Amino-Terminal Polymorphisms of the Human β_2-Adrenergic Receptor Impart Distinct Agonist-Promoted Regulatory Properties†

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ABSTRACT: We have recently delineated three naturally occurring polymorphisms of the human β_2-adrenergic receptor caused by missense mutations encoding for amino acids 16 and 27 of the extracellular N-terminus of the receptor. We have studied the functional consequences of these polymorphisms by site-directed mutagenesis and the recombinant expression of these receptors in Chinese hamster fibroblasts. The polymorphisms consist of substitutions of Gly for Arg at amino acid 16 (Arg16→Gly), Glu for Gln at amino acid 27 (Gln27→Glu), and a combination of both substitutions. All three mutated receptors displayed normal agonist binding and functional coupling to G_s, resulting in the stimulation of adenylyl cyclase activity. However, these mutations markedly altered the degree of agonist-promoted downregulation of receptor expression. After 24-h exposure to 10 μM isoproterenol, wild-type β_2AR underwent a 26 ± 3% reduction in receptor density. In contrast, Arg16→Gly underwent a 41 ± 3% reduction. Gln27→Glu, on the other hand, was found to be completely resistant to downregulation. Arg16→Gly + Gln27→Glu also underwent an increased downregulation compared to wild-type β_2AR (39 ± 4%). The rates of new receptor synthesis after irreversible alkylation were not different between these receptors, nor were the rates of agonist-promoted receptor internalization to the intracellular pool. Gln27→Glu cellular mRNA minimally increased during agonist exposure, and wild-type β_2AR and the other mutated receptor mRNAs did not change, which infer that the aberrant downregulation patterns of these polymorphisms may be due to the altered degradation of receptor protein. On Western blots, the Gln27→Glu receptor did not appear to be expressed in the fully mature form as compared to wild-type β_2AR, suggesting that conformational alterations of this receptor lead to depressed receptor degradation during agonist exposure. These results indicate that common polymorphisms of the β_2AR at the N-terminus impart distinct phenotypes of agonist-promoted regulation and may explain the observed heterogeneity of *in vivo* responsiveness and regulation of β_2AR.

β_2-Adrenergic receptors (β_2ARs)[1] are membrane-bound receptors which upon binding the endogenous catecholamines epinephrine and norepinephrine signal to the interior of cells via the stimulatory guanine nucleotide-binding protein, G_s (Liggett & Raymond, 1993; Dohlman et al., 1991). The presumed membrane topology of the β_2AR is typical of the family of G protein-coupled receptors, with an extracellular N-terminus, seven transmembrane spanning regions, three intracellular and three extracellular loops, and a cytoplasmic C-terminus (Liggett & Raymond, 1993; Dohlman et al., 1991). Recently, we have identified in the general population several different polymorphic forms of the human β_2AR (Reishaus et al., 1993), including three which are comprised of one or two homozygous missense mutations encoding for amino acids in the N-terminus of the receptor. These N-terminal polymorphisms (Figure 1) consist of substitutions of glycine for arginine at amino acid position 16 (Arg16→Gly) and glutamine for glutamic acid at position 27 (Gln27→Glu) and a receptor with both substitutions (Arg16→Gly + Gln27→Glu). In this paper, we have mimicked these polymorphisms by site-directed mutagenesis of the wild-type human β_2AR cDNA, expressed these receptors in Chnese hamster fibroblast cells (CHW), and delineated the relevance of these mutations to receptor function and regulation.

EXPERIMENTAL PROCEDURES

Constructs, Transfections, and Cell Culture. cDNA encoding the human β_2AR was subcloned into the mammalian expression vector pcDNA 1/Neo at the *Xho*1 site as described previously (Green et al., 1993). Site-directed mutagenesis (Kunkel, 1985) was employed to mutate nucleic acids 46 (adenine to guanine) and 79 (cytosine to guanine) or both, creating constructs encoding for substitutions at amino acid residues 16 (Arg16→Gly), 27 (Gln27→Glu), or both (Arg16→Gly + Gln27→Glu). These were individually ligated into pcDNA 1/Neo at the exact same site as the wild-type β_2AR construct. Authenticity of the mutations was confirmed by dideoxy sequencing. Transfection of CHW-1102 fibroblasts was performed by calcium phosphate precipitation (Green et al., 1992, 1993). Cells resistant to 300 μg/mL G418 were screened for β_2AR expression by a [^{125}I]CYP binding assay as described below. Recombinant clones expressing approximately equivalent amounts of β_2AR (500–1000 fmol/mg of protein) were selected for further study. Cells were maintained at 37 °C, in a 5% CO_2 atmosphere in DMEM supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 80 μg/mL G418. All studies were performed with cells at 90% confluency.

