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Agonist-Promoted Sequestration of the β_2-Adrenergic Receptor Requires Regions Involved in Functional Coupling with G_s

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SUMMARY

The molecular basis for the desensitization of β_2-adrenergic receptors was investigated by oligonucleotide-directed mutagenesis. β-Adenergic receptor mutants containing deletions within the sixth hydrophilic domain that failed to couple to G_s and stimulate adenylyl cyclase did not undergo agonist-mediated sequestration. In contrast, all receptor mutants that displayed G_s coupling were sequestered away from the cell surface in response to isoproterenol. Progressive truncation of the C-terminus of the receptor resulted in decreases in the initial rates of receptor sequestration and functional uncoupling, although the final extent of these desensitization processes was not affected by the mutations. These data suggest that structural features of the β_2-adrenergic receptor that are involved in receptor activation are also essential for mediating the subsequent inactivation caused by the sequestration of the receptor from the cell surface.

The β_2AR is a member of a family of receptors that mediate their intracellular actions by activation of G proteins. The cloning of the genes for several of the G protein-linked receptors has revealed a high degree of sequence homology among these proteins, suggesting a structural basis for their similar mechanisms of action (1–9). By analogy with the structural model proposed for rhodopsin (10), which activates the G protein transducin, all of these receptors are postulated to consist of seven transmembrane hydrophobic helices connected by alternating extracellular and intracellular hydrophilic loops. Upon binding agonists, these receptors interact with specific G proteins, promoting the exchange of GTP for bound GDP on the G protein and leading to defined intracellular effector functions. Continued exposure of cells to agonists then leads to a loss of functional activity, corresponding to a desensitization of the receptors.

Both the activation and inactivation responses of the β_2AR, which interacts with the G protein G_s to stimulate the adenylyl cyclase effector system, have been extensively investigated. Some insight into the structural features involved in the mechanism of action of the β_2AR has arisen from genetic analysis of the receptor protein, expressed in mammalian cells. The ligand-binding domain of the β_2AR involves residues within the hydrophobic core of the protein that are highly conserved among the G protein-linked receptors (11-14). In contrast, deletion mutagenesis analysis suggests that the interaction between the β_2AR and the G_s/adenylyl cyclase system involves residues within the sixth hydrophilic domain of the receptor, a putatively intracellular region that is not highly conserved within the G protein-linked receptor family (11, 15).

The desensitization of the β_2AR is characterized by a loss of receptor function resulting from prolonged exposure to agonists. This phenomenon arises from two related processes, 1) a sequestration of receptors away from the cell surface, making them inaccessible to exogenous agonists, and 2) a loss of functional coupling between the receptor and the G_s/adenylyl cyclase effector system (16). The observation that β_2AR desensitization correlates with receptor phosphorylation in some systems led to the hypothesis that both of these processes are mediated by the phosphorylation of the serine/threonine-rich C-terminus of the β_2AR by a receptor-specific kinase (17–19), in a manner analogous to the phosphorylation of the C-terminus of the light-activated form of rhodopsin by rhodopsin kinase (10). However, previous mutagenesis studies have suggested that the C-terminal region of the β_2AR is not required for receptor sequestration or uncoupling to occur (20, 21). In contrast, correlations between G_s coupling and agonist-promoted sequestration have been noted, (20, 22, 23), prompting the suggestion that a single region of the β_2AR may be involved in both receptor activation and inactivation.

In the present study, we present a detailed genetic analysis of the structural requirements for G_s coupling and receptor desensitization. Progressive truncation of the C-terminus of the β_2AR attenuated the initial rate but not the final level of receptor sequestration and uncoupling. In contrast, deletions within the third intracellular loop, which abolished G_s coupling,

ABBREVIATIONS: β_2AR, β_2-adrenergic receptor; ^{125}I-CYP, ^{125}I-cyanopindolol; G protein, GTP-binding protein; Gpp(NH)p, 5′-guanylyl-imidophosphate; PBS, phosphate-buffered saline.

also abolished the sequestration response to agonists. These data provide evidence for a correlation between structural features of the β_2AR involved in receptor activation and those involved in receptor sequestration and give further support to our hypothesis that the C-terminus of the β_2AR is not required for desensitization to occur.

Materials and Methods

Expression and mutagenesis. The nucleotide sequence and expression vector for the hamster β_2AR gene have been published (2, 11). Oligonucleotide-directed mutagenesis was used to introduce nucleotide substitutions into the gene, as previously described (11). The identities of the mutant plasmids were confirmed by dideoxy sequencing (24), and purity was determined by retransformation of each mutant into *Escherichia coli* and hybridization at high stringency with the oligonucleotide used to create the mutation. Each of the mutant receptors used in the present study was determined to be at least 99% pure (i.e., 100 out of 100 colonies examined contained the mutation).

