$, 0.1 mM ascorbic acid, 2 mM EDTA, with 30–75 pM ^{125}I-CYP and 0–100 μM ISO in the presence or absence of 100 μM Gpp(NH)p (agonist competition), or with 1–400 pM ^{125}I-CYP in the presence or absence of 10 μM (−)-alprenolol (^{125}I-CYP saturation), at 25° for 90–120 min. Binding reactions were terminated by filtration over Whatman GF/C glass fiber filters. Protein was measured by the method of Bradford (42), using bovine serum albumin as standard.

Adenylyl cyclase assays. Adenylyl cyclase activities were measured in the presence of water (basal), 100 μM ISO, or 100 μM forskolin, by the method of Salomon *et al.* (43), in a buffer consisting of 30 mM Tris·HCl (pH 7.4), 2 mM $MgCl_2$, and 0.8 mM EDTA. Assay mixtures contained ~ 10μg of membrane protein, 0.012 mM ATP, 0.1 mM cAMP, 0.053 mM GTP, 2.7 mM phosphoenolpyruvate, 0.2 units of pyruvate kinase, 1 unit of myokinase, and ~1 μCi of [α-^{32}P]ATP, in a final volume of 50 μl. Reactions were initiated by the addition of membranes, incubated for 20 min at 37°, and terminated by the addition of 1 ml of an ice-cold solution of 0.4 mM ATP, 0.3 mM cAMP, and [^{3}H]cAMP (~0.01 μCi). [^{32}P]cAMP was determined following sequential chromatography over Dowex AG 50W-X4 and aluminum oxide.

Data analyses. The dissociation constant (K_d) for binding of ^{125}I-CYP was determined by weighted, nonlinear, least-squares regression analysis of saturation binding curves, using the computer program

[4] M. Hnatowich, S. B. Liggett, B. F. O'Dowd, M. G. Caron, and R. J. Lefkowitz. Manuscript in preparation.

DNRP53 (44). A nonlinear least-squares regression curve-fitting procedure described by Kent *et al.* (45) and DeLean *et al.* (46) was used to analyze data from agonist competition binding assays. Statistical analyses comparing "goodness-of-fit" between one- and two-affinity state models were used to determine the appropriate model for each curve. Binding parameters for a two-site model are shown only when this constitutes a significant (i.e., $p < 0.01$) improvement over a one-site model. Statistical comparisons were by paired or unpaired two-tailed Student's t test. A value of $p < 0.05$ was considered to be statistically significant. The Bonferroni method was used to adjust for multiple comparisons when appropriate.

Results

Mutant β_2AR. To evaluate the role of β_2AR-G_s coupling and receptor phosphorylation in the processes of sequestration and down-regulation, eight mutant forms of the receptor were constructed and stably transfected into CHW cells (Fig. 1). To assess the integrity of β_2AR-G_s coupling, we determined both receptor-mediated adenylyl cyclase activity and agonist competition binding parameters for the various receptors. Both methods were used because it has recently been proposed that agonist binding characteristics reflect the state of "physical" coupling between the receptor and its G protein, whereas agonist-stimulated adenylyl cyclase activity appears to correspond to "functional" coupling or activation of G_s mediated by the receptor, occurring downstream of the "physical" coupling process (40, 47). In confirmation of previous results (40, 48)[4] D267–273, S263–274, S341, and S-III showed impaired functional activation of G_s (Table 1, Fig. 2). In contrast, a different subset of mutant receptors exhibited abnormal agonist-binding characteristics (Table 2). Compared with β_2AR, S216–237 formed a lower percentage of receptors in the high affinity agonist-binding state (Table 2), S263–274 (Table 2) and S341 (48) exhibited a single, low affinity binding state for ISO, and S-III (Table 2) showed substantially higher affinity for ISO in its low affinity agonist-binding conformation. Examination of the data in Tables 1 and 2 clearly shows that differences in receptor expression levels (i.e., B_{max}) cannot account for these impairments in receptor-G_s coupling.