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[1] Abbreviations: β_2AR, β_2-adrenergic receptor; G_s, stimulatory guanine nucleotide regulatory binding protein; [^{125}I]CYP, [^{125}I]cyanopindolol; BIM, N^8-(bromoacetyl)-N^1-[3′-(4-indolyloxy)-2′-hydroxypropyl]-(*Z*)-1,8-diamino-*p*-menthane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; TBS, 20 mM Tris, 500 mM NaCl, pH 7.5; CHW, Chinese hamster fibroblasts; DMEM, Dulbecco's modified Eagle's medium.

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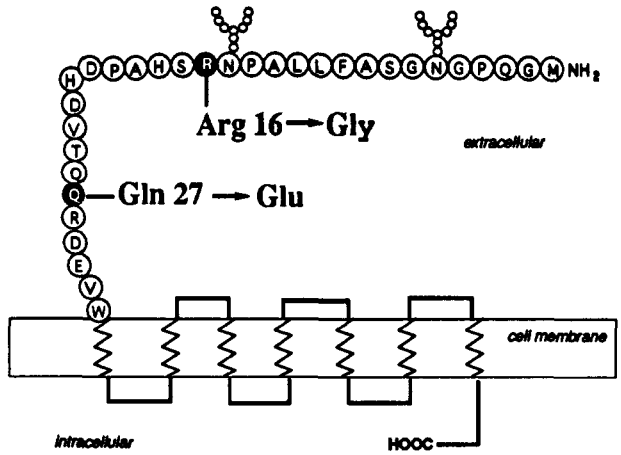

FIGURE 1: Location of β_2AR polymorphisms. Indicated is the primary amino acid sequence of the N-terminus of the human β_2AR with the remainder of the receptor depicted in a schematic form typical of that proposed for G protein-coupled receptor membrane topology. Shown are the amino acid substitutions at position 16 and 27.

Radioligand Binding. Radioligand binding was performed exactly as previously described (Green et al., 1992, 1993) using particulate preparations from confluent cultures of recombinant CHW cells. Briefly, particulates were prepared by mechanical detachment in lysis buffer (5 mM Tris, pH 7.4, 2 mM EDTA), centrifuged at 39000*g* for 10 min, homogenized in a Brinkman polytron, and recentrifuged. For the determination of maximal receptor density (B_{max}), membranes (approximately 100 μg/mL in 75 mM Tris, pH 7.4, 12.5 mM $MgCl_2$, and 2 mM EDTA) were incubated in a final volume of 250 μL for 2 h at 22 °C with a saturating concentration (400 pM) of [^{125}I]CYP; nonspecific binding was defined by the binding of this same concentration of radioligand in the preence of 1 μM propranolol. In preliminary studies, we found a good correlation between receptor expression determined with [^{125}I]CYP binding versus immunoblotting using β_2AR antisera. For competition studies, membranes were incubated with 30 pM [^{125}I]CYP, 100 μM GppNHp, and 0–100 μM of unlabelled ligand as indicated. Reactions were stopped by dilution and rapid filtration over glass fiber filters which were then counted in a gamma counter. Bound radioligand was generally <10% of that added to reaction tubes. Data were analyzed by nonlinear techniques as previously described (Green et al., 1992, 1993).

Adenylyl Cyclase. Adenylyl cyclase activity was determined in membranes prepared as described above, using a modification (Green et al., 1993) of the method of Salomon (Salomon et al., 1974). Briefly, membranes were incubated in 30 mM Tris, 2 mM $MgCl_2$, 0.8 mM EDTA, 0.12 mM ATP, 0.06 mM GTP, 2.8 mM phosphoenolpyruvate, 2.2 μg of myokinase, 0.1 mM cAMP, and 1.0 μCi of [α-^{32}P]ATP in a final volume of 50 μL for 45 min at 37 °C. Reactions were stopped by the addition of excess unlabeled ATP. The [^{32}P]cAMP formed was separated by sequential chromatography over Dowex and alumina columns and counted in a scintillation counter. Column efficiency was determined by [^{3}H]cAMP, which was included in the stop buffer. Activities were measured in the presence of water (basal) or various concentrations of agonist as indicated. Results are expressed as picomoles of cAMP formed minute^{-1} (milligram of protein)$^{-1}$.

Receptor Downregulation. Receptor expression following long-term exposure to agonist was assessed by incubating nearly confluent CHW cells expressing either wild-type or mutated β_2AR with fresh media containing 10% fetal calf serum, 10 μM isoproterenol, and 0.1 mM ascorbic acid for 0–24 h in a 37 °C, 5% CO_2 incubator. Control cells were incubated with serum and ascorbic acid alone. Under these conditions, the level of confluency after agonist exposure was found to be no different as compared to treatment with ascorbic acid alone. Cells were then washed five times with ice-cold PBS, particulates were prepared, and [^{125}I]CYP binding was performed as described above.

