Role of the Amino Terminus of the Third Intracellular Loop in Agonist-Promoted Downregulation of the α_{2A}-Adrenergic Receptor†

Elizabeth A. Jewell-Motz, Elizabeth T. Donnelly, Margaret G. Eason, and Stephen B. Liggett*

Departments of Medicine and Molecular Genetics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

Received March 3, 1997; Revised Manuscript Received May 19, 1997⊗

ABSTRACT: A prominent feature of long-term regulation of the α_{2A}-adrenergic receptor (α_{2A}AR) is a loss of cellular receptors over time (downregulation). The molecular determinants of downregulation were sought by targeting regions of the receptor involved in G protein coupling and phosphorylation. Mutated receptors, consisting of chimeric substitutions of analogous β_2-adrenergic receptor (β_2AR) and serotonin 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) receptor sequence into the second intracellular loop (ICL2) (residues 113−149), the amino terminus (residues 218−235) and carboxy terminus (residues 355−371) of ICL3, and a deletion of the β-adrenergic receptor kinase (βARK) phosphorylation sites in the third intracellular loop (ICL3) (residues 293−304), were expressed in Chinese hamster ovary (CHO) cells. Wild-type α_{2A}-AR underwent 31% ± 3% downregulation after 24 h of exposure to 100 μM epinephrine. Loss of downregulation was observed with some mutants, but this was not related to functional coupling to inhibitory or stimulatory guanine nucleotide regulatory binding proteins (G_i or G_S) or to phosphorylation. Rather, any mutant with a substitution of the amino terminus of ICL3 (regardless of whether the substitution was with β_2AR or 5-HT$_{1A}$ sequence) resulted in upregulation. Studies with an inhibitor of protein synthesis indicated that the primary mechanism of downregulation of the α_{2A}AR is agonist-promoted degradation of receptor protein which requires a destabilization sequence in the amino terminus of ICL3. Thus, in contrast to other G protein-coupled receptors, in which G protein coupling or phosphorylation are critical for long-term agonist regulation, the α_{2A}AR has a specific structural domain distinct from these other functional regions that serves to direct agonist-promoted downregulation.

The phenomenon of desensitization, or tachyphylaxis, occurs with many G protein-coupled receptor systems and is defined as the waning of signal transduction despite continued presence of agonist. Several distinct processes have been found within the superfamily that are responsible for desensitization (reviewed in refs *1* and *2*). In some receptors, phosphorylation of serine or threonine residues by G protein-coupled receptor kinases (GRKs)[1] or second messenger-dependent kinases, such as protein kinase A or C, leads to rapid uncoupling of receptor from G protein. With agonist exposures on the order of minutes to hours, some G protein-coupled receptors undergo internalization (sequestration) of receptors into an intracellular compartment. Depending on the extent of sequestration and degree of receptor reserve, sequestration may result in further dampening of the signal. Sequestration has also been proposed to provide a mechanism by which phosphorylated receptors can become dephosphorylated and thereby recycled back to the cell surface for reactivation. Finally, after prolonged agonist exposure (i.e., hours), the net number of cellular receptors can decrease, a process that has been termed downregulation.

While many G protein-coupled receptors have been examined for their ability to undergo agonist promoted downregulation, relatively little is known about the molecular determinants within these receptors that are required for the process. For the β_2-adrenergic receptor (β_2AR), which is one of the most extensively studied, it has been shown that the ability to couple to the stimulatory G protein (G_S) is required for full downregulation of the receptor to occur (*3*). It has also been suggested that sites for protein kinase A (*4*), a region of the cytoplasmic tail (*5*), and cytoplasmic tyrosine residues (*6*) are necessary for downregulation of the β_2AR. Recent studies have also indicated that GRK-mediated phosphorylation and β-arrestin binding are important components of agonist-mediated β_2AR trafficking (*7*). With the m3 muscarinic acetylcholine receptor, carboxy terminal threonine residues appear to be necessary for agonist-promoted downregulation of this receptor (*8*), but whether this is due to phosphorylation at these sites is not known. In the m1 muscarinic receptor, a serine-rich region in the midportion of the third intracellular loop has been shown to be critical for agonist-promoted sequestration and/or downregulation (*9*, *10*). Little is known regarding the structural requirements for agonist-promoted downregulation of any of the three α_2AR subtypes. In the only study published to date, the palmitoylcysteine of the α_{2A}AR has been shown to be necessary for downregulation of this receptor (*11*). However, since other receptors also contain this cysteine but do not undergo downregulation, it has been concluded that this covalent modification may be necessary, but is not sufficient, for downregulation to occur. In the present study, we have investigated both the structural and