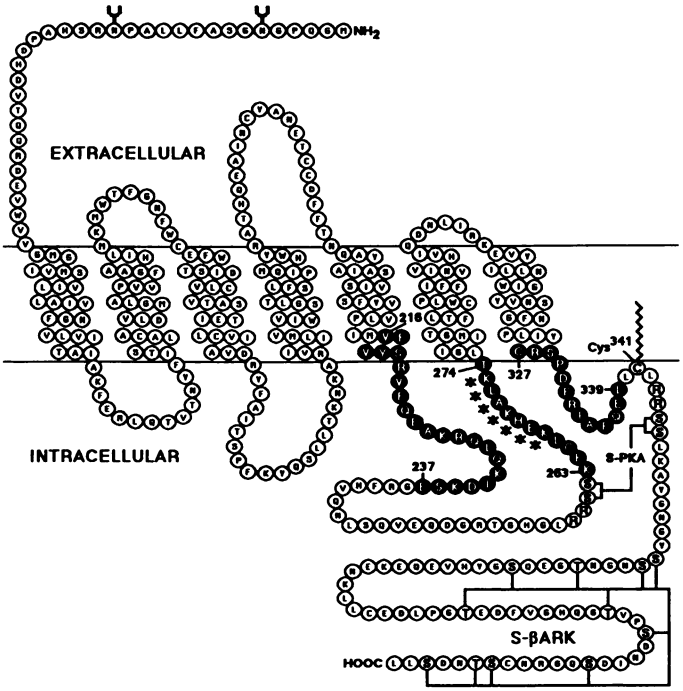

Fig. 1. Schematic representation illustrating the sites of mutation in various β_2AR constructs. *Black circles*, amino acid residues that were substituted with the corresponding residues from the human α_{2A}AR (i.e., S216–237, S263–274, and S327–339, plus S-III, which comprises the first three); *asterisks*, residues that were deleted (D267–273); Cys^{341}, substitution with glycine (S341); *S-βARK*, putative βARK phosphorylation site substitutions (glycine or alanine; see Experimental Procedures); *S-PKA*, putative PKA phosphorylation site substitutions (alanine).

Role of G_s coupling in receptor sequestration. The role of G_s coupling in the process of receptor sequestration is controversial (31–33, 40). We, therefore, determined the rate and extent of agonist-induced sequestration of wild-type and of five mutant β_2AR, which range from normally coupled to almost totally uncoupled (Fig. 2, Table 2). Following exposure of cells to 2 μM ISO, a rapid sequestration of ~30% of the receptors was observed, which was maximal within 15 min (Fig. 3). This level of sequestration was essentially maintained throughout the 24-hr time course of the experiment. All mutant receptors exhibited receptor sequestration patterns equivalent to that of β_2AR (Fig. 3).

Role of G_s coupling in receptor down-regulation. To evaluate the role of β_2AR-G_s coupling in the process of receptor down-regulation, cells expressing wild-type or mutant β_2AR were treated with 2 μM ISO for 1–24 hr, and total cellular receptor binding was determined. In cells expressing β_2AR, ^{125}I-PIN binding was markedly reduced, in a time-dependent fashion, with ~50% lost after exposure to ISO for 6 hr. Ultimately, ~80% of the β_2AR down-regulated following exposure to agonist for 24 hr (Fig. 4). Three mutant receptors (i.e., S263–274, S341, and S-III) with marked impairment in both their physical and their functional abilities to couple to G_s (44) (Fig. 2, Tables 1 and 2) each exhibited a dramatically delayed onset and reduced extent of down-regulation (Fig. 4). Although S263–274 and S-III exhibited an apparent initial increase in receptor binding over the first 3 hr of exposure to ISO, this was not a consistent finding and was statistically insignificant. S216–237, which exhibited an impaired ability to bind agonist (Table 2), showed a delayed and blunted down-regulation pattern (Fig. 4). In contrast, S327–339 and D267–273, both of which had normal agonist-binding characteristics, showed down-regulation patterns indistinguishable from that of β_2AR (Fig. 4).