Receptor Sequestration. Confluent cells in 24-well tissue culture plates were exposed at 37 °C to fresh serum-free media containing 10 μM isoproterenol for the indicated times. Cells were washed five times with ice-cold PBS and then incubated in monolayers for 3 h at 4 °C with the radioligand [^{3}H]-CGP12177 (6 nM), a hydrophilic βAR antagonist that does not cross the cell membrane and thus detects only cell surface receptors. Cells were then washed three times with cold PBS and solubilized in SDS (1% in PBS) before counting in a scintillation counter. Nonspecific binding was defined by 1 μM propranolol. In separate experiments, recovery from sequestration was determined by exposing cells to 10 μM isoproterenol for 30 min, washing extensively with PBS, and incubating at 37 °C in fresh media for the indicated times. [^{3}H]CGP12177 binding was then carried out as described above. The number of internalized receptors in the basal state was determined using whole cell [^{125}I]CYP binding in the absence and the presence of the hydrophobic antagonist propranolol (0.3 μM) or unlabeled CGP12177 (0.1 μM) as previously described (Green et al., 1993). In addition, internalized receptors were identified by differential centrifugation over 35% sucrose gradients as described elsewhere (Suzuki et al., 1992). The intracellular light vesicular fractions and the plasma membrane fractions were then assayed for the presence of β_2AR by [^{125}I]CYP binding and by Western blots.

Receptor Alkylation and Recovery. Wild-type and mutated β_2AR were irreversibly alkylated using the agent BIM (alkylating pindolol) (Box, 1989). Confluent CHW cells in culture flasks were exposed for 2 h at 37 °C to 10 nM BIM. Typically, this approach resulted in the alkylation of 50–70% of the total number of cellular receptors. Cells were then washed five times with warm PBS and returned to the incubator for the indicated times. Control cells were treated as above, but not exposed to BIM. At the indicated times, cells were washed three times with PBS, particulates were prepared, and receptor density was determined by [^{125}I]CYP binding as described above. In preliminary studies, we found that the appearance of new receptors over time was blocked with the protein synthesis inhibitor cyclohexamide (data not shown).

Northern Hybridization Analysis. Total RNA was prepared from confluent CHW cultures by an acid phenol method (Chomczynski & Sacchi, 1987). A total of 25 μg of total RNA was then electrophoresed in 1.2% agarose/formaldehyde gels and transferred to nylon membranes (Gene Screen, Du Pont NEN) by standard capillary transfer techniques. RNA was fixed to the membranes by UV cross-linking. Commercially available oligonucleotide probes for β_2AR and β-actin were 3′-end-labeled with [α-^{32}P]dATP using terminal transferase (Du Pont-NEN). Hybridizations were performed with these probes at concentrations of 2×10^6 DPM/mL at 45 °C overnight, the membranes were washed twice, and the signals were quantitated using a phosphor imaging system (Molecular Systems, Inc). Changes in β_2AR mRNA were normalized to any changes which occurred in β-actin mRNA.

Western Blots. For Western blots, membranes were prepared from each cell line as described above with the following protease inhibitors included in all steps: 2 μg/mL leupeptin, 0.5 μg/mL aprotinin, 100 μg/mL PMSF, 2 μg/mL benzamidine, and 10 μg/mL soybean trypsin inhibitor.

Pellets were resuspended in electrophoresis sample buffer (50 mM Tris, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) and electrophoresed through 10% SDS–polyacrylamide gels. Proteins were transferred to nitrocellulose (0.45-mm pore size) using the semi-wet technique and probed for β_2AR or Na^+/K^+ ATPase. The β_2AR antiserum was directed against a fusion protein consisting of 86 amino acids (Arg^{328}-Leu^{413}) of the C-terminal tail of the receptor fused with glutathione *S*-transferase using techniques previously described (Liggett et al., 1992) and was provided by R. Lefkowitz. In Western blots, the antiserum identifies several bands in membranes derived from CHW cells transfected with the human β_2AR, consistent with this receptor's multiple glycosylated states. In nontransfected CHW cells, which do not naturally express β_2AR, the antiserum does not identify any proteins (see Results). Antiserum directed against sheep kidney Na^+/K^+ ATPase was used as a positive control and was provided by J. Lingrel. The nitrocellulose membranes were cut into strips, washed with TBS, and blocked by incubation with 3% gelatin in TBS. Strips were then incubated with primary antibody (1:2000 dilution in TBS with 1% gelatin and 0.05% Tween 20) for 4 h at room temperature. Strips were washed twice with TBS and 0.05% Tween 20 and once with TBS and then incubated with the second antibody (goat anti-rabbit IgG complexed to horseradish peroxidase) at a titer of 1:3000 for 1 h. Strips were washed again as above, incubated with Chemoluminescence Reagent (Dupont NEN) for 1 min, dried, and exposed to X-ray film for 20 s.