†Supported by NIH Grants HL53436 and HL07382.

* Correspondence should be addressed to this author at University of Cincinnati College of Medicine, 231 Bethesda Ave., ML 0564, Room 7511, Cincinnati, OH 45267. Phone 513-558-4831; Fax 513-558-0835.

⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1997.

[1] Abbreviations: AR, adrenergic receptor; 5-HT, 5-hydroxytryptamine; GRK, G protein-coupled receptor kinase; βARK, β-adrenergic receptor kinase; G_i, inhibitory guanine nucleotide regulatory binding protein; G_S, stimulatory guanine nucleotide regulatory binding protein; CHO cells, Chinese hamster ovary cells; ICL, intracellular loop.

S0006-2960(97)00487-X CCC: $14.00

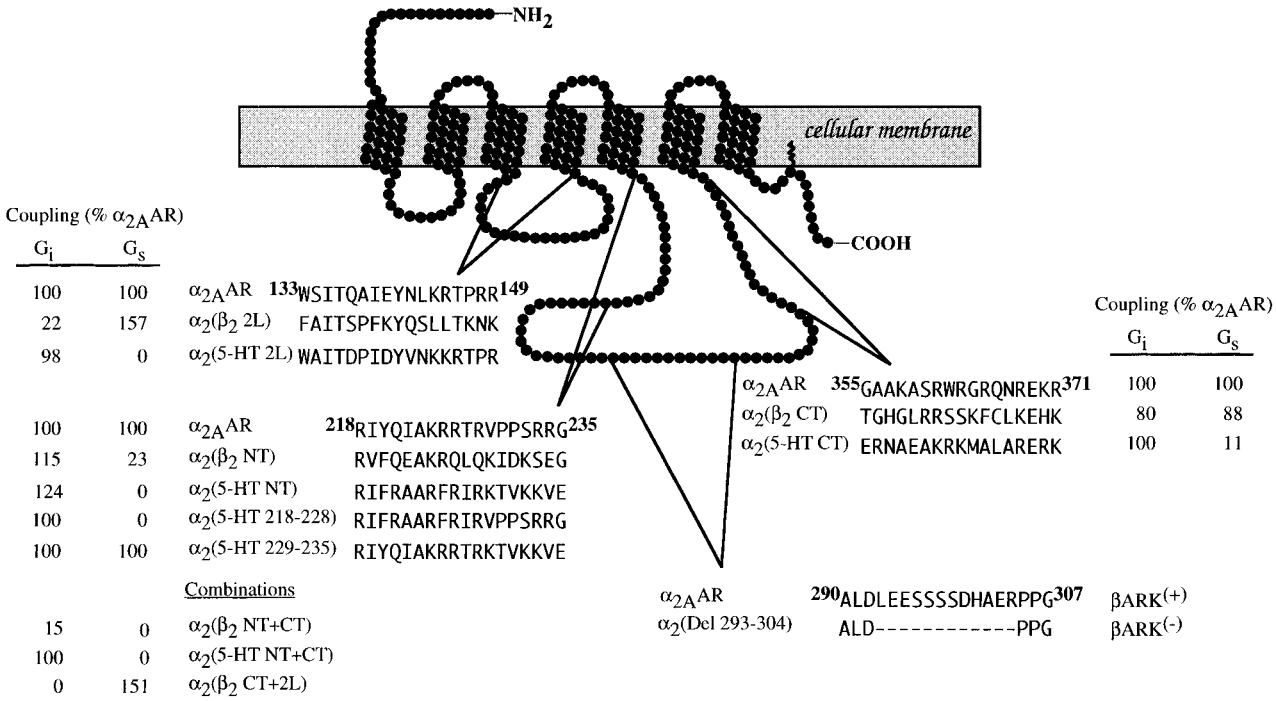

FIGURE 1: Summary of mutated α_{2A}ARs and their functional characteristics. As indicated, amino acid sequences from the β_2AR or the serotonin 5-HT_{1A} receptor were substituted into the α_{2A}AR at either the second intracellular loop (amino acids 133−149) or the extreme amino or carboxy termini (amino acids 218−235 or 355−371, respectively). As shown, α_{2A}AR with β_2AR or 5-HT_{1A} receptor sequences substituted for the second intracellular loop are denoted $\alpha_2(\beta_2$ 2L) or α_2(5-HT 2L), respectively. α_{2A}AR with either the amino or carboxy termini of the third intracellular loop substituted with the corresponding amino acids from the β_2AR are referred to as $\alpha_2(\beta_2$ NT) or $\alpha_2(\beta_2$ CT), respectively, and those substituted with 5-HT_{1A} receptor sequences are called α_2(5-HT NT) or α_2(5-HT CT). Two additional substitutions were made in the amino-terminal region of the third intracellular loop such that only amino acids 218−228 of the α_{2A}AR were substituted with 5-HT_{1A} sequences [α_2(5-HT 218−228)] or only amino acids 229−235 were substituted [α_2(5-HT 229−235)]. Additionally, three mutant receptors containing combinations of these substitutions were made and are listed under Combinations. The functional consequences of each of these substitutions with respect to G protein coupling are indicated adjacent to the descriptions of these substitution mutants expressed as the percent of wild-type coupling to G_i or G_S as reported (*13*, *16*). One additional mutated α_{2A}AR utilized in this study contains a 12 amino acid deletion within the third intracellular loop from amino acids 293−304 [α_2(Del 293−304)]. This deletion encompasses the four serines that have previously been shown to be sites for βARK phosphorylation (*12*).