It is important to reiterate that D267–273 down-regulates normally, despite the marked impairment in its ability to mediate adenylyl cyclase stimulation, suggesting that no simple correlation exists between functional activation of G_s and down-regulation. However, if cAMP production is required for agonist-induced down-regulation, then sufficient levels must have been generated via D267–273 to support this process. Conversely, the cAMP levels generated via the more severely uncoupled mutant receptors may not have been sufficient to allow down-regulation to proceed. To test this hypothesis, we treated cells expressing β_2AR, S263–274, or S-III with 2 μM ISO plus 1 mM dibutyryl-cAMP plus 0.1 mM IBMX, for 1–24 hr, and assessed receptor down-regulation. The addition of dibutyryl-cAMP and IBMX had no statistically significant effect on the pattern of agonist-induced down-regulation for either the wild-type or mutant receptors (Fig. 5).

Role of phosphorylation in receptor sequestration and down-regulation. To assess the role of receptor phosphorylation in the processes of receptor sequestration and down-

TABLE 1

Summary of adenylyl cyclase activation parameters and receptor expression levels for wild-type and mutant β_2AR

Data obtained with cells expressing different levels of β_2AR are shown in the first two rows. Data are mean ± standard error of the number of independent experiments (*n*) shown. Adenylyl cyclase activity and ^{125}I-CYP binding units refer to mg of membrane protein. Details are as described in Experimental Procedures.

Receptor	*n*	Adenylyl cyclase activity			^{125}I-CYP binding
		Basal	100 μM ISO	100 μM Forskolin	
		pmol of cAMP/(min × mg)			*fmol/mg*
β_2AR	8	4.36 ± 1.52	24.1 ± 2.7	54.2 ± 6.6	719 ± 86
β_2AR	3	4.17 ± 0.69	21.3 ± 5.4	42.6 ± 8.9	1882 ± 512
S327–339	5	3.30 ± 0.57	24.5 ± 3.8	56.3 ± 9.7	765 ± 98
S216–237	5	3.62 ± 0.71	22.2 ± 6.2	52.5 ± 13.1	1431 ± 198
S-PKA	3	2.79 ± 0.87	25.3 ± 1.5	46.0 ± 9.8	1138 ± 180
S-βARK	4	4.19 ± 1.46	23.0 ± 4.0	67.9 ± 12.2	1059 ± 235
D267–273	3	5.75 ± 0.85	16.2 ± 3.9	56.0 ± 15.4	706 ± 130
S263–274	4	3.41 ± 0.68	6.74 ± 1.33	63.1 ± 11.0	476 ± 279
S341	3	2.25 ± 0.46	4.29 ± 0.98	40.2 ± 9.8	932 ± 335
S-III	5	2.34 ± 0.53	4.98 ± 0.68	55.3 ± 10.3	2301 ± 397

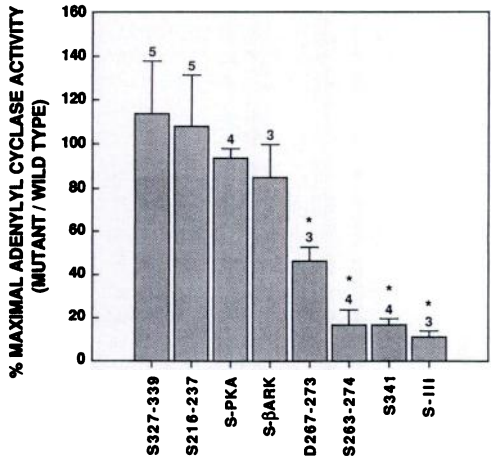

Fig. 2. ISO-stimulated adenylyl cyclase activity in cells expressing wild-type and mutant β_2AR. Maximal ISO-stimulated adenylyl cyclase activity was measured as described in Experimental Procedures and is expressed as a percentage of that for β_2AR. Basal and forskolin-stimulated activities were not significantly different between cells expressing wild-type or mutant receptors (see Table 1). Data are mean ± standard error of the number of independent experiments indicated above the *error bars*. *, ISO-stimulated activities that are significantly different from β_2AR ($p < 0.05$).