Materials. [α-^{32}P]ATP, [α-^{32}P]dATP, [^{125}I]cyanopindolol, [^{3}H]CGP12177, and [^{3}H]cAMP were from Du Pont NEN. DMEM and fetal calf serum were from JRH Bioscience. G418 was from GIBCO/BRL. BIM was from Research Biochemicals, Inc. Oligonucleotide probes for β_2AR and β-actin were from Dupont NEN. CHW-1102 cells were from American Type Culture Collection.

Statistical Analysis. Data are presented as means ± SEM. All comparisons were made using paired or unpaired *t*-tests as appropriate. *p* values ≤0.05 were considered significant.

RESULTS

Table 1 shows the pharmacologic properties of Arg16→Gly, Gln27→Glu, and Arg16→Gly + Gln27→Glu compared to wild-type β_2AR. As can be seen, these receptors had agonist binding affinities for isoproterenol and epinephrine that were comparable to those of wild-type β_2AR. Similarly, no impairment of functional coupling to G_s was imparted by these mutations. Basal and maximal isoproterenol-stimulated adenylyl cyclase activities were similar between each mutated receptor and wild-type β_2AR as were the k_{act}'s for isoproterenol stimulation.

Given the potential importance of the N-terminus in membrane insertion and receptor trafficking of G protein-coupled receptors [reviewed in Dohlman et al. (1991)], we focused our attention on these processes, particularly as they relate to agonist regulation of the receptor. Downregulation of net receptor expression, the predominant mechanism in long-term agonist-promoted desensitization of β_2AR, was examined after the exposure of cells expressing each receptor polymorphism to 10 μM isoproterenol for the indicated times. Cell homogenates were then prepared, and β_2AR density was delineated using [^{125}I]CYP radioligand binding. Despite normal agonist binding and coupling to G_s, we found marked differences in the degrees of agonist-promoted downregulation between the different mutated receptors. As shown in Figure 2, wild-type β_2AR underwent 26 ± 3% (n = 6) downregulation at 24 h under these conditions. As compared to wild-type β_2AR, Arg16→Gly underwent a greater degree of down-

Table 1: Pharmacologic Properties of N-Terminal Mutations of β_2AR[a]

	wild-type β_2AR	Arg16→Gly	Gln27→Glu	Arg16→Gly + Gln27→Glu
	Adenylyl Cyclase (pmol min^{-1} mg^{-1})			
basal	1.51 ± 0.19	0.95 ± 0.23	1.65 ± 0.37	3.40 ± 1.00
maximal, iso	5.29 ± 0.88	6.41 ± 0.52	7.49 ± 1.12	9.07 ± 1.79
maximal minus basal	3.78 ± 0.80	5.46 ± 0.45	5.84 ± 0.87	5.66 ± 1.12
K_{act} (nM)	32.8 ± 2.3	59.4 ± 10.7	33.6 ± 5.9	11.3 ± 3.6
	[^{125}I]CYP Binding (K_i, nM)			
isoproterenol	390 ± 160	312 ± 153	250 ± 33	376 ± 139
epinephrine	1070 ± 272	946 ± 260	766 ± 150	930 ± 163

[a] Membranes were prepared from transfected CHW cells expressing wild-type β_2AR or the indicted mutated β_2AR, and adenylyl cyclase and competition binding studies were carried out as described in Experimental Procedures. iso, 10 μM isoproterenol.

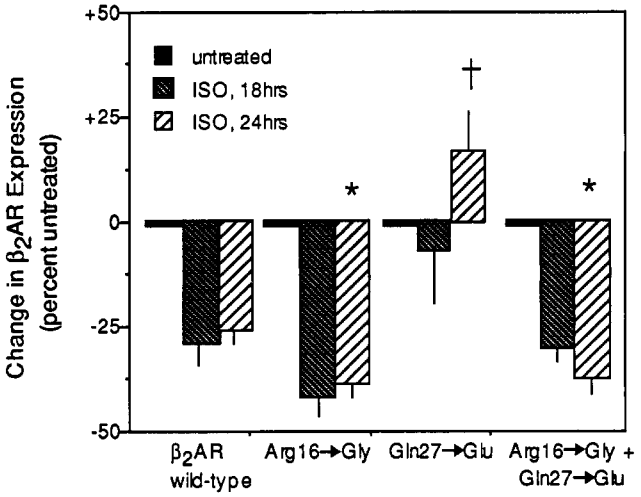

FIGURE 2: Long-term agonist-promoted downregulation of wild-type and mutated β_2AR. Permanently transfected CHW cells expressing the indicated receptors were exposed to 10 μM isoproterenol for the indicated times and washed, and membranes were prepared. Receptor density was determined using [^{125}I]CYP as described in Experimental Procedures. Shown are mean data ± SEM for six experiments: (*) = $p < 0.05$; (†) = $p < 0.01$ as compared to wild-type β_2AR.