functional requirements for downregulation of the α_{2A}AR. By utilizing chimeric receptors consisting of mutations in G protein coupling domains (the second intracellular loop and the amino-and carboxy-terminal portions of the third intracellular loop) and the GRK phosphorylation sites, we were able to determine a critical region of the receptor that is required for agonist-promoted downregulation, potentially acting as a destabilizing motif.

EXPERIMENTAL PROCEDURES

Construction of Chimeric or Mutated Receptor cDNAs. The construction of the chimeric α_{2A}AR and a third intracellular loop deletion mutant have been previously described (*12*, *13*). Briefly, using a combination of site-directed mutagenesis and cassette substitution of annealed oligonucleotides, the second intracellular loop, the amino terminus of the third intracellular loop or the carboxy terminus of the third intracellular loop were substituted either individually or in combination with analogous regions of the 5-HT_{1A} receptor or β_2AR. A PCR-based strategy was used to construct the deletion mutant Del(293−304), which removes the four serines that undergo agonist-promoted phosphorylation by βARK. Mutations were verified by sequence analysis and the coding block was subcloned into the expression vector pBC12BI as described. Figure 1 summarizes the mutations and the nomenclature utilized in the current study.

Tissue Culture and Transfection. Permanent CHO cell lines expressing wild-type or mutated receptors were generated using a calcium phosphate precipitation transfection procedure (*13*). Cells were transfected with the appropriate α_{2A}AR construct and pSV_2neo and selected in 1 mg/mL G418. Transfectants were then screened for receptor expression via radioligand binding. Transfected cells were maintained as monolayers in HAM's F12 medium supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 80 μg/mL G418 at 37 °C in a 5% CO_2 atmosphere.