Transfections of L cells were performed as previously described (11). L cells were grown in monolayer culture in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in a 5% CO_2 atmosphere at 37° and were used at confluence for the experiments described here.

Membrane preparation and receptor assays. L cell membranes were prepared by hypotonic lysis and resuspended in 75 mM Tris, 12.5 mM $MgCl_2$, 1.5 mM EDTA, pH 7.5 (TME buffer) as previously described (11). For ^{125}I-CYP binding, 20 μg of membrane protein was suspended in 0.25 ml of TME buffer containing 10–400 pM ^{125}I-CYP (New England Nuclear, Boston, MA) for 90 min at 23° before bound radioactivity was separated on GF/C filters. Nonspecific binding, representing <5% of the total, was determined in the presence of 10 μM alprenolol. Data were analyzed by nonlinear regression. The binding of the agonist isoproterenol was determined in 0.25 ml of TME buffer, containing 5–10 pM β_2AR, 35 pM ^{125}I-CYP, and increasing concentrations of isoproterenol in either the absence or the presence of 100 μM Gpp(NH)p for 90 min at 23° before bound radioactivity was separated with GF/C filters. The data were analyzed using the iterative program LIGAND (25).

Adenylyl cyclase assays were performed by the method of Salomon *et al.* (26), exactly as previously described (11). Isoproterenol stimulation curves were analyzed by nonlinear regression.

Receptor sequestration and desensitization. For sequestration experiments, L cells grown in monolayer culture in 24-well plates were incubated in their growth medium with (−)-isoproterenol under the conditions outlined in the legend to Fig. 2 (20). At the end of the incubation time, the plates were transferred to an ice bath to stop the reaction and washed three times with ice-cold PBS. The binding of [^{3}H]-CGP 12177 (Amersham, Arlington Heights, IL) was measured directly on the monolayer with 20 nM[^{3}H]-CGP in a total volume of 0.25 ml of PBS containing 0.1% bovine serum albumin for 4 hr at 4°. The monolayers were then washed three times with cold PBS and removed from the plate in 1% sodium dodecyl sulfate, and the radioactivity was measured in a liquid scintillation counter. Nonspecific binding was assessed in the presence of 10 μM alprenolol and represented <10% of total bound radioactivity.

For desensitization experiments, L cells growing in monolayer culture were incubated with (−)-isoproterenol for the times indicated. At the end of the incubation, the cells were washed three times with ice-cold PBS, then membranes were prepared and the adenylyl cyclase activation and ^{125}I-CYP binding were measured as described above.

Results

We have previously shown that a mutant β_2AR having a large deletion (residues 239–272) from the putative third intracellular loop failed to couple to G_s to stimulate adenylyl cyclase activity and exhibited no agonist-promoted receptor sequestration (11, 15, 20). In contrast, these responses were unaffected by the deletion of the C-terminus of the protein. To analyze the structural features of the β_2AR required for activation and desensitization, other deletion mutations were made within these hydrophilic internal domains of the receptor and the G_s coupling and desensitization responses of these mutant β_2ARs were assessed.

G_s coupling of mutant β_2AR. The coupling of the β_2AR to G_s can be demonstrated by the ability of GTP or its nonhydrolyzable analog Gpp(NH)p to decrease the affinity of the receptor for agonists. As shown in Table 1, the agonist affinities of three mutant receptors having deletions within the third intracellular loop were insensitive to Gpp(NH)p, reflecting altered coupling to G_s. In the absence of Gpp(NH)p, D(230–262)βAR, containing a large deletion from the middle of this loop, bound isoproterenol with low affinity, similar to that seen for the uncoupled form of the wild-type receptor in the presence of Gpp(NH)p (Table 1). The affinity of this mutant receptor was not further decreased upon addition of Gpp(NH)p, suggesting that it was not strongly coupled to G_s. D(222–229)βAR, previously identified as not activating G_s, and D(258–270)βAR, which couples poorly with G_s, each also displayed a single GTP-insensitive agonist binding site, but the affinity of this site for isoproterenol was higher than that determined for the uncoupled form of the wild-type β_2AR. In contrast, mutant receptors having truncated C-termini exhibited normal coupling to G_s, as evidenced by their affinities for isoproterenol in the presence and absence of Gpp(NH)p (Table 1). A mutant receptor combining the largest deletion of the third intracellular loop with a long truncation at the C-terminus [D(230–262)T(345)βAR] ressembled D(230–262)βAR in that it exhibited only low affinity binding of isoproterenol both in the absence and presence of Gpp(NH)p (Table 1).

