Articles

Mutation of Tyrosine-350 Impairs the Coupling of the β_2-Adrenergic Receptor to the Stimulatory Guanine Nucleotide Binding Protein without Interfering with Receptor Down-Regulation†

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Received November 2, 1992; Revised Manuscript Received March 2, 1993

ABSTRACT: Long-term stimulation of the β_2-adrenergic receptor (β_2AR) leads to an internalization and degradation of the receptor. This down-regulation of the β_2AR number contributes to the desensitization of the adenylyl cyclase activity induced by chronic exposure to agonists. It was proposed that two tyrosine residues (Tyr-350 and Tyr-354) located in the cytoplasmic tail of the β_2AR play a crucial role in agonist-induced down-regulation. In addition to perturbation of the down-regulation, the substitution of these tyrosines for alanines also led to a functional uncoupling of the receptor from Gs [Valiquette et al. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5089–5093]. To further characterize the relative contribution of Tyr-350 and Tyr-354 to the receptor interaction with Gs and agonist-promoted down-regulation, both tyrosines were individually replaced by alanines and mutant receptors expressed in CHW cells. We show here that mutation of Tyr-350 but not that of Tyr-354 significantly decreased the ability of the β_2AR to be functionally coupled to Gs and thereby to stimulate the adenylyl cyclase. Moreover, in contrast to the double tyrosine mutation, neither of the single-point mutations affected the agonist-induced down-regulation pattern. These data suggest that the presence of either Tyr-350 or Tyr-354 is sufficient to maintain normal agonist-induced down-regulation whereas the integrity of Tyr-350 is required for an appropriate coupling to Gs. We therefore conclude that a normal interaction of the receptor with Gs is not a prerequisite for the agonist-promoted down-regulation of the β_2AR and that distinct molecular determinants are involved in these two processes.

Down-regulation of the β_2-adrenergic receptor (β_2AR)[1] number contributes to the desensitization of the β-adrenergic-stimulated adenylyl cyclase activity which follows sustained agonist stimulation [reviewed in Benovic et al. (1988) and Dohlman et al. (1991)]. Phosphorylation of the receptor on serine and threonine residues, which plays an important role in the rapid desensitization of the β_2AR (Clark et al., 1989; Bouvier et al., 1988a; Hausdorff et al., 1989), appears to have a marginal role in the long-term down-regulation (Strader et al., 1987b; Bouvier et al., 1988a, 1989). A cAMP-mediated reduction in the steady-state level of the β_2AR mRNA has been proposed to contribute to this process (Hadcock & Malbon, 1988; Bouvier et al., 1989; Hadcock et al., 1989; Collins et al., 1989). In addition, internalization and degradation of the receptor protein have also been shown to play an important role in the down-regulation [reviewed in Perkins et al. (1990)].

Recently, analogous to receptors such as low-density lipoprotein (Davis et al., 1986), polyimmunoglobulin (Vega & Strominger, 1989), and mannose 6-phosphate (Lobel et al., 1989), two tyrosine residues (Tyr-350 and Tyr-354) located in the carboxyl tail of the β_2AR were proposed as important determinants in the receptor down-regulation (Valiquette et al., 1990). Replacement of the two tyrosines by alanine residues considerably slowed and reduced the agonist-induced down-regulation of the receptor. These tyrosine residues may be part of a common molecular motif involved in receptor internalization and degradation (Valiquette et al., 1990; Ktistakis et al., 1990). The mutation also resulted in a partial uncoupling of the β_2AR from Gs and led to a decreased ability of the receptor to maximally stimulate the production of cAMP (Valiquette et al., 1990). Although the depressed stimulation of cAMP production by the mutant β_2AR did not account for the impaired down-regulation, it was hypothesized that the physical uncoupling from Gs could lead to such a blunted down-regulation (Valiquette et al., 1990). Previous studies using mutant S49 cyc$^-$ or UNC cells had also suggested a link between the ability of the receptor to interact with Gs and to undergo down-regulation (Su et al., 1980). More recently, Campbell et al. (1991) reported a good correlation between the deficit in β_2AR–Gs coupling and the impairment in the down-regulation observed in cells expressing various β_2AR mutants.

In the present study, we further characterized the role of Tyr-350 and Tyr-354 in the β_2AR down-regulation and interaction with Gs. Thus, β_2AR mutants in which either

† This work has been supported by grants from the Medical Research Council of Canada and the Heart and Stroke Foundation of Canada. M.V. holds a studentship from the Medical Research Council of Canada, and M.B. is a scholar from the Medical Research Council of Canada.

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[1] Abbreviations: β_2AR, β_2-adrenergic receptor; Gs, stimulatory guanine nucleotide binding protein; PKA, cAMP-dependent protein kinase; βARK, β-adrenergic receptor kinase; DMEM, Dulbecco's modified Eagle's medium; PBS, Dulbecco's phosphate-buffered saline; IBMX, isobutylmethylxanthine; Bt_2cAMP, N^6,$O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate; [^{125}I]PIN, [^{125}I]iodopindolol; [^{125}I]CYP, [^{125}I]iodocyanopindolol; CHW, Chinese hamster fibroblasts; AlF_3, aluminum fluoride; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); EDTA, ethylenediaminetetraacetic acid.

0006-2960/93/0432-4979$04.00/0

Tyr-350 or Tyr-354 was replaced by alanine were expressed in CHW, and their ability to productively interact with Gs and to undergo agonist-promoted down-regulation was assessed. The quantitative contribution of the "tyrosine-dependent" down-regulation in the long-term desensitization of the β-adrenergic-stimulated adenylyl cyclase activity was also investigated.