Assessment of Receptor Downregulation. CHO cells expressing wild-type or mutant receptors at ~95% confluency were placed in serum-free HAM's F12 medium plus 100 μM ascorbic acid and exposed to the indicated concentrations of epinephrine for the indicated times up to 24 h. The plates were then washed five times with room-temperature PBS, scraped in cold 5 mM Tris, pH 7.4, and 2 mM EDTA, and centrifuged at 40000*g* for 10 min at 4 °C. The pellet was resuspended in 5 mL of the same buffer and homogenized with a Brinkman polytron for 15 s at 50% maximal speed. The sample was then brought up in 10 mL of the same buffer, centrifuged again, and resuspended in 75 mM Tris, 12 mM $MgCl_2$, and 2mM EDTA, pH 7.4. Receptor expression was then determined in the presence of 100 μM GTP using saturating concentrations (25 nM) of [^{3}H]-yohimbine. Phentolamine (100 μM) was used to define

nonspecific binding. Incubations were carried out in triplicate at 37 °C for 30 min, the reactions were terminated by dilution in ice-cold 10 mM Tris buffer, pH 7.4, and then filtered over Whatman GF/C glass filters to separate bound from free radioligand. The filters were then counted in the presence of a xylene-based cocktail in a liquid scintillation counter. Specific binding was defined as the difference between total and nonspecific binding and was normalized to protein. As previously shown, agonist-promoted downregulation of the α_{2A}AR is not associated with a change in the k_D for the radioligand, and the use of a single saturating concentration of [^{3}H]yohimbine accurately quantitates changes in receptor number (*11*, *14*). Agonist-promoted sequestration was assessed using a whole-cell [^{3}H]yohimbine binding assay as described (*11*). At least two separate clonal isolates (expression levels between ~700 and 2000 fmol/mg) were examined for each receptor to control for any potential clonal variation.

Western Analysis. CHO cells expressing the indicated receptors were exposed to vehicle alone or vehicle plus 100 μM epinephrine for 24 h. Cells were washed five times with ice-cold PBS, scraped in 5 mM Tris and 2 mM EDTA, pH 7.4, which included protease inhibitors (10 μg/mL benzamidine, 10 μg/mL soybean trypsin inhibitor, and 5 μg/mL leupeptin) and centrifuged at 40000*g* for 10 min at 4 °C. Pellets were resuspended in a volume of buffer to equalize the protein concentrations and then an equal volume of 2× SDS−PAGE stop buffer was added. Samples were sonicated and equal amounts of protein were fractionated on an SDS−10% polyacrylamide gel. Proteins were transferred to nitrocellulose (Protran, Schleicher & Schuell) overnight at 30 V using a Bio-Rad transfer apparatus. The Western blot was performed by blocking the filter with 5% nonfat dry milk and then incubating with a 1:6000 dilution of an α_{2A}-AR-specific polyclonal antiserum as previously described (*15*). The blot was then incubated with an anti-rabbit horseradish peroxidase-conjugated second antibody (1:1000) and developed using enhanced chemiluminescence (Dupont−NEN).

Miscellaneous. Data were analyzed by paired or unpaired *t*-tests as indicated, with significance imparted when *p* values were less than 0.05. The sources of materials not specifically indicated are as indicated previously (*13*, *15*, *16*).

RESULTS

We have previously shown that α_{2A}AR expressed in CHO cells undergo ~30−40% downregulation due to agonist exposure for 24 h (*11*, *14*). Time-course and dose−response studies indicated that downregulation is maximal by ~18 h of exposure to epinephrine at concentrations between 10 and 100 μM (data not shown). Thus for the current study 24 h exposures to 100 μM epinephrine were routinely used to study downregulation. Figure 1 depicts the mutated receptors that were studied. Based on the possibility that downregulation is dependent on functional G protein coupling, the majority of these mutations disrupted potential coupling domains. As previously delineated, the α_{2A}AR couples not only to G_i with inhibition of adenylyl cyclase but also weakly to G_S, resulting in stimulation of adenylyl cyclase (*16*, *17*). The rationale behind construction of the chimeric receptors was to substitute analogous regions of the 5-HT$_{1A}$ receptor (which couples to G_i but not G_S) or the β_2AR (which couples to G_S but not G_i) into the second intracellular loop and the amino and carboxy termini of the third intracellular loop of the human α_{2A}AR. The results of such perturbations on G protein coupling of the α_{2A}AR as previously published (*13*, *16*) are summarized in Figure 1. As can be seen, the panel of mutated receptors used in the current study allows us, in some instances, to separate the importance of functional coupling versus structural localization within the receptor. For example, G_S coupling can be disrupted by substitution of the second intracellular loop or the amino terminus of the third intracellular loop. It should be noted that agonist binding affinities are preserved in all these mutated receptors (*13*).