Coupling of the mutant β_2ARs to G_s was also investigated by examining the ability of isoproterenol to stimulate adenylyl cyclase in membranes prepared from cells expressing these mutant receptors. Truncation of the C-terminus of the receptor at different positions did not affect its ability to stimulate adenylyl cyclase (Fig. 1) (11, 20). In contrast, D(230–262)βAR and D(230–262)T(345)βAR, which bound isoproterenol with low affinity, stimulated adenylyl cyclase activity with correspondingly higher K_{act} values. The isoproterenol-mediated stimulation by these two mutant receptors was only approximately 50% the level of the maximal response measured with NaF, reminiscent of the action of partial agonists on the wild-type receptor. As previously reported (15), D(258–270)βAR also showed attenuated adenylyl cyclase activity, with the maximal stimulation by isoproterenol reaching only 40% that seen with NaF, but with a normal K_{act}, reflecting the normal affinity of this mutant receptor for isoproterenol. D(222–229)βAR failed to mediate any detectable isoproterenol-stimulated adenylyl cyclase activity (Fig. 1) (15).

Sequestration of mutant β_2AR. The ability of these mutant β_2ARs to undergo agonist-mediated sequestration away from the cell surface was measured after treatment with isoproterenol, using the hydrophilic antagonist [^{3}H]-CGP 12177, which does not penetrate the surface of intact cells (27). As shown in Fig. 2A, treatment of cells expressing the wild-type β_2AR with isoproterenol resulted in a rapid loss of 70% of the cell-surface receptors, with a half-time of receptor loss of <5 min. D(258–270)βAR was also rapidly lost from the cell surface

TABLE 1

Characteristics of mutant β_2AR

Ligand binding properties and adenylyl cyclase stimulation by β_2AR mutants were measured and the data analyzed as described in Materials and Methods. K_1 and K_2 for isoproterenol binding indicate the K_d values for the first and second affinity states, respectively. The data are presented for two classes of affinity states only in cases in which the binding data are significantly better described by a two-site fit than by a one-site fit, according to the program LIGAND ($p < 0.02$). Only one affinity site was observed in the presence of Gpp(NH)p for wild-type or mutant β_2AR. Adenylyl cyclase stimulation was measured as described in Materials and Methods and the data were analyzed by nonlinear regression. The data for mutant β_2AR marked with an *asterisk* have been presented previously (15) and are presented here for comparison.

	^{125}I-CYP B_{max}	K_d (isoproterenol) −Gpp(NH)p K_1	K_d (isoproterenol) −Gpp(NH)p K_2	K_d (isoproterenol) +Gpp(NH)p	Adenylyl cyclase K_{act}
	fmol/mg		M		
Wild Type*	100	$3 \pm 2 \times 10^{-9}$	$2 \pm 2 \times 10^{-7}$	$2 \pm 2 \times 10^{-7}$	$2 \pm 0.4 \times 10^{-8}$
D(258–270) βAR*	200	$2 \pm 1 \times 10^{-8}$		$2 \pm 1 \times 10^{-8}$	$3 \pm 2 \times 10^{-9}$
D(230–262) βAR	300		$4 \pm 3 \times 10^{-7}$	$5 \pm 1 \times 10^{-7}$	$8 \pm 3 \times 10^{-7}$
D(222–229) βAR*	150	$5 \pm 0.5 \times 10^{-9}$		$6 \pm 1 \times 10^{-9}$	
T(376) βAR*	350	$7 \pm 1 \times 10^{-9}$	$3 \pm 1 \times 10^{-7}$	$3 \pm 2 \times 10^{-7}$	$8 \pm 2 \times 10^{-9}$
T(354) βAR*	240	$2 \pm 1 \times 10^{-9}$	$2 \pm 1 \times 10^{-7}$	$2 \pm 0.1 \times 10^{-7}$	$1 \pm 0.2 \times 10^{-8}$
T(345) βAR	55	$2 \pm 1 \times 10^{-9}$	$6 \pm 0.1 \times 10^{-7}$	$7 \pm 3 \times 10^{-8}$	$7 \pm 0.3 \times 10^{-8}$
D(230–262)T(345) βAR	210		$2 \pm 0.1 \times 10^{-7}$	$8 \pm 2 \times 10^{-7}$	$1 \pm 0.1 \times 10^{-5}$