EXPERIMENTAL PROCEDURES

Materials. [^{125}I]Iodocyanopindolol ([^{125}I]CYP), [^{125}I]-iodopindolol ([^{125}I]PIN), [α-^{32}P]ATP, and [^{3}H]cAMP were obtained from New England Nuclear. (−)-Isoproterenol, (−)-alprenolol, (−)-propranolol, ATP, GTP, cAMP, phosphoenolpyruvate, myokinase, forskolin, and isobutylmethylxanthine (IBMX) were purchased from Sigma. Pyruvate kinase was from Calbiochem. G418, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, fungizone, glutamine, trypsin, penicillin, streptomycin, and Dulbecco's phosphate-buffered saline (PBS) were obtained from GIBCO. CGP-12177 was generously provided by CIBA-Geigy.

Site-Directed Mutagenesis. Two mutant human β_2AR, ([Ala^{350}]β_2AR and [Ala^{354}]β_2AR), in which Tyr-350 and Tyr-354 were respectively replaced by an alanine, were constructed as follows. The β_2AR coding sequence plus 40 and 570 base pairs of 5′ and 3′ untranslated sequence, respectively, was cloned between the *Eco*RI and *Hin*dIII sites of pTZ18R (Pharmacia). Single-stranded DNA of the noncoding strand was generated using VCS-M13 helper phage (Stratagene) and served as a template for oligonucleotide-directed mutagenesis (Amersham). The mutation was confirmed by dideoxynucleotide sequencing. For expression in eukaryotic system, the fragments between the *Bgl*II and *Eco*RV sites of [Ala^{350}]β_2AR and [Ala^{354}]β_2AR in pTZ18R were subcloned between the *Bgl*II and *Eco*RV sites of the wild-type β_2AR in the expression vector pBC12BI (Cullen, 1987). Constructions for wild-type β_2AR and [Ala^{350},Ala^{354}]β_2AR were as described (Valiquette et al., 1990).

Cell Transfection and Culture. Wild-type and mutant β_2-AR constructs were cotransfected with pSV2-neo (Southern & Berg, 1982) into transformed Chinese hamster fibroblasts (CHW) by calcium phosphate precipitation (Mellon et al., 1981). Neomycin-resistant cells were selected in DMEM supplemented with 10% (v/v) fetal bovine serum and G418 (150 μg/mL). Resistant clones were then screened for β_2AR expression with a [^{125}I]CYP binding assay. Cloned cell lines expressing similar numbers (0.7–1 pmol/mg of membrane protein) of receptors were selected for the study and were grown as monolayers in 75 cm^2 flasks containing DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), fungizone (0.25 μg/mL), and glutamine (1 mM) in an atmosphere of 95% air/5% CO_2 at 37 °C.

Agonist-Induced Down-Regulation and Whole-Cell Radioligand Binding Assays. Nearly confluent cells were incubated at 37 °C for the indicated periods of time with DMEM supplemented as above with or without isoproterenol (1 μM). Following the incubation, the cells were washed twice with PBS and detached with trypsin (0.25%) at room temperature. The action of trypsin was stopped by the addition of 5 mL of supplemented DMEM. The cells were then centrifuged at 500*g* for 5 min and resuspended in PBS at a concentration of 0.1 mg of protein/mL. For radioligand binding assays, 50 μL of cell suspension was used. Binding saturation isotherms were obtained using [^{125}I]PIN concentrations ranging from 2 to 400 pM. In such experiments, it was determined that the affinity of [^{125}I]PIN for the β_2AR was not altered by pretreatment of the cells with isoproterenol (data not shown). Therefore, on a routine basis, binding was performed as previously described to assess down-regulation (Bouvier et al., 1988b; DeBlasi et al., 1985) using a saturating concentration (250 pM) of [^{125}I]PIN. Binding reactions were performed in DMEM at 25 °C for 1.5 h in a final volume of 0.5 mL and terminated by rapid filtration over Whatman GF/C glass fiber filters. No reversal of the down-regulation is likely to occur under these binding conditions since no such reversal was observed when down-regulated cells where incubated in agonist-free media for up to 2 h at 37 °C (data not shown). Specific binding was defined as the amount of radioligand binding inhibited by 0.3 μM (−)-propranolol. Cell-surface receptor number was defined as the amount of specific [^{125}I]PIN binding inhibited by 0.1 μM CGP-12177 at 13 °C as previously described by Hausdorff et al. (1989). This concentration of CGP-12177 was chosen because it displaces approximately 95% of the [^{125}I]PIN bound to control cells (data not shown). The validity of this technique to detect cell-surface receptors is confirmed by the fact that identical receptor distribution is found using differential centrifugation of the membrane fractions (Suzuki et al., 1992). Binding data were analyzed by nonlinear least-squares regression using the computer program LIGAND (De Lean et al., 1980).

Desensitization, Membrane Preparations, and Adenylyl Cyclase Assays. Nearly confluent cells were incubated at 37 °C for 24 h with isoproterenol (1 μM) or medium alone. After incubation, cells were washed twice with 5 mL of PBS at room temperature and mechanically detached in 10 mL of ice-cold 5 mM Tris-HCl (pH 7.4)/2 mM EDTA. Cells were lysed with a polytron homogenizer, Ultra-Turrax T25 (one 5-s burst at maximum speed). The lysate was centrifuged at 45000*g* for 20 min at 4 °C. The pelleted membranes were resuspended in 2 mL of a buffer containing 75 mM Tris-HCl (pH 7.4)/12.5 mM $MgCl_2$/2 mM EDTA.