The results from downregulation studies are shown in Figure 2, with the receptors grouped according to their G protein coupling status in the top panel. (Note that for Figure 2, the asterisk denotes a difference compared to the extent of receptor loss observed with the wild-type α_{2A}AR.) α_2(5HT CT), α_2(Del 293−304), α_2(5HT 2L), α_2(β_2 CT + 2L), and α_2(β_2 2L) all displayed agonist-promoted downregulation, albeit the latter receptor had a somewhat attenuated response as compared to wild-type α_{2A}AR. In contrast, α_2(β_2 NT + CT), α_2(5HT NT), α_2(β_2 NT), α_2(β_2NT + CT), α_2(5HT NT + CT), and α_2(5HT 218−228) failed to undergo downregulation and in fact displayed upregulation to various extents. From this analysis it is clear that downregulation was not dependent upon whether the receptor functionally coupled to G_i or to G_S, nor was it dependent on the sequence (5HT receptor or β_2AR) that was substituted. When grouped according to the location of the mutation, however, a pattern becomes apparent (Figure 2, bottom panel). As can be seen, any receptor with substitutions in the amino terminus of the third loop lacked downregulation. This included those that comprised substitutions with 5-HT$_{1A}$ or β_2AR sequence and was independent of whether G_i or G_S coupling was altered. Two additional chimeras contain smaller substitutions of 5-HT$_{1A}$ sequences. One mutant substituted only the first 10 amino acids [α_2(5-HT 218−228)] of the 17 substituted in the α_2(5-HT NT) mutant and the other substituted the last seven residues [α_2(5-HT 229−235)]. Neither of these chimeras underwent downregulation, suggesting that the structural integrity of the entire region is important for evoking downregulation. Also, removal of the GRK phosphorylation sites, in mutant α_2(Del293−304), had no effect on downregulation (Figure 2, bottom panel).

With a critical region established for agonist regulation, further studies were carried out to investigate the mechanisms by which amino-terminal substitutions altered downregulation. First, in order to be assured that true upregulation of receptor expression occurred in the amino terminus mutants and to assess whether the potentially newly synthesized protein consisted of a single species, Western blots were carried out with cells expressing wild-type and the α_2(β_2-NT) mutant receptors in the absence or presence of a 24 h exposure to agonist. As shown in Figure 3, wild-type α_{2A}-AR migrates as a somewhat broad band centered at ~73 kDa, and downregulation of receptor protein as identified by immunoblotting occurs with long-term agonist exposure. Consistent with the agonist-promoted upregulation observed in radioligand binding studies, the α_2(β_2NT) receptor displayed an increase in receptor protein with agonist exposure as detected by Western blotting, with no evidence of immature or aberrant forms.