regulation, we examined two receptor species in which putative PKA and βARK phosphorylation sites had been destroyed, S-PKA and S-βARK, respectively. As compared with β_2AR, both mutants exhibited normal receptor sequestration (2, 4) and coupling abilities (Table 1, Fig. 2). With S-PKA, the agonist-induced down-regulation over the first 6 hr was indistinguishable from that in cells expressing β_2AR. Beyond this time, however, the rate of down-regulation of this mutant was significantly blunted, with ~40% of receptor binding still being detectable by 24 hr (Fig. 4). In contrast, S-βARK had a down-regulation pattern equivalent to that of the wild-type receptor (Fig. 4).

Discussion

In this study, we demonstrate that agonist exposure of cells expressing either wild-type or severely uncoupled mutant β_2AR produces an equivalent degree of receptor sequestration, suggesting that sequestration is independent of receptor-G_s coupling. In contrast, the abilities of several mutant forms of the β_2AR to undergo agonist-induced down-regulation correlate

TABLE 2

Agonist-binding parameters for wild-type and mutant β_2AR

Radioligand binding was performed as described in Experimental Procedures. Data are mean ± standard error of three or four independent experiments. K_{iH} and K_{iL}, high and low affinity inhibition constants for agonist binding, respectively; R_H, proportion of agonist binding in the high affinity state; K_i, inhibition constant for agonist binding in the presence of 100 μM Gpp(NH)p. Receptor expression levels for β_2AR, S216–237, S327–339, S263–274, and S-III were 1013 ± 116, 1431 ± 198, 765 ± 98, 298 ± 29, and 2007 ± 343 fmol/mg of membrane protein, respectively. K_d values for ^{125}I-CYP binding to both wild-type and mutant β_2AR were ~40 pM. Binding parameters for mutant receptors not listed were previously published, as described in Results.

Receptor	ISO competition binding			
	K_{iH}	K_{iL}	R_H	+Gpp(NH)p K_i
	nM	*nM*	%	*nM*
β_2AR	3.2 ± 1.3	215 ± 28	38 ± 2	245 ± 45
S216–237	1.2 ± 0.2	183 ± 17	27 ± 2[a]	217 ± 68
S327–339	3.8 ± 1.3	220 ± 50	40 ± 4	226 ± 27
S263–274	0[a]	158 ± 16	0[a]	188 ± 23
S-III	2.3 ± 1.2	53 ± 4[a]	36 ± 9	43 ± 2[a]

[a] Binding parameters for mutant receptors that are significantly different from those for β_2AR ($p < 0.05$).