Adenylyl cyclase activities were measured by the method of Salomon et al. (1974). Assay mixtures contained 0.02 mL of membrane suspension (10–15 μg of protein), 0.012 mM ATP, 0.1 mM cAMP, 0.053 mM GTP, 2.7 mM phosphoenolpyruvate, 0.2 unit of pyruvate kinase, 1 unit of myokinase, and 0.13 μCi of [α-^{32}P]ATP in a final volume of 0.05 mL. Enzyme activity was determined in the absence of activators (i.e., basal activity) or in the presence of 100 μM isoproterenol. Reactions were initiated by the addition of the membranes, and the assay mixture was incubated for 30 min at 37 °C. The assay was terminated by the addition of 1 mL of an ice-cold solution containing 0.4 mM ATP, 0.3 mM cAMP, and [^{3}H]-cAMP (25 000 cpm), and cAMP was isolated by sequential chromatography on a Dowex cation-exchange resin and aluminum oxide. For whole-cell and membrane preparations, protein concentrations were measured by the method of Bradford (1976), using bovine serum albumin as standard.

[^{125}I]CYP Binding Competition Assays with Isoproterenol. Competition experiments were conducted using 0.01 mL of membrane suspension (5–10 μg of protein), prepared as described above. Duplicate assay tubes contained 80 pM [^{125}I]-CYP and 1×10^{-10} to 1×10^{-4} M of isoproterenol. Binding reactions were performed in a buffer containing 75 mM Tris-HCl (pH 7.4/12.5 mM $MgCl_2$/2 mM EDTA at 25 °C for 1.5 h in a final volume of 0.5 mL and terminated by rapid filtration over Whatman GF/C glass fiber filters. Data from competition experiments were analyzed by nonlinear least-squares regression using the computer program LIGAND (De Lean et al., 1980).

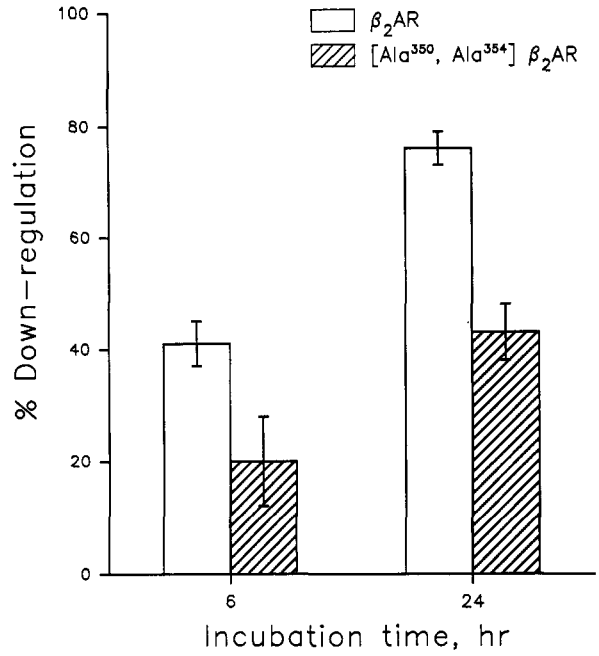

FIGURE 1: Isoproterenol-induced down-regulation of β_2AR and [Ala^{350},Ala^{354}]β_2AR in CHW cells. Cells were incubated with isoproterenol (1 μM) for 6 or 24 h at 37 °C, and whole-cells β_2AR number was determined by radioligand binding assay using [^{125}I]-PIN as described under Experimental Procedures. Data are means ± SEM of four determinations.

RESULTS

Contribution of Tyrosine-Dependent Down-Regulation to Overall β-Adrenergic Desensitization. Exposure of CHW cells expressing the wild-type human β_2AR to isoproterenol for a prolonged period of time led to a down-regulation of the total number of β_2AR measured by [^{125}I]PIN binding in whole cells. As seen in Figure 1, incubation with isoproterenol for 6 and 24 h reduced the number of β_2AR by 41 ± 4% and 76 ± 3%, respectively. Under the same conditions, the down-regulation in cells expressing [Ala^{350},Ala^{354}]β_2AR is considerably reduced and reaches only 20 ± 8% and 43 ± 5%. Thus, in agreement with our previous study (Valiquette et al., 1990), the mutation of Tyr-350 and Tyr-354 in β_2AR perturbed the down-regulation in CHW cells. The influence of such a blunted down-regulation on the long-term desensitization of the β-adrenergic-stimulated adenylyl cyclase was then evaluated. While a 76 ± 2% desensitization of the isoproterenol-stimulated adenylyl cyclase activity was observed following a 24-h incubation with isoproterenol in cells expressing wild-type β_2AR, the same treatment resulted in a desensitization of only 48 ± 11% in cells expressing [Ala^{350},Ala^{354}]β_2AR (Figure 2). Cells expressing wild-type and mutant receptors were also incubated with isoproterenol in the presence of Bt$_2$-cAMP and IBMX. Even in the presence of a saturating concentration of Bt$_2$cAMP, the desensitization observed in the cells expressing [Ala^{350},Ala^{354}]β_2AR was significantly less than that observed for the wild-type β_2AR (Figure 2). Therefore, the down-regulation process which is affected by the mutation of Tyr-350 and Tyr-354 plays a significant role in the long-term regulation of the responsiveness of the β_2-adrenergic-stimulated adenylyl cyclase.