regulation (41 ± 3%, n = 6, $p < 0.05$) in parallel studies. A completely different effect was imparted by the mutation in Gln27→Glu, which displayed no downregulation whatsoever (−15.5 ± 9.3%, n = 6, $p < 0.01$ compared to wild-type β_2AR). The receptor with both mutations (Arg16→Gly + Gln27→Glu) also displayed a greater degree of downregulation as compared to that found with wild-type β_2AR (39.0 ± 3.5%, n = 6, $p < 0.05$).

The mechanisms behind these altered degrees of downregulation were explored along several lines. Agonist-promoted downregulation of β_2AR is due to changes both in the amount of cellular β_2AR steady-state mRNA levels and by receptor protein degradation (Collins et al., 1992). As shown in Figure 3, when under the control of the promoter elements in the expression vector pcDNA 1/Neo, wild-type β_2AR downregulation in transfected CHW cells is not accompanied by significant decreases of β_2AR mRNA. Despite the enhanced downregulation of Arg16→Gly and the combination mutation after 24 h of agonist exposure, we observed no differences in this mRNA pattern (Figure 3). Thus, with CHW cells transfected with the β_2AR in this expression vector, receptor degradation appears to be the major mechanism of agonist-promoted downregulation of the wild-type and these two mutated receptors. With the Gln27→Glu polymorphism, a small (<2-fold) increase in β_2AR mRNA

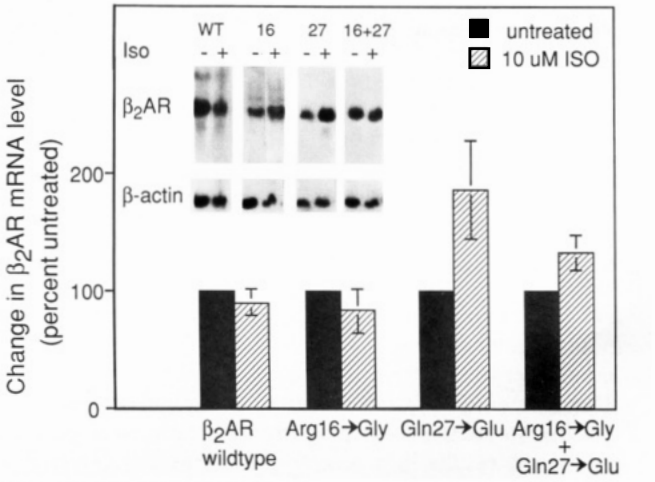

FIGURE 3: Agonist-promoted changes in cellular β_2AR mRNA. CHW cells expressing the indicated receptors were exposed to 10 μM isoproterenol for 24 h, and RNA was prepared, electrophoresed, transferred, and hybridized to ^{32}P-labeled oligonucleotide probes for β_2AR or β-actin as described in Experimental Procedures. Quantitation of hybridization was performed using a phosphor imager. Wild-type β_2AR mRNA did not significantly change after agonist exposure nor did Arg16→Gly or Arg16→Gly + Gln27→Glu. A (1.87 ± 0.43)-fold increase in β_2AR mRNA was observed for the Gln27→Glu β_2AR (p = 0.05 compared to wild type). Shown are mean data ± SEM for three experiments. A typical autoradiograph is shown in the inset.

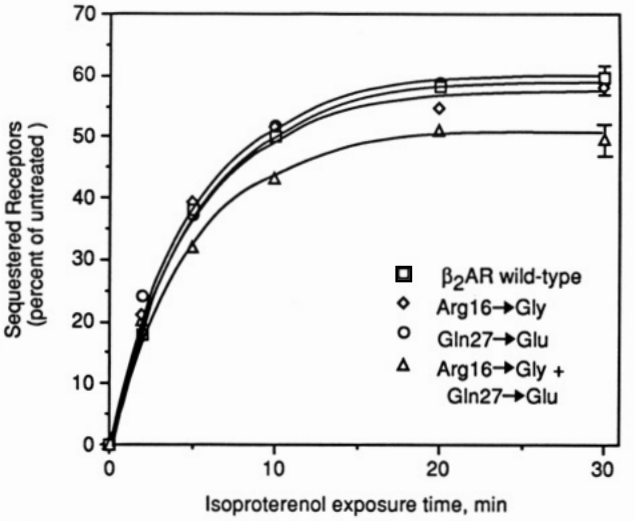

FIGURE 4: Agonist-promoted sequestration (internalization) of wild-type and mutated β_2AR. CHW cells expressing the indicated receptors were exposed to 10 μM isoproterenol for the times shown, and surface β_2AR was determined with a whole cell [^{3}H]CGP12177 radioligand binding assay. The rates of sequestration were identical between all receptors. The maximal extent of sequestration (~59%) was the same between Arg16→Gly, Gln27→Glu, and wild-type β_2-AR. Shown are mean data ± SEM for four experiments. The Arg16→Gly + Gln27→Glu mutation displayed slightly less maximal sequestratin (49 ± 3%, $p < 0.01$) as compared to wild-type β_2AR.

was observed after 24-h agonist exposure (Figure 3).