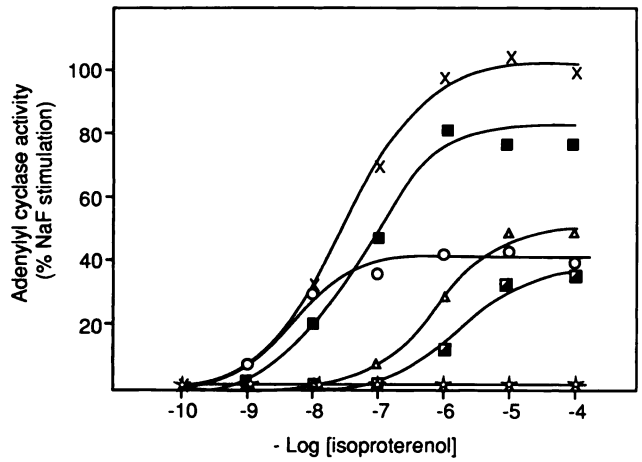

Fig. 1. Adenylyl cyclase stimulation by wild-type and mutant β_2AR. Adenylyl cyclase stimulation was measured in the presence of increasing concentrations of isoproterenol as described in Materials and Methods and is expressed as percentage of the maximal stimulation by 10 mM NaF. For the experiment shown, the adenylyl cyclase activity for the various mutant receptors expressed as basal/10^{-5} M isoproterenol-stimulated/10 mM NaF-stimulated, in pmol of [^{32}P]cAMP/mg of protein/min, was as follows: wild-type β_2AR, 6/39/37 (×); T(345)βAR, 10/46/54 (■); D(258–270)βAR, 16/27/42 (○); D(230–262)βAR, 8/39/70 (△); D(230–262)T(345)βAR, 4/12/28 (◪); and D(222–229)βAR, 9/9/102 (☆). The data shown are representative of 2 to 5 separate experiments.

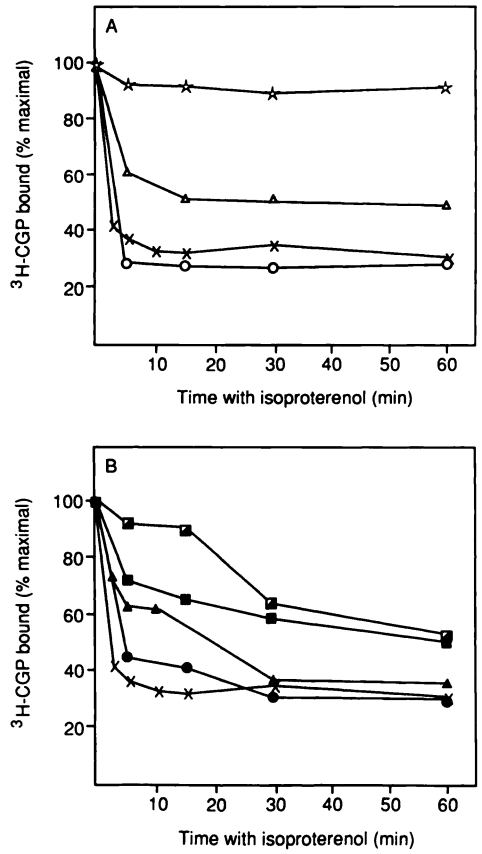

Fig. 2. Sequestration of cell-surface wild-type and mutant β_2AR. Monolayers of L cells expressing wild-type β_2AR (×), D(222–229)βAR (☆), D(230–262)βAR (△), D(258–270)βAR (○), T(376)βAR (●), T(354)βAR (▲), T(345)βAR (■), or D(230–262)T(345)βAR (◪) were incubated at 37° in the presence of 10 μM isoproterenol for the times indicated along the *x* axis and washed with PBS and [^{3}H]CGP binding to the cell-surface receptors was determined at 4° as described in Materials and Methods. The data shown are the means of 2 to 5 separate experiments, with each determination done in duplicate.

upon exposure to isoproterenol, with a final level of sequestration of approximately 70% of the receptors (Fig. 2A). Within the time resolution of these experiments, agonist-promoted sequestration of this mutant receptor appeared to proceed at least as rapidly as that of the wild-type β_2AR. Exposure of cells expressing the mutant D(230–262)βAR to isoproterenol also resulted in sequestration of receptors from the surface of the cell, although the response was attenuated, with only 50% of the surface receptors sequestered during the first 60 min. In contrast, D(222–229)βAR was not sequestered away from the cell surface in response to agonists. This mutant β_2AR showed only a slight (5–10%) decrease in cell surface receptor number after treatment with 10 μM isoproterenol (Fig. 2A), even at times up to 5 hr (data not shown).