Effect of Tyr-350 and Tyr-354 Mutations on β_2AR Coupling. Table I illustrates the β_2-adrenergic-stimulated adenylyl cyclase activity observed in cells expressing either the wild-type β_2AR or the mutant [Ala^{350},Ala^{354}]β_2AR. Although no significant difference in the basal activity was

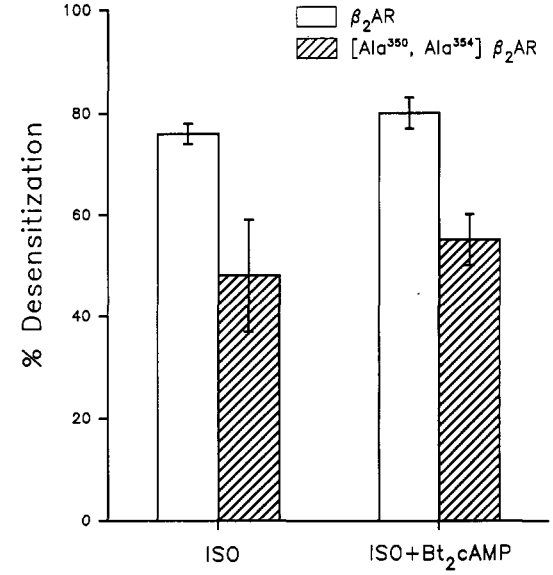

FIGURE 2: Isoproterenol- and (isoproterenol + Bt$_2$cAMP)-induced desensitization of β_2AR and [Ala^{350},Ala^{354}]β_2AR in CHW cells. Cells were incubated for 24 h at 37 °C with isoproterenol (1 μM) alone or in the presence of Bt$_2$cAMP (1 mM) and IBMX (0.1 mM) to prevent Bt$_2$cAMP degradation. Desensitization was measured as the percent loss of adenylyl cyclase stimulation by isoproterenol (100 μM). Data are means ± SEM of three independent experiments.

Table I: Receptor Number and Adenylyl Cyclase Activity for Wild-Type and Mutant β_2AR[a]

receptor	receptor no. (fmol/mg of protein)	adenylyl cyclase act. [pmol of cAMP min^{-1} (mg of protein)$^{-1}$]	
		basal	isoproterenol-stimulated
β_2AR	644 ± 52	7.2 ± 2.4	20.3 ± 8.0
[Ala^{350},Ala^{354}]β_2AR	624 ± 95	4.9 ± 0.6	8.7 ± 2.4

[a] Membranes were prepared from cells expressing wild-type β_2AR or [Ala^{350},Ala^{354}]β_2AR, and basal or stimulated (isoproterenol, 100 μM) adenylyl cyclase activity was measured and expressed as picomoles of cAMP per minute per milligram of membrane protein. Data are means ± SEM of five determinations.

observed between the two cell lines, [Ala^{350},Ala^{354}]β_2AR showed a greatly decreased ability to promote isoproterenol stimulation of the enzyme. Indeed, while isoproterenol induces a (2.6 ± 0.1)-fold stimulation of the adenylyl cyclase activity in cells expressing the wild-type receptor, the same treatment only caused a (1.6 ± 0.2)-fold ($p < 0.05$) stimulation in cells expressing [Ala^{350},Ala^{354}]β_2AR. This observation, which suggests an uncoupling of the mutant receptor from Gs, is consistent with our previous report (Valiquette et al., 1990). To assess the respective role of Tyr-350 and Tyr-354 in this phenotype, these residues were individually replaced by alanines (Figure 3). CHW cells were transfected with [Ala^{350}]β_2AR or [Ala^{354}]β_2AR cDNAs, and cell lines expressing similar levels of either wild-type or mutant receptors (1 pmol/mg of protein) were selected for the study.

As shown in Figure 4, the mutation of Tyr-350 produced a significant reduction in the receptor-mediated stimulation of the adenylyl cyclase activity. Indeed, the maximal isoproterenol stimulation of the adenylyl cyclase in cells expressing [Ala^{350}]β_2AR was less than 60% of that observed in cells expressing the same number of wild-type receptor. In contrast, replacement of Tyr-354 by an alanine had no effect on the ability of the receptor to mediate the agonist stimulation of adenylyl cyclase activity (Figure 4).

The agonist binding characteristics of the β_2AR are believed to reflect the state of physical coupling between the receptor