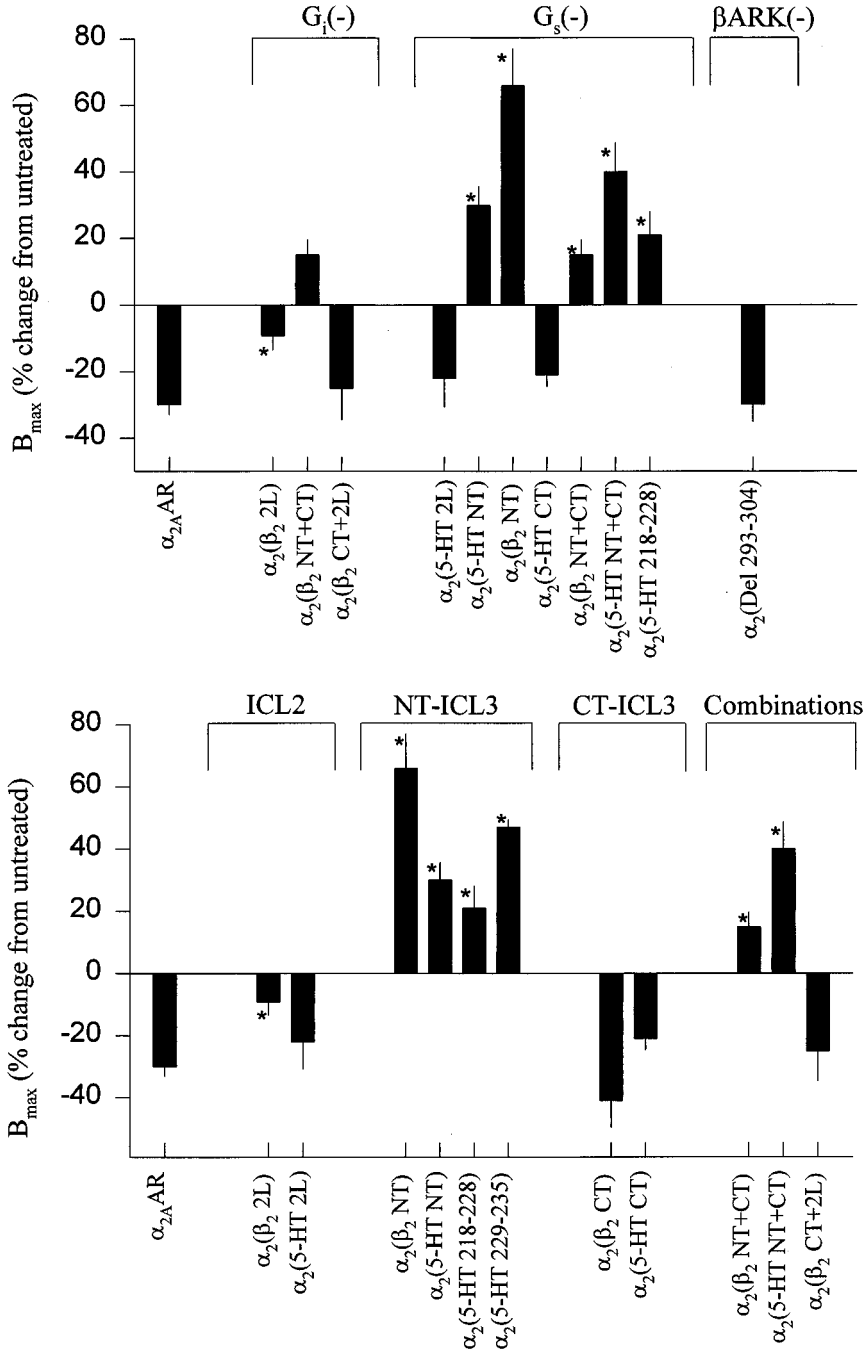

FIGURE 2: Consequences of functional (top) or structural (bottom) alterations on downregulation of the α_{2A}AR. Cells were treated with and without agonist for 24 h prior to determination of receptor density. Results are shown as percent change in B_{max} for agonist-treated cells relative to that for nontreated cells. In the top graph, the α_{2A}AR substitution mutations studied have been grouped according to their functional characteristics [i.e., $G_i(-)$, $G_S(-)$, or βARK(−)]. No relationship was found between receptor function and downregulation. In the bottom graph, the α_{2A}AR substitution mutants have been grouped according to the location of the substitutions. While second loop substitutions (indicated by ICL2) and substitutions localized at the carboxy terminus of the third intracellular loop (indicated by CT-ICL3) do not establish a pattern of a loss of downregulation, substitutions at the amino terminus of the third intracellular loop (NT-ICL3) clearly affect downregulation. Furthermore, downregulation is also abolished in any combination mutant receptor containing an amino-terminal substitution. All receptors displayed agonist-induced changes (increases or decreases) in receptor number that were statistically significant as compared to the untreated condition. Asterisks indicate $p < 0.05$ *as compared to downregulation of the wild-type* α_{2A}*AR.*

One potential mechanism by which downregulation could be altered in these mutants would be if agonist-promoted sequestration was perturbed. However, the α_{2A}AR undergoes very little agonist-promoted sequestration as compared, for example, to the β_2AR or the α_{2B}AR (*18*). We nevertheless assessed this parameter with the $\alpha_2(\beta_2$NT) receptor and wild-type α_{2A}AR and found no difference in the small degree of sequestration that is evident after 30 min of agonist exposure (15% ± 5% vs 14% ± 1%, respectively).