We have previously shown that β_2AR mutants with the C-terminus truncated at positions 395 or 354 showed normal sequestration responses to agonist (20). In the present study, a series of C-terminal truncations was made to further examine the structural requirements of this region of the receptor in

mediating sequestration. Treatment of cells expressing each of these mutant β_2ARs with isoproterenol resulted in loss of cell surface receptors, as measured with [^{3}H]-CGP (Fig. 2B). Interestingly, as longer regions were truncated from the C-terminus, the initial rate of the sequestration process became significantly slower than that of the wild-type protein. Thus, T(395)βAR (20) and T(376)βAR (Fig. 2B) showed rates of sequestration not significantly different from that of the wild-type β_2AR at the resolution of the present experiments. In contrast, the sequestration of T(354)βAR was measurably slower during the first 10 min of exposure to agonist, whereas T(345)βAR was sequestered still more slowly, with differences from the wild-type β_2AR apparent throughout the first 60 min of agonist treatment (Fig. 2B). The final level of total surface T(345)βAR observable after a 5-hr exposure to isoproterenol was indistinguishable from that seen for the wild-type β_2AR, with only 30–40% of the receptors remaining on the cell surface (data not shown).

Mutant D(230–262)T(345)βAR, in which a large truncation of the C-terminal tail was combined with a large deletion from the third intracellular loop, was sequestered upon agonist exposure, with characteristics reflecting contributions from both deletions. The sequestration of this mutant receptor was markedly slower even than that observed for T(345)βAR. As with all of the mutant receptors, the extent of sequestration reached 70% after exposure to isoproterenol for 5 hr (data not shown).

Functional desensitization of receptors. To investigate the functional consequences of exposure of mutant β_2ARs to agonists, membranes were prepared from cells that had been exposed to isoproterenol and both ligand binding and adenylyl cyclase activation were measured. For the wild-type β_2AR expressed in L cells, exposure to isoproterenol has been shown to cause a time-dependent loss of both ^{125}I-CYP binding (down-regulation) and agonist-stimulated adenylyl cyclase activation (uncoupling) measured in membrane preparations, with significant inactivation apparent after 15 min of incubation with agonist (20) (Fig. 3). A maximal 80% decrease in isoproterenol-stimulated adenylyl cyclase activity was measured after 16 hr with the agonist (Table 2). There was no corresponding loss of NaF-stimulated activity, indicating that homologous desensitization was occurring under these conditions. The effects on the desensitization process of noninactivating deletions from the third intracellular loop and truncations of various lengths at the C-terminus were investigated with the results summarized in Table 2 and Fig. 3. The rate of down-regulation, as assessed by ^{125}I-CYP binding to membrane preparations from isoproterenol-treated cells, proceeded more slowly than in cells expressing the wild-type receptor. This decreased rate was apparent over the first 15 min of exposure to isoproterenol and correlated well with the slower rates of sequestration observed for these mutant receptors. After a 15-min exposure to isoproterenol, the levels of ^{125}I-CYP binding in membranes preparations containing T(345)βAR and D(230–262)T(345)βAR were 80% of the original levels, whereas the cells expressing the wild-type β_2AR retained 67% of their original binding activity. In addition, the maximal levels of down-regulation measured for both of these mutant receptors were significantly less than that of the wild-type receptor (Table 2).

The decreased rates of down-regulation and sequestration observed for these mutant receptors were accompanied by a decrease in the rate of functional uncoupling of the receptor