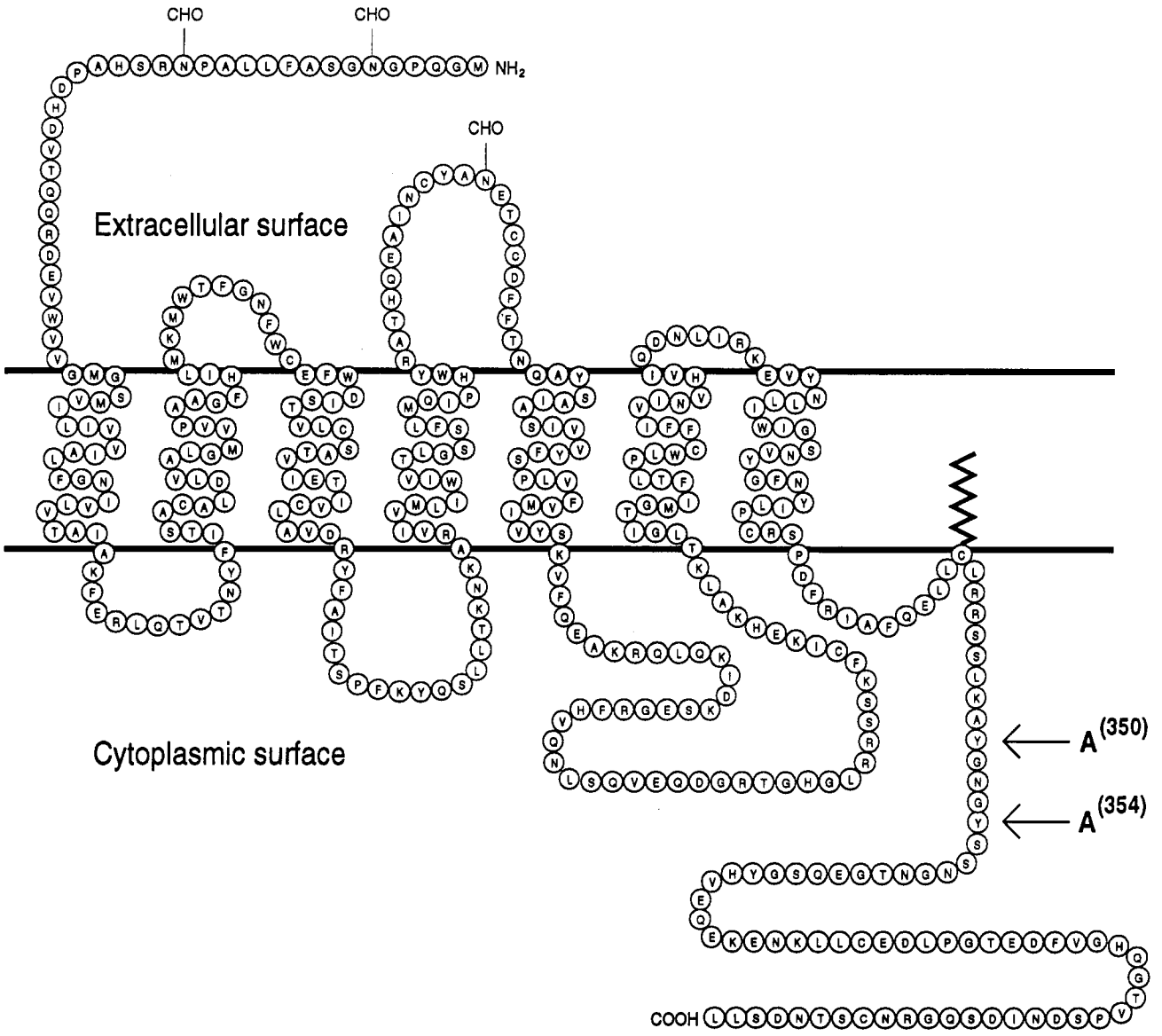

FIGURE 3: Schematic representation of human β_2AR. Tyr-350 and Tyr-354 (indicated by arrows) were replaced by alanine residues to generate two mutant receptors termed [Ala^{350}]β_2AR and [Ala^{354}]β_2AR, respectively. The single-letter amino acid code is used.

and Gs (De Lean et al., 1980; Hausdorff et al., 1990). As shown in Figure 5, isoproterenol displacement of [^{125}I]CYP binding to wild-type receptor, [Ala^{350}]β_2AR, or [Ala^{354}]β_2AR was characterized by a biphasic curve which was best resolved by a two-affinity-state model using iterative nonlinear least-squares fitting of the nontransformed data (De Lean et al., 1980). The agonist binding parameters obtained from three to nine competition assays are summarized in Table II. The proportion of receptors found in the guanyl nucleotide-sensitive state of high affinity for isoproterenol was identical for the wild-type receptor and [Ala^{354}]β_2AR. However, this proportion was reduced by more than 50% for [Ala^{350}]β_2AR. Thus, the mutation of Tyr-350 has the same effect as the mutation of both Tyr-350 and Tyr-354 on the ability of the receptor to stabilize the high-affinity state (Table II). These data therefore suggest that the mutation of Tyr-350 alone is sufficient to provoke a physical uncoupling of the receptor from Gs, consistent with the reduced adenylyl cyclase stimulation mediated by this receptor. The same binding and adenylyl cyclase phenotypes indicative of an uncoupled receptor were observed in several distinct clonal cell lines expressing this mutant receptor, excluding the possibility that it reflected the properties of a unique clone (data not shown).

Effect of Tyr-350 and Tyr-354 Mutations on β_2AR Agonist-Mediated Down-Regulation. Incubation of the cells expressing wild-type β_2AR with isoproterenol for 4 and 24 h induced a down-regulation of 31% and 67% of the receptor number, respectively. As shown in Figure 6, the agonist treatment led to nearly identical down-regulation profiles in cells expressing [Ala^{350}]β_2AR and [Ala^{354}]β_2AR. This contrasts with the significant effect of the double mutation on the down-regulation and suggests that the presence of one tyrosine residue is sufficient to maintain a normal down-regulation pattern. To further document that [Ala^{350}]β_2AR which is uncoupled from Gs maintained down-regulation properties identical to that of wild-type, the effect of increasing concentration of agonist on its down-regulation was assessed. As shown in Figure 7, the dose-dependent down-regulation process was identical for both receptors. Such a normal down-regulation pattern was observed in several distinct cell lines expressing between 0.4 and 1.3 pmol of receptors/mg of proteins. As previously reported (Campbell et al., 1991), we found that when expressed as a percentage of the total receptor number, the ability of a given species to undergo down-regulation was independent of its expression level.