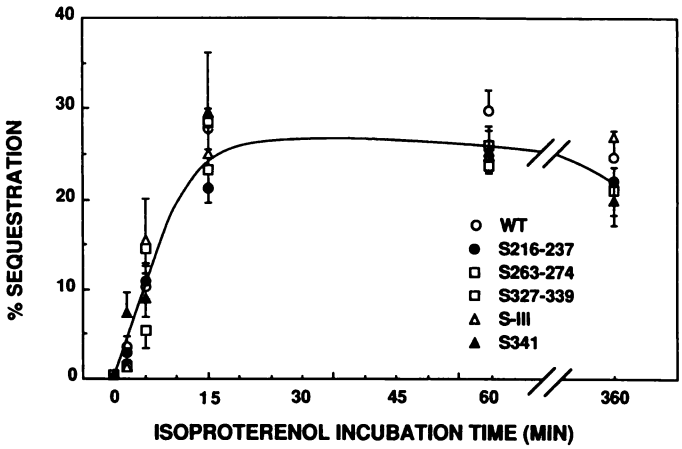

Fig. 3. ISO-induced sequestration of wild-type (*WT*) and mutant β_2AR. Cells were incubated with 2 μM ISO for the indicated times, and the percentage of sequestered receptors was assessed in a whole-cell radioligand binding assay (see Experimental Procedures). Data are mean ± standard error from three to eight independent experiments. Mutant receptor sequestration was statistically indistinguishable from that of β_2AR at each time point measured.

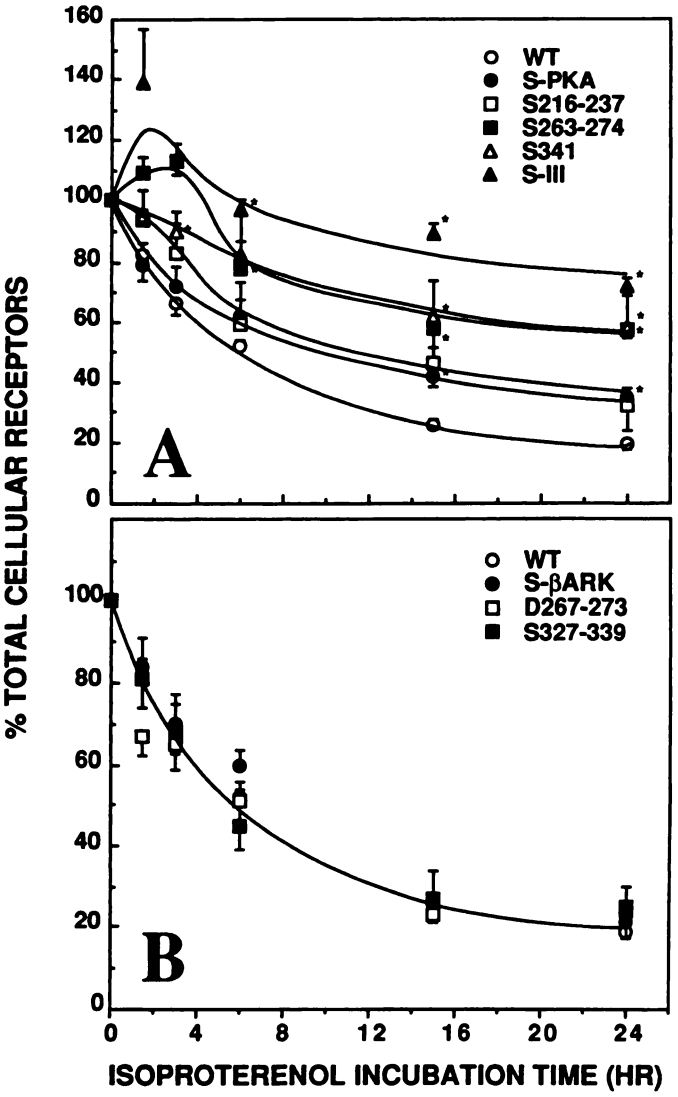

Fig. 4. ISO-induced down-regulation of wild-type (*WT*) and mutant β_2AR. Cells were incubated with 2 μM ISO for the indicated times, and the percentage of down-regulated receptors was assessed in a whole-cell radioligand binding assay (see Experimental Procedures). Data are mean ± standard error from three to 10 independent experiments. *, Significant difference between β_2AR and the particular mutant receptor at the indicated time ($p < 0.05$).