To delineate whether these mutant receptors underwent different rates of synthesis, receptors were irreversibly alkylated with BIM, and new receptor appearance was quantitated with [^{125}I]CYP binding. The $t_{1/2}$ of new receptor synthesis for wild-type β_2AR was found to be 18.8 ± 4.9 h (n = 4). The N-terminal mutated receptors, when compared to wild-type β_2AR, showed no significant differences in this parameter (Arg16→Gly = 11.8 ± 2.5 h, Gln27→Glu = 15.9 ± 4.6 h, and Arg16→Gly + Gln27→Glu = 22.1 ± 5.8 h).

An early event that occurs during agonist-promoted downregulation of β_2AR is an internalization of receptors (termed sequestration) to a subcellular compartment where they are inaccessible to hydrophilic ligands (Perkins et al., 1991; Liggett & Lefkowitz, 1993). Although the specific pathways have not been fully elucidated, there is evidence to suggest that receptors in this sequestered pool may be trafficked to a degradation pathway under conditions of prolonged agonist exposure (Perkins et al., 1991; von Zastrow & Kobilka, 1992). We therefore examined agonist-promoted sequestration of these mutated receptors using the hydrophilic radioligand [^{3}H]-CGP-12177. As shown in Figure 4, the rates of agonist-promoted sequestration were identical between the different mutated receptors and wild-type β_2AR. The rate constants for wild-type β_2AR, Arg16→Gly, Gln27→Glu, and the combination mutation were 0.19 ± 0.01, 0.23 ± 0.01, 0.22 ± 0.02, and 0.22 ± 0.02 min^{-1}, respectively (n = 4–6, p = NS). The maximal extents of sequestration were also the same between wild-type β_2AR (59 ± 2%), Arg16→Gly (58 ± 1%), and Gln27→Glu (59 ± 3%) and statistically slightly less for the combination mutation (49 ± 3%, n = 6, $p < 0.01$). Since mutations in other regions of the β_2AR N-terminus have been reported to alter the distribution of cell surface versus internalized receptor in the basal state (Dixon et al., 1987; Rands et al., 1990), we examined this distribution using several approaches. Using differential centrifugation, light vesicle fractions (representing internalized receptors) and plasma membrane fractions were assayed for β_2AR using [^{125}I]CYP binding (Table 2). As shown, the majority of wild-type β_2AR were expressed on the cell surface. The polymorphic forms

Table 2: Cellular Distribution of β_2AR in Transfected CHW Cells[a]

cell fraction	wild-type β_2AR	Arg16→Gly	Gln27→Glu	Arg16→Gly + Gln27→Glu
intracellular (light vesicle)	103 ± 1	96 ± 4	77 ± 2	69 ± 4
cell surface (plasma membrane)	9450 ± 300	9140 ± 160	6200 ± 220	6390 ± 160

[a] Membrane and vesicular fractions were separated by centrifugation over sucrose gradients as described in Experimental Procedures. β_2AR in each fraction was quantitated by radioligand binding, and the results were expressed as fmol of [^{125}I]CYP binding sites/fraction. Shown are the mean ± SEM from three experiments.

of β_2AR showed no deviation from this pattern. Similar findings were obtained when the two fractions were subjected to Western blots using β_2AR antisera (data not shown). Finally, using whole cell radioligand binding studies with [^{125}I]CYP and the hydrophobic antagonist propranolol and the hydrophilic antagonist CGP12177, we also detected no accumulation of the intracellular receptor with any of the polymorphisms (data not shown).