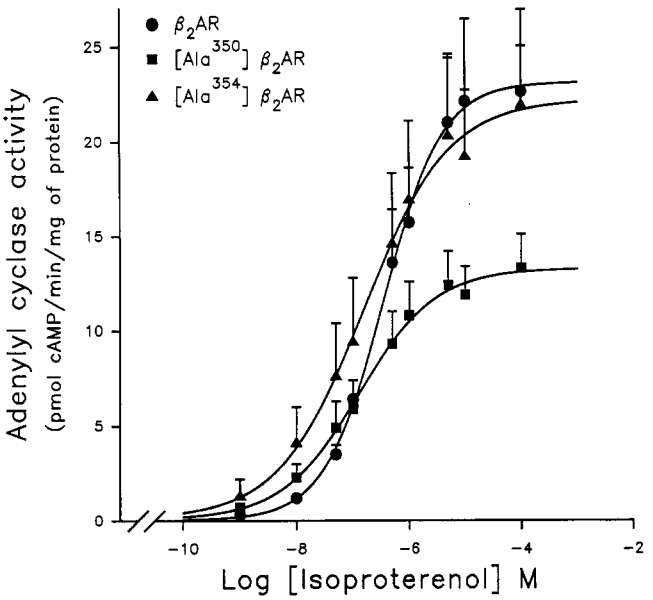

FIGURE 4: Dose response curves of isoproterenol-stimulated adenylyl cyclase in membranes from cells expressing β_2AR, [Ala^{350}]β_2AR, and [Ala^{354}]β_2AR. The adenylyl cyclase activity was measured in membrane preparations as described under Experimental Procedures and expressed as picomoles of cAMP per minute per milligram of protein. The data were analyzed using nonlinear least-squares regressions, and the EC_{50} values calculated were 3.5×10^{-7}, 1.3×10^{-7}, and 1.6×10^{-7} M for β_2AR, [Ala^{350}]β_2AR, and [Ala^{354}]β_2AR, respectively. Data are means ± SEM of six or seven independent experiments.

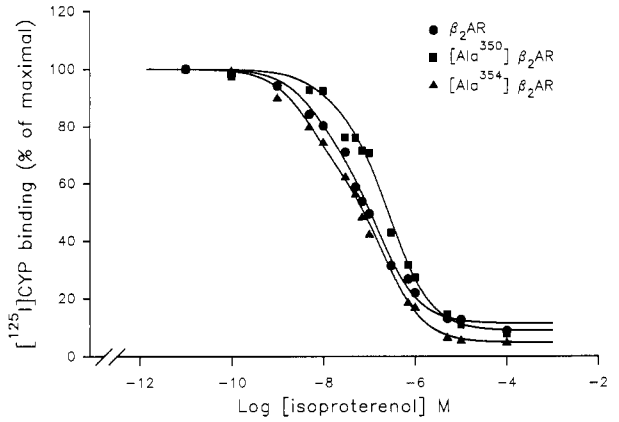

FIGURE 5: Competition of [^{125}I]CYP binding with isoproterenol in cells expressing β_2AR, [Ala^{350}]β_2AR, and [Ala^{354}]β_2AR. Membranes were prepared, and binding of [^{125}I]CYP was assessed in the presence of varying concentrations of isoproterenol. The data were analyzed using the nonlinear least-squares regression computer program LIGAND (De Lean et al., 1980). The results shown are representative of three to nine distinct experiments conducted in duplicates.

Shorter stimulation of the β_2AR (minutes) is known to induce a sequestration of the receptor away from the cell surface where it is still detectable by hydrophobic but not hydrophilic ligands. Figure 8 shows that exposure of cells expressing human β_2AR to isoproterenol for 5, 10, and 15 min significantly increased the proportion of receptors not accessible to the hydrophilic ligand CGP-12177. As previously observed for the double mutation, the replacement of either Tyr-350 or Tyr-354 by alanine residues did not affect the agonist-promoted sequestration of the receptor (Figure 8).

DISCUSSION

The results presented here suggest that although Tyr-350 alone is important to allow normal coupling of the β_2AR to Gs, the presence of either Tyr-350 or Tyr-354 is sufficient to preserve the normal receptor down-regulation and desensitization. More importantly, we report here that a mutant β_2AR ([Ala^{350}]β_2AR) which is largely uncoupled from Gs

Table II: Agonist Parameters for Wild-Type and Mutant β_2AR[a]

receptor	$K_{i(H)}$ (nM)	$K_{i(L)}$ (nM)	$R_{(H)}$ (%)	n
β_2AR	19 ± 5	377 ± 64	46 ± 4	9
[Ala^{354}]β_2AR	6 ± 2	146 ± 32	47 ± 2	4
[Ala^{350}]β_2AR	4 ± 1	289 ± 123	22 ± 3	4
[Ala^{350},Ala^{354}]β_2AR	4 ± 1	291 ± 78	28 ± 5	3

[a] Competition binding was performed as described under Experimental Procedures. The data were analyzed using the nonlinear least-squares regression curve-fitting program LIGAND. $K_{i(H)}$ and $K_{i(L)}$ are the high- and low-affinity inhibition constants for isoproterenol binding. $R_{(H)}$ is the proportion of agonist binding in the high-affinity state. n is the number of independent experiments.

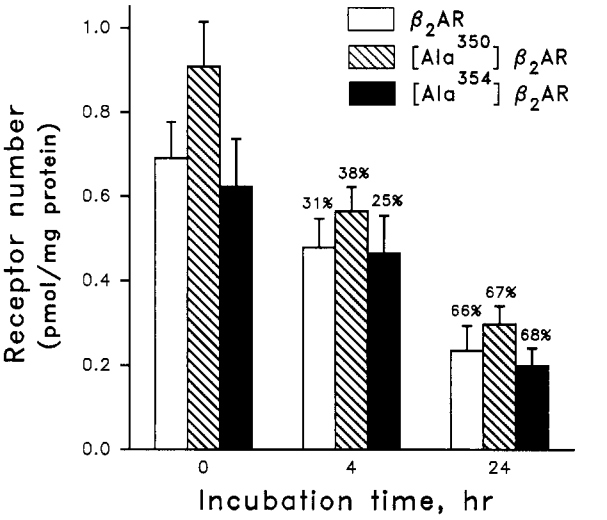

FIGURE 6: Isoproterenol-induced down-regulation of β_2AR, [Ala^{350}]β_2AR, and [Ala^{354}]β_2AR in CHW cells. Cells were incubated with isoproterenol (1 μM) for 4 or 24 h at 37 °C, and whole cell β_2AR number was determined by radioligand binding assay using [^{125}I]PIN as described under Experimental Procedures. The percentage of down-regulation is indicated above each column. Data are means ± SEM of four to eight independent determinations.