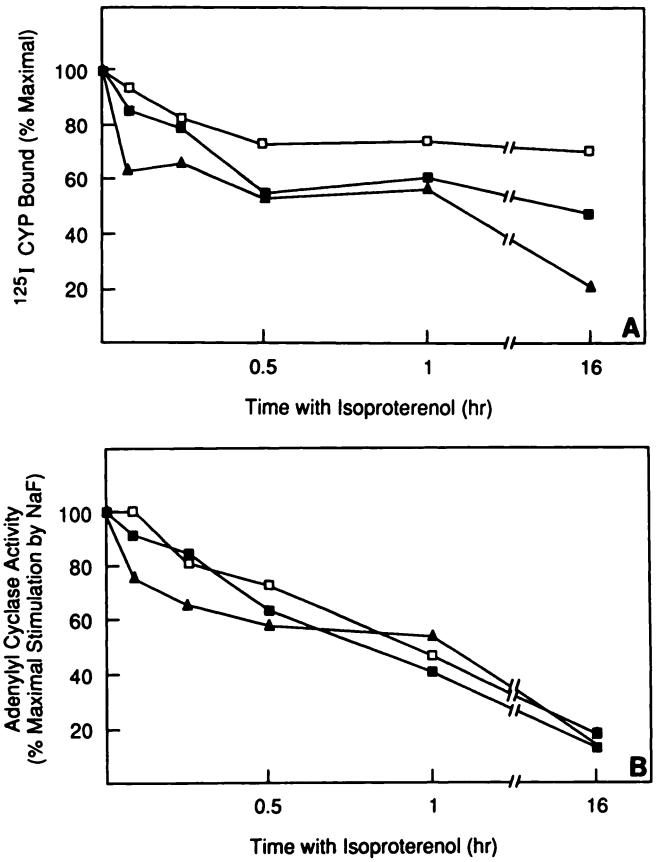

Fig. 3. Desensitization and down-regulation of wild-type and mutant β_2AR. Cells expressing wild-type β_2AR (▲), T(345)βAR (■), or D(230–262)T(345)βAR (□) were exposed to isoproterenol for the times indicated along the *x* axis, before membranes were prepared for assays, as described in Materials and Methods. A, Down-regulation of receptors, expressed as percentage of maximal ^{125}I-CYP bound. B, Uncoupling of adenylyl cyclase activity. The data are presented as percentage of the maximal level of stimulation obtained with NaF, normalized to 100% at $t = 0$.

from adenylyl cyclase stimulation. As mentioned above, both D(230–262)βAR and D(230–262)T(345)βAR showed only partial adenylyl cyclase activation in response to isoproterenol (Fig. 1). However, both of these mutant receptors were desensitized in response to isoproterenol treatment, with a 20% decrease in agonist-mediated adenylyl cyclase activation after 15 min, a 50% loss in activity after 1 hr, and a 75–85% loss of the original activity after a 16-hr exposure to the agonist (Table 2). Likewise, T(345)βAR, which fully stimulated adenylyl cyclase, was fully desensitized in response to isoproterenol. The initial rates of desensitization of these three mutant receptors were slower than that of the wild-type β_2AR over the first 15 min of exposure to the agonist (Fig. 3B). However, after 30 min of exposure to the agonist, the rate and level of desensitization of the mutant receptors were indistinguishable from those of the wild-type protein (Fig. 3B).

Discussion

The deletion of amino acid residues 239–272 from the third intracellular loop of the β_2AR was previously shown to remove the ability of the receptor both to couple to G_s and to undergo agonist-promoted sequestration, whereas smaller deletions within the central portion of this region affected neither param-

TABLE 2

Functional desensitization of mutant β_2AR

Monolayers of cells expressing either β_2AR or mutant β_2AR were either untreated (control) or incubated with 10^{-5} M isoproterenol for 16 hr under normal growth conditions (desensitized) as described in Materials and Methods. Cell membranes were isolated and ^{125}I-CYP binding and adenylyl cyclase activity were measured as described. *B* indicates basal activity; *I*, activity stimulated by 10^{-5} M isoproterenol; and F^-, activity stimulated by 10 mM NaF. Values given are the means of three to five separate experiments.

Mutant	Control				Desensitized			
	^{125}I-CYP binding	Adenylyl cyclase			^{125}I-CYP binding	Adenylyl cyclase		
		B	I	F^-		B	I	F^-
	fmol/mg	*pmol/min/mg*			*fmol/mg*	*pmol/min/mg*		
Wild-type βAR	128.6	3.2	20.0	21.0	26.6	2.7	5.4	22.2
D(230–262) βAR	381.0	9.1	23.8	56.1	118.5	9.3	13.3	55.3
T(345)βAR	58.3	6.3	17.4	29.3	28.3	5.0	6.5	27.6
D(230–262)T(345) βAR	209.8	4.2	9.9	22.6	150.2	3.4	4.4	26.1