We considered, then, that the upregulation of receptors such as the $\alpha_2(\beta_2$NT) mutant is due to a perturbation of the rates of synthesis and degradation of this receptor in the presence of agonist. Agonist exposure studies in the presence of the protein synthesis inhibitor cycloheximide are shown in Figure 4. With wild-type receptor, exposure of cells to cycloheximide alone results in a loss of receptor number. This indicates that in the basal state there is some degree of new receptor synthesis that is necessary to maintain a

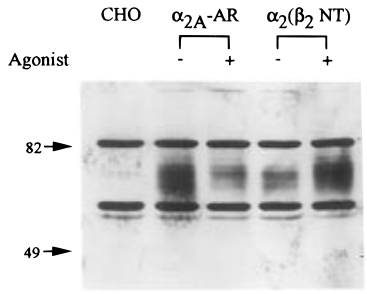

FIGURE 3: Western analysis of wild-type α_{2A}AR and $\alpha_2(\beta_2$ NT) following treatment with and without agonist for 24 h. Shown is a representative experiment illustrating the decrease in total receptor number from the cell membrane for the wild-type receptor and the apparent increase in receptor for $\alpha_2(\beta_2$ NT). The lane marked CHO contains an equivalent amount of protein from nontransfected CHO cells.

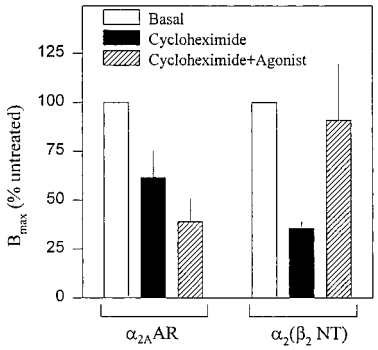

FIGURE 4: Agonist-exposure studies of the wild-type α_{2A}AR and the $\alpha_2(\beta_2$ NT) mutant in the presence of cycloheximide. Cells were treated with and without 10 μg/mL cycloheximide for 2 h prior to the addition of agonist for 24 h. Cells were then harvested and maximal binding determined as described under Experimental Procedures. The results are expressed as percent basal B_{max}. Relative to the basal state, cycloheximide alone and with agonist decreased receptor number with the wild-type α_{2A}AR. In contrast, cycloheximide + agonist had no statistically significant ($P = 0.66$) effect on receptor number with the $\alpha_2(\beta_2$ NT) mutant. See Results and Discussion sections for interpretation.

constant level of expression and, by inference, there is a basal level of ongoing degradation that is present under these conditions. In the presence of cycloheximide and agonist, a further downregulation of ~40% occurred, indicating that, with the wild-type receptor in CHO cells, downregulation represents enhanced protein degradation rather than a decrease in receptor synthesis. These same studies carried out with the $\alpha_2(\beta_2$NT) mutant indicated that about the same level of receptor synthesis and degradation is present in the basal state (indicated by the loss of receptor expression in the presence of cycloheximide) of this receptor as in wild type. However, downregulation fails to occur in the presence of agonist during synthesis inhibition, indicative of a lack of agonist-promoted receptor degradation with this mutant in the agonist-bound state.

We wondered whether the depressed receptor degradation observed with the upregulation mutants might be due to these receptors being stabilized against degradation by bound ligand. Such stabilization would be consistent with our observation that agonist-dependent upregulation is not dependent on G protein coupling. We considered, then, that since achievement of receptor activation is not required, upregulation in these mutant receptors might also be evoked via stabilization by antagonist binding. The results from studies exploring this are shown in Figure 5. Incubation of