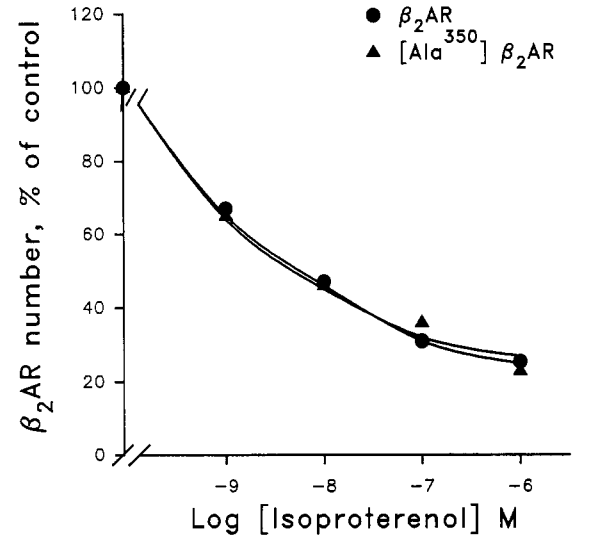

FIGURE 7: Dose dependence of isoproterenol-induced down-regulation of β_2AR and [Ala^{350}]β_2AR in CHW cells. Cells were incubated with different concentrations of isoproterenol for 24 h at 37 °C, and whole-cell β_2AR number was determined by radioligand binding assay using [^{125}I]PIN as described under Experimental Procedures. Data are means of two independent determinations.

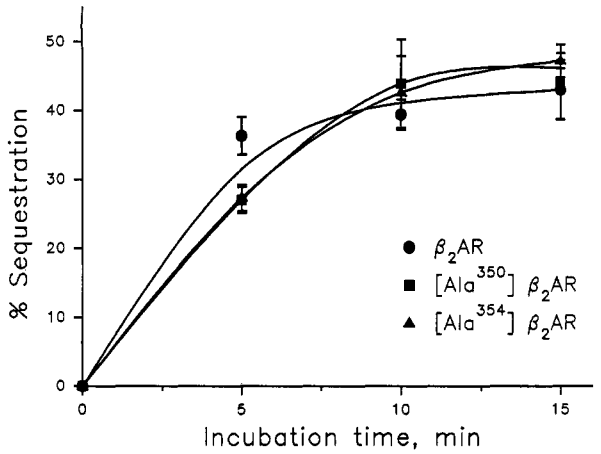

FIGURE 8: Isoproterenol-induced sequestration of β_2AR, [Ala^{350}]β_2AR, and [Ala^{354}]β_2AR in CHW cells. Cells were incubated with isoproterenol (1 μM) for 0, 5, 10, and 15 min at 37 °C, and radioligand binding was conducted in whole-cell preparations as described under Experimental Procedures. Sequestration was defined as the difference between the total number of specific [^{125}I]PIN binding sites and the cell-surface sites accessible to the hydrophilic antagonist CGP-12177. Sequestered receptor number is expressed as percent of total β_2AR number. Data are means ± SEM of four independent experiments.

displays a normal profile of down-regulation, challenging the existence of a strict relationship between receptor coupling to Gs and down-regulation.

The observation that the impaired down-regulation of [Ala^{350},Ala^{354}]β_2AR is accompanied by a significant reduction of the agonist-promoted desensitization supports the notion that the down-regulation process, which depends on the integrity of the two tyrosine residues, plays a significant role in controlling the β-adrenergic responsiveness. In agreement with our previous study (Valiquette et al., 1990), the mutation of Tyr-350 and Tyr-354 decreases the ability of the β_2AR to productively interact with Gs and stimulate cAMP production. Given the role of the cAMP-dependent protein kinase (PKA) in agonist-promoted desensitization (Bouvier et al., 1988a; Clark et al., 1989; Hausdorff et al., 1989), it could be hypothesized that the incomplete desensitization observed results from a reduced PKA-mediated uncoupling rather than from a reduced down-regulation. However, the failure of a saturating concentration of Bt$_2$cAMP to restore normal down-regulation and desensitization argues against this possibility.