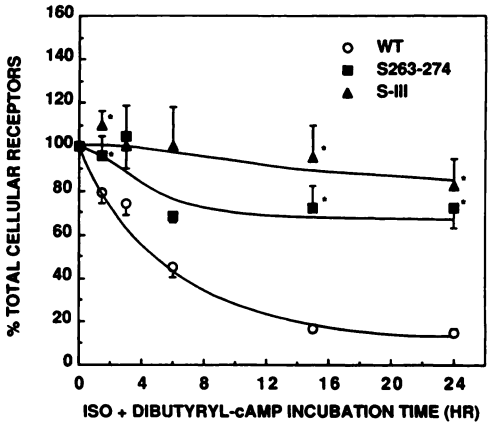

Fig. 5. ISO- plus dibutyryl-cAMP-induced down-regulation of wild-type (*WT*) and mutant β_2AR. Cells were incubated with 2 μM ISO plus 1 mM dibutyryl-cAMP plus 0.1 mM IBMX for the indicated times, and the percentage of down-regulated receptors was assessed in a whole-cell radioligand binding assay (see Experimental Procedures). Data are mean ± standard error from three independent experiments. *, Significant difference between β_2AR and the particular mutant receptor at the indicated time ($p < 0.05$).

with their respective capacities to couple to G_s, as assessed by alterations in their agonist-binding parameters. These results suggest that the phenomena of sequestration and down-regulation are governed by distinct molecular mechanisms and are likely mediated by different aspects of the receptor molecule. In this regard, our findings are consistent with conclusions derived from earlier studies with mutant S49 and HC-1 cell lines that either lack or express defective components of this signal transduction pathway other than the receptor (33–36).

A consistent finding in this study is that the two most commonly accepted indicators of the integrity of receptor-G_s coupling (i.e., agonist binding and receptor-mediated adenylyl cyclase stimulation) differ in their abilities to serve as predictors of the down-regulation abilities of the various mutant receptors we have examined. Several lines of evidence suggest that the extent of adenylyl cyclase activation is not closely associated with the capacities of the various receptors to undergo down-regulation, whereas the latter does appear to correlate with their agonist-binding characteristics. First, only those mutant receptors with altered agonist-binding parameters (S216–237, S263–274, S341, and S-III) consistently exhibited impaired down-regulation. Second, a mutant β_2AR (D267–273) with a substantially impaired ability to mediate ISO-stimulated adenylyl cyclase activity, but with normal agonist-binding characteristics, exhibited the β_2AR pattern of down-regulation. Third, no improvement in the abnormal down-regulation patterns was observed when the more severely uncoupled mutants (S263–274 and S-III) were exposed to a combination of ISO, dibutyryl-cAMP, and IBMX, suggesting that the impairment of down-regulation seen with these mutants is not secondary to a deficiency in the ability of the receptors to mediate the production of cAMP. The findings described above indicate that stimulated levels of cAMP are not involved in the ISO-induced down-regulation process, at least over the first 6 hr of agonist exposure. These results are also consistent with those previously observed with the S49 mutant H21a and the HC-1 hepatoma cell lines. Both cell lines are unable to mediate G_s stimulation of adenylyl cyclase, either because the interaction of G_s with adenylyl cyclase is impaired (H21a) or because the adenylyl cyclase moiety itself is absent (HC-1). Nevertheless, βAR in both cell lines down-regulate normally (33, 36, 37).

It is not yet clear why the ultimate capacity of the receptor to physically couple to G_s correlates with the down-regulation properties of the receptor. There are, however, at least three possible interpretations. First, molecular determinants of the receptor required for physical coupling [e.g., third intracellular loop plus proximal carboxyl tail (25, 28–30)] may happen to coincide with those involved in down-regulation. Second, the physically coupled β_2AR-G_s ternary complex may be a substrate for the down-regulation pathway. In this regard, it may be relevant that G_s has recently been reported to bind with high affinity to tubulin (49). Alternatively, formation of the ternary complex may trigger down-regulation through a non-cAMP second messenger system (37) not measured here.