eter (11, 15, 20). Three other deletion mutations within this hydrophilic domain that interfere with the normal coupling of the β_2AR to G_s have now been identified. The mutant D(222–229)βAR mimics D(239–272)βAR, in that G_s coupling and adenylyl cyclase stimulation are not observed, whereas D(258–270)βAR couples only weakly to this effector system. D(230–262)βAR, encompassing a large deletion from the middle of this hydrophilic loop, also couples poorly to G_s, as evidenced by the absence of an effect of Gpp(NH)p on agonist binding and the submaximal stimulation of adenylyl cyclase by this mutant receptor. The affinity of D(230–262)βAR expressed in L cells was lower than the wild-type receptor, with a correspondingly higher K_{act} for adenylyl cyclase stimulation, whereas its affinity in COS-7 cells (in which G_s coupling is not observed) was indistinguishable from that of the wild-type protein (12). These data suggest that the deletion of this large segment of the third intracellular loop of the β_2AR may act to destabilize the high affinity complex between the β_2AR and G_s without affecting the intrinsic affinity of the receptor for the agonist. This destabilization of the β_2AR-G_s complex may be a conformational effect of the large deletion, because the region deleted from this mutant is almost entirely spanned by the combination of mutants D(229–236)βAR, D(238–251)βAR, D(250–259)βAR, and D(259–262)βAR, all of which show normal agonist binding and adenylyl cyclase stimulation parameters (11).

The regions of the β_2AR found by deletion analysis to be required for G protein coupling are all located within a region proposed to form the third intracellular loop of the receptor. The intracellular exposure of this region has been demonstrated using antibodies to a peptide corresponding to residues 226–239 of the receptor[1] (28). The region encompassing amino acids 222–229, which is absolutely required for G_s coupling, would be predicted to form the junction between the fifth hydrophobic transmembrane helix and the N-terminus of this intracellular loop. However, the sequence of this segment of the receptor is not absolutely conserved among β_1 and β_2 receptors from various sources, all of which interact with G_s to stimulate adenylyl cyclase. The lack of sequence identity in this functionally important region would suggest that some physical-chemical feature of the receptor other than its primary sequence might play an important role in mediating the interaction between the β_2AR and G_s. Interestingly, residues 222–229 of the β_2AR would be predicted to form an amphiphilic α-helix, both according to the helical wheel model of Schiffer and Edmundson (29) (Fig. 4) and by the method of Chou and Fasman (30) (data not shown). The amphiphilic nature of the helix that would be predicted to be formed by this region of the protein is conserved among all of the G protein-linked receptors whose sequences are known. Likewise, residues 258–273 at the C-terminal end of the third intracellular loop, which also appear to be involved in G_s coupling, would be predicted to form an amphiphilic α-helix. Higashijima and co-workers (31) have recently reported that mastoparan, a peptide that has been shown to assume an amphiphilic α-helical structure, can directly stimulate the G protein G_o. We hypothesize that the activation of G proteins by receptors is characterized by the involvement of amphiphilic α-helices at the N- and C-termini of the third cytoplasmic loops of the receptors.

We had previously determined that D(239–272)βAR, which does not couple to the G_s/adenylyl cyclase system, fails to undergo sequestration away from the cell surface upon binding agonist (20). A correlation between G_s coupling and β_2AR sequestration has also been noted in mutant S49 cell lines (22, 23). In the present study, examination of this sequestration response for the other β_2AR mutants that were found to have altered G_s coupling showed that D(222–229)βAR, which, like D(239–272)βAR, showed no G_s coupling or adenylyl cyclase stimulation, also failed to be sequestered upon agonist binding.

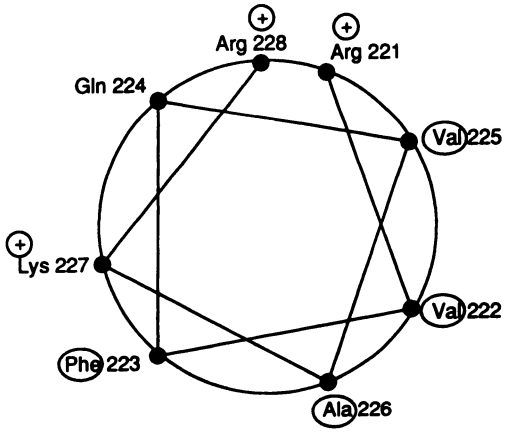

Fig. 4. Schematic representation of the sequence 221–228 of the β_2AR, superimposed on a helical wheel diagram. The *solid circles* indicate the positions of the amino acid residues, with the identity of each residue and its position number indicated. Residues carrying a net positive charge are designated with a *plus sign* whereas hydrophobic residues are circled in the diagram.

[1] Aoki, C. B. A. Zemcik, C. D. Strades, and V. M. Pickel. Cytoplasmic loop of β-adrenergic receptors: Synaptic and intracellular localization and relation to catecholaminergic neurons in the nuclei of the solitary tracts, manuscript in preparation.