We also considered that these N-terminal mutations may have caused changes in the conformation of the receptor, which could result in altered movement into the degradation pathway or altered rates of degradation during agonist-promoted downregulation. To address this, Western blots were performed using antisera directed against the C-terminal tail of the human β_2AR. As depicted in Figure 5, several specific bands were identified in cells transfected with wild-type β_2-AR. (As is shown, nontransfected CHW cells, which do not express endogenous β_2AR, gave no signal.) Predominant bands at 91, 72, 67, and 53 kDa were identified with wild-type β_2AR, and the same pattern was observed with Arg16→Gly. However, the electrophoretic mobility of Gln27→Glu under these conditions was markedly different than that of the wild-type β_2AR as shown in Figure 5. Predominant bands were at 62, 58, and 46 kDa. The combination mutation showed bands in yet another pattern, composed of six bands at the molecular masses shown. The altered electrophoretic mobilities of these two polymorphic β_2AR proteins are in contrast

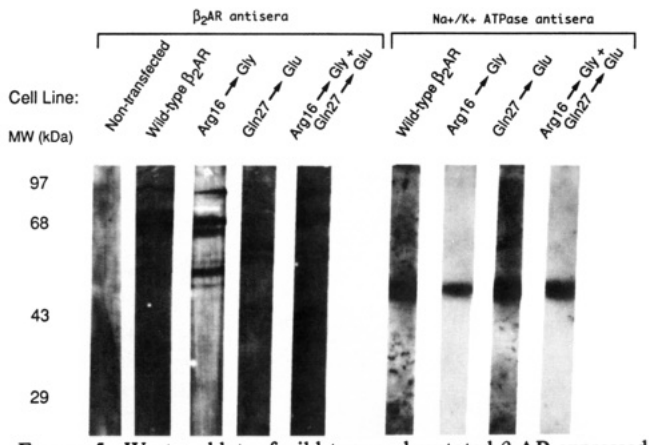

FIGURE 5: Western blots of wild-type and mutated β_2AR expressed in CHW cells. An antibody against the β_2AR indicates an altered mobility of the Gln27→Glu and Arg16→Gly + Gln27→Glu mutation compared to wild-type β_2AR. In contrast, the Na^+/K^+ ATPase (probed as a control) migrated at identical molecular masses for all four cell lines. Shown are data from a single experiment representative of four such studies performed.

to what was observed in Western blots using an antibody directed against the Na^+/K^+ ATPase, which was used as a control for each cell line. As is shown, in parallel experiments membranes from all four cell lines expressing the different receptors displayed a band at the same molecular mass of 48 kDa.

DISCUSSION

β_2ARs are dynamically regulated by a number of processes in virtually every system studied, including *in vivo* human and animal settings and cell culture systems (Liggett & Lefkowitz, 1993; Hausdorff et al., 1990). One important form of regulation is that induced by agonists whereby, during continuous exposure, mechanisms are evoked that serve to limit the cellular response over time, a phenomenon termed desensitization. A major mechanism responsible for long-term agonist-promoted desensitization of β_2AR is receptor downregulation (Liggett & Lefkowitz, 1993; Collins et al., 1992). Under conditions of increased endogenous agonist, such as prolonged neuronal firing or elevated systemic catecholamines, this process may serve to limit the end-organ response *in vivo*. Desensitization may also limit the utility of administered therapeutic agonists. Even under steady-state conditions (i.e., when the sympathetic nervous system is not extensively activated), *in vivo* studies have shown that β_2ARs appear to be under constant dynamic regulation (Insel, 1987). Interestingly, for many years it has been known that there is a wide variation in β_2AR expression between apparently matched normal human subjects (Liggett et al., 1989; Rosen et al., 1984). The data that we have presented in the current study may partially explain this interindividual variation in β_2AR expression.

We have recently identified six different polymorphic forms of β_2AR in man (Reishaus et al., 1993). These consist of the wild-type as delineated in the original cloning of the human β_2AR (Kobilka et al., 1987), two polymorphisms or a combination of the two in the N-terminus as described above, and two polymorphisms in transmembrane regions (Green et al., 1993). Val34→Met (in the first transmembrane spanning region) appears to be a silent mutation, while Thr164→Ile (in the fourth transmembrane spanning region) results in a receptor with markedly altered ligand binding and coupling properties (Green et al., 1993). Here, we now report the consequences of the N-terminal polymorphisms at amino acids 16 and 27. As expected, these mutations did not result in significant alterations in agonist binding or activation of the receptor, since the transmembrane spanning regions and the intracellular portions of the receptor appear to be the primary determinants of these functions, respectively (Liggett & Raymond, 1993; Dohlman et al., 1991). The N-terminus of β_2AR has not been as extensively studied as these other receptor domains. Studies have suggested though that the N-terminus plays an important role in cell sorting and membrane insertion of the receptor. Mutated β_2AR lacking amino acids 21–30 have been reported to be poorly processed by the cell to the mature protein (Dixon et al., 1987). In another series of mutations, where amino acid residues 6–15 were deleted, no immunoreactive protein of the mature receptor was detected (Dixon et al., 1987). Removal of asparagine-linked glycosylation sites of the N-terminus by substitution of alanine for asparagine results in a receptor which accumulates intracellularly, implying an altered ability of the receptor to be inserted into the membrane (Rands et al., 1990). We found no such accumulation in the internal pool with any of our N-terminal mutated receptors nor an alteration in the rate or extent of agonist-promoted receptor internalization.