Following substitutions of various domains of the third cytoplasmic loop and carboxyl tail of the β_2AR for corresponding domains of the α_{2A}AR, Campbell et al. (1991) proposed the existence of a close relationship between the ability of the receptor to interact with Gs and to undergo agonist-promoted down-regulation. They proposed that the physical interaction of the receptor with Gs rather than the production of cAMP determines the down-regulation profile. Studies using variants of the S49 lymphoma cell series also suggested that receptor–Gs coupling, rather than the generation of cAMP, is a key determinant in down-regulation. Indeed, several mutant cell lines like Kin$^-$ (lacking PKA activity), H21a (impaired Gs–adenylyl cyclase coupling), or HC-1 (lacking the catalytic unit of adenylyl cyclase) displayed normal β_2AR down-regulation (Mahan et al., 1985; Su et al., 1980; Shear et al., 1976). In contrast, the mutant cell lines cyc$^-$ and UNC with perturbed receptor–Gs coupling showed blunted down-regulation (Su et al., 1980). In the present study, mutation of Tyr-350 alone affected the ability of the β_2AR to interact with Gs, as illustrated by the lower proportion of this mutated receptor in the high-affinity state for agonists and its decreased ability to stimulate the adenylyl cyclase, but did not alter the down-regulation. In fact, the level of uncoupling caused by the mutation of Tyr-350 is equal to that provoked by the double tyrosine mutation whereas the mutation of Tyr-354 is without effect. This suggests that only Tyr-350 is required for the proper interaction of the β_2AR with Gs and that a decreased interaction with Gs is not sufficient to alter down-regulation. Such absence of a strict correlation between the ability of the β_2AR to couple to Gs and to undergo agonist-induced down-regulation suggests that normal coupling to Gs is not required for down-regulation to occur. Our observations may be reconciled with previous findings by suggesting that similar regions of the receptor but not the same residues are involved both in coupling Gs and in mediating down-regulation. Following this model, mutation of Tyr-350 would perturb the interaction with Gs without affecting the molecular structure responsible for down-regulation whereas other mutations such as those reported by Campbell et al. (1991) or the mutation of the two tyrosines would affect both processes. However, we cannot exclude the possibility that the poor coupling of [Ala^{350}]β_2AR is still sufficient to allow normal down-regulation.

A distinct possibility is that a nonproductive interaction (which does not lead to the stabilization of the high-affinity state) of [Ala^{350}]β_2AR with Gs could still be involved in the down-regulation. Mutation of a discrete region of the β_2AR has already been shown to impair specific aspects of the receptor–Gs interaction. Indeed, a seven amino acid deletion in the third cytoplasmic loop alters agonist activation of the adenylyl cyclase without affecting high-affinity agonist binding (Hausdorff et al., 1990). It is noteworthy that GTP binding proteins have recently been proposed to play a role in endocytotic processes. Interestingly, Gi-like proteins were found in endocytotic vesicles (Ali et al., 1989), and GTPγS, AlF_3, and mastoparan interfered with the fusion of these vesicles (Wessling-Resnick & Braell, 1990; Mayorga et al., 1989; Colombo et al., 1992). The direct contribution of such a GTP binding protein to the down-regulation of the β_2AR remains to be investigated.

Recent studies (Ktistakis et al., 1990; Canfield et al., 1991; Bansal & Gierasch, 1991; Eberle et al., 1991; Lobel et al., 1989; Davis et al., 1986; Vega & Strominger, 1989) proposed the existence of a "tyrosine internalization signal" for many proteins which are internalized and down-regulated. This signal would be formed by a short amino acid sequence, with polar or basic residues flanking a tyrosine and by residues frequently found in reverse turns (Ktistakis et al., 1990; Eberle et al., 1991). It was suggested that the essential tyrosine residue could be properly positioned by a tight turn. Analysis by Chou–Fassman secondary structure prediction algorithms of the sequence surrounding Tyr-350 and Tyr-354 in the β_2-AR predicts the formation of a β-turn in this region of the protein. The mutation of either Tyr-350 or Tyr-354 does not affect the prediction, and in both mutants, the β-turn is preserved. Thus, the presence of only one tyrosine appears sufficient to maintain a putative "tyrosine internalization signal". Helin et al. (1991) also reported that whereas single or double mutations of three tyrosine residues in the cytoplasmic tail did not affect the rate of internalization of the EGF receptor, the triple-point mutant significantly slowed this process. In contrast, the mutation of either two tyrosines located in the cytoplasmic domain of the γ subunit of the type III receptor for IgG blocks internalization (Amigorena et al., 1992). In several cases, only one tyrosine residue seems to be required in the internalization signal (Eberle et al., 1991;

Bansal & Gierasch, 1991). These observations might indicate that a general molecular context rather than a linear sequence is important in defining an internalization signal.

Also supporting a role for a "tyrosine internalization signal" in the agonist-promoted down-regulation of the β_2AR is the recent observation that in human 293 cells, the internalized β_2AR is colocalized with the internalized transferrin receptor (Zastrow & Kobilka, 1992). Indeed, an internalization sequence which includes a tyrosine has been shown to be involved in the endocytosis of the transferrin receptor (Gironès et al., 1991; Collawn et al., 1990).

Our study also points to Tyr-350 as an important residue contributing to the interaction of β_2AR with Gs. Most studies identified the N-terminal and the C-terminal portions of the third cytoplasmic loop as important determinants of the interaction of the receptor with Gs (Hausdorff et al., 1990; Strader et al., 1987a; O'Dowd et al., 1988). However, O'Dowd et al. (1989) reported that the presence of the palmitoylated cysteine-341 (Cys-341) is required for proper coupling to Gs, thus suggesting a role for the N-terminal portion of the cytoplasmic tail in the receptor–Gs interaction. A larger mutation of this domain supported such a role (Liggett et al., 1991). Tyr-350, located nine amino acids downstream from Cys-341, might be an important residue for the interaction with Gs, or else its mutation to alanine might modify the structure of this domain such that the coupling is perturbed.

In conclusion, the molecular determinants involved in the formation of the high-affinity ternary complex (hormone/receptor/Gs) and activation of Gs are not identical to those responsible for the agonist-promoted down-regulation. However, the small domain which includes Tyr-350 and Tyr-354 appears to be involved in both processes, and further studies will be required to define the exact molecular motif specific to each of them.

ACKNOWLEDGMENT

We are grateful to Nicole Robinson and Stéphane St-Onge for their help in the preparation of the manuscript.

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