A role for β_2AR phosphorylation by PKA in the process of

agonist-induced down-regulation is also indicated by our data. In cells expressing S-PKA, down-regulation following prolonged exposure to agonist was significantly blunted in extent, despite normal sequestration and down-regulation at early times (i.e., <6 hr). Similar findings were obtained with two other mutant receptors (T-365 in Ref. 2 and "Mutant AB" in Ref. 4) that do not undergo PKA phosphorylation.[5] This blunting is similar to what has previously been shown for dibutyryl-cAMP-induced down-regulation of this mutant β_2AR (15). One possible explanation is that receptor phosphorylation by PKA increases the extent of down-regulation by enhancing the susceptibility of the receptors to degradation. This would be analogous to what has been observed in photoreceptor signal transduction systems, where phosphorylation of rhodopsin has been proposed to induce conformational changes that promote the action of several proteases (50, 51). In contrast, we found no alteration in the pattern of receptor down-regulation in cells expressing S-βARK, suggesting that this process is probably not dependent on βARK phosphorylation.

Two recent studies using mutant β_2AR have postulated the existence of a causal relationship between receptor-G protein coupling and sequestration, and have further suggested that regions of the β_2AR responsible for functional coupling with G_s are identical to those that mediate receptor sequestration (31, 32). The differences in the conclusions reached in the aforementioned studies and in the present work may reflect the types of mutations used in each. For example, large deletions within the third cytoplasmic loop and extensive truncations of the carboxyl tail of β_2AR (31, 32) may not only uncouple the receptor from G_s but may also radically alter the tertiary conformation of the receptor, so as to nonspecifically interfere with the sequestration process. In the mutants we describe, substitution of smaller portions of the third intracellular loop with corresponding sequences from the human α_{2A}AR (e.g., S263–274) markedly impairs receptor-G_s coupling but still evidently maintains the structural requirements for receptor sequestration. Similarly, a recent study has shown that substitution of tyrosines 350 and 354 in the cytoplasmic tail of β_2AR produces a marked impairment of the ability of the receptor to functionally and physically couple to G_s but has no detectable effect on agonist-induced sequestration (38). A second difference lies in the way receptor sequestration and down-regulation are defined in the various studies. Reliance solely on measurements of [^{3}H]CGP 12177 binding to assess changes in receptor distribution (31, 32) makes it difficult to distinguish between loss of total cellular β_2AR binding versus loss of cell surface receptor binding only. This is because a decrease in cell surface receptor binding due to either sequestration or down-regulation results in a loss of total [^{3}H]CGP 12177 binding. In contrast, we (2, 4) and others (9, 33, 38) have discriminated between the two processes by simultaneously comparing the whole-cell binding of the hydrophilic ligand CGP 12177 (or its radiolabeled derivative) with that of hydrophobic radioligands (e.g., ^{125}I-PIN).

We present several lines of evidence to support the hypothesis that coupling of the β_2AR to G_s is related to the process of agonist-induced receptor down-regulation. The apparently fundamental role of G proteins in this process may be analogous to that recently determined for intracellular vesicular transport, where it has been shown that "low molecular weight" G proteins (i.e., M_r ~20,000) are essential components in the transport of protein between secretory compartments (52). Further studies of down-regulation will need to integrate the contributions of G protein coupling with the previously demonstrated or postulated involvement of agonist-induced alterations in βAR gene transcription rate, mRNA stability, protein synthesis, and receptor degradation (15–18). On the other hand, only agonist occupancy is clearly required for receptor sequestration; neither receptor phosphorylation by PKA or βARK nor coupling to G_s appear to be involved. A full understanding of this process will require more specific receptor mutants that selectively alter sequestration.

Note added in proof: More direct evidence for a distinction between physical and functional coupling of receptors to G-proteins was recently reported (Franke, R. R., König, B., Sakmar, T. P., Khorana, H. G., and Hofman, K. P. (1990) Science *250* 123–125.

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