In the current study, we have shown that the Arg16→Gly, Gln27→Glu, and Arg16→Gly + Gln27→Glu polymorphisms result in clearly different properties of agonist-promoted downregulation. With the Arg16→Gly polymorphism, agonist-promoted downregulation was enhanced compared to wild-type β_2AR. In contrast, the Gln27→Glu polymorphism was found to be resistant to such downregulation. The combination polymorphism displayed enhanced downregulation. These differences could not be accounted for by alterations in ligand binding, coupling to G_s, the rates of receptor synthesis, or the rates of agonist-promoted sequestration. We found no significant agonist-promoted decreases of steady-state mRNA levels with wild-type β_2AR or the Arg16→Gly and Arg16→Gly + Gln27→Glu polymorphisms. Thus, downregulation of cellular β_2AR in CHW cells transfected with constructs based on the vector pcDNA 1/Neo is not due to changes in mRNA and is likely to be due to protein degradation. Interestingly, a small increase (<2-fold) in mRNA was noted with the Gln27→Glu polymorphic β_2AR during agonist exposure. Whether this small difference is responsible for the lack of downregulation of this polymorphism during agonist exposure seems unlikely. Given that each of these receptor cDNAs was inserted in the exact same location within the expression vector pcDNA 1/Neo, transcriptional differences are likewise unlikely to be responsible for the altered degrees of agonist-promoted downregulation observed in these transfected cells. Similarly, in cells which naturally express β_2-AR, long-term agonist-promoted downregulation does not appear to be regulated at the transcriptional level (Hadcock et al., 1992). In addition, the Gln27→Glu receptor, based on an altered electrophoretic mobility, does not appear to reach the mature wild-type conformation, and this may result in an altered ability of this receptor to be degraded. The mechanisms for the more profound downregulation found with Arg16→Gly and the combination mutation are not clear, but given that both of these receptors underwent this exaggerated response, it is suggested that the presence of Gly in position 16 overcomes any effects on downregulation imparted by the mutation at position 27.

The findings of specific molecular regions of the β_2AR protein that are important for agonist-promoted downregulation is not without precedence. Using site-directed mutagenesis of wild-type β_2AR with expression in CHW cells, several groups have identified regions that appear to play roles in the downregulation process. These include a serine within

the PKA consensus sequence in the third intracellular loop (Bouvier et al., 1989), tyrosines at positions 350 and 354 of the cytoplasmic tail (Valiquette et al., 1990, 1993), the palmitoylated cysteine at position 341 of the cytoplasmic tail (Campbell et al., 1990), and the N- and C-terminal portions of the third intracellular loop (Campbell et al., 1990). In none of these cases though has a mechanism by which a mutation imparts impaired downregulation been delineated. This is primarily because the events involved in the protein degradation pathway of agonist-promoted downregulation of β_2AR are poorly understood. We provide evidence here that regions in the N-terminus of the receptor also appear to be important for this process and, furthermore, that common polymorphisms in the N-terminus either enhance or impair agonist-promoted downregulation.

The current study has several limitations. First, the *in vitro* design of the study raises questions regarding the applicability of our results to β_2AR expressed on native cells. Our primary goal was to compare the function and regulation of the various β_2AR variants at the receptor level. Since naturally occurring cell lines expressing each polymorphic β_2AR are not currently available, such comparisons necessitated using the recombinant approach described herein. Secondly, we chose to make our comparisons using cell lines expressing β_2AR at equivalent levels. Because some cell lines did not express these polymorphic receptors at low receptor densities, this resulted in studying regulation events at expression levels somewhat higher than that observed in most human tissues. However, our preliminary studies demonstrated that, even over wide ranges of wild-type receptor density (300–2700 fmol/mg), the extent of agonist-promoted downregulation was not related to the basal receptor expression. A similar lack of dependency of downregulation on receptor expression in CHW cells has also been reported by others (Campbell et al., 1990). Finally, we recognize that the use of viral promoters in the current study limits the applicability of detailed transcriptional analysis in this recombinant system. This limitation does not in and of itself detract from our study, since each polymorphic β_2AR was always compared to an otherwise identical wild-type construct.

In summary, naturally occurring polymorphisms of the human β_2AR impart altered agonist-promoted receptor downregulation. This altered regulation included an enhancement of downregulation in two polymorphisms and a complete absence of downregulation in one as compared to wild-type β_2AR. These alterations were of a distinct nature, in that ligand binding and functional activation of these receptors were not affected. These polymorphisms, which occur at frequencies ranging from ~10% to ~50% of the population (Reishaus et al., 1993), may be responsible for the *in vivo* interindividual variation in expression, regulation, and function of β_2AR.

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