In contrast, the sequestration response of D(230–262)βAR, which appeared to form an active but unstable complex with G_s, was only slightly attenuated and D(258–270)βAR, which coupled weakly to G_s, showed a normal sequestration response to agonist binding. These observations are consistent with the concept that some G_s coupling is necessary to stimulate the sequestration of the β_2AR and that similar structural features of the receptor are essential for both processes. Whether receptor activation and desensitization also share a common mechanistic basis or simply involve residues within the same region of the protein remains to be determined. Inasmuch as only those deletions (residues 222–229 and 239–272) that completely abolish G_s coupling result in a loss of receptor sequestration, the β_2AR-G_s coupling may be more sensitive to receptor conformation than is receptor sequestration.

The mutant D(230–262)βAR undergoes agonist-mediated receptor sequestration and functional desensitization, even though all of the serine residues within the third intracellular loop of the protein were removed by the deletion. Likewise, deletion of the serine- and threonine-rich C-terminus does not prevent the desensitization response. Bouvier *et al.* (21) have recently reported that truncation or substitution of serine residues in the C-terminal tail up to position 365 of the human β_2AR, forming a receptor equivalent to the hamster T(376)βAR used in the present study, removed all the sites of *in vivo* phosphorylation when the mutant receptors were expressed in CHW cells. In that study, the rate of functional uncoupling of the mutant receptor, truncated at position 365, was decreased over the first 10 min of exposure to agonists, although, as observed in the present study, the maximal level of desensitization was not affected (21). The rate was further decreased when the serine residues were removed by substitution rather than truncation (21). Whereas we saw no effect of truncation at position 376 on the rate of receptor desensitization in L cells, we did observe that progressive truncation of the C-terminal region of the receptor beyond residue 376 was associated with a decrease in the initial rates of receptor sequestration and functional desensitization. These rates were further decreased by the concomitant deletion of the large region encompassing residues 230–262 from the third intracellular loop of the receptor. The slower initial rate of desensitization was accompanied by a decrease in the rate of degradation of the protein, suggesting differences between the desensitized wild-type and mutant receptors in intracellular cycling. Furthermore, a larger truncation at the C-terminus, at position 338, resulted in a failure of the receptor to bind antagonists, apparently reflecting improper folding of the receptor (12). These results are consistent with a conformational effect of large C-terminal truncations on receptor desensitization in both L cells and CHW cells. The observed effects of these mutations on the rate of receptor desensitization are unlikely to arise from removal of the serine residues that serve as sites of phosphorylation, because the most striking effects were seen with truncations larger than those required to remove all of the *in vivo* phosphorylation sites.

In contrast to the results reported here, no correlation between receptor sequestration and the presence of serine residues was observed in the CHW cell system (21). In that study, the level of sequestration was moderately *increased* for the mutant receptor in which the serine residues were removed by truncation but not when they were removed by amino acid substitution. These differences between the two studies may reflect differences in the mechanisms of desensitization in the expression systems used. In the L cell system, only homologous desensitization is observed in response to isoproterenol even after 16 hr with the agonist, with the basal and NaF-stimulated responses remaining unaffected by agonist treatment, whereas heterologous desensitization appears to occur at the later time points in the CHW cells. In addition, in L cells the sequestration response is much faster ($t_{1/2} < 5$ min) than the uncoupling of adenylyl cyclase stimulation ($t_{1/2} \sim 1$ hr), suggesting that receptor sequestration is the primary desensitization event in this system. This does not appear to be the case in the CHW cells, in which only low levels of sequestration are observed, perhaps due to the overexpression of the receptor in that system (21).

The data presented here suggest that phosphorylation of the β_2AR on the hydrophilic third intracellular loop and C-terminal tail does not represent the primary mechanism for homologous receptor desensitization. However, we cannot rule out the involvement of this process in desensitization events occurring at times shorter than those experimentally accessible in the present series of studies. Such a mechanism could be important under physiological conditions where rapid release of epinephrine occurs, for example, at points of synaptic contact at which epinephrine is acting as a neurotransmitter. In contrast, other mechanisms appear to be involved in mediating receptor desensitization on the time scale of the tachyphylactic response to pharmacological agonists (minutes to hours), more likely to be significant in peripheral tissues where epinephrine functions as a hormone.

The data presented here support the involvement of regions within the third intracellular loop of the β_2AR in the desensitization process and suggest that the structural features of the β_2AR involved in its interaction with G_s are also required for receptor sequestration. The specific biophysical interactions involved in these processes will await further genetic and biochemical characterization of these regions of the receptor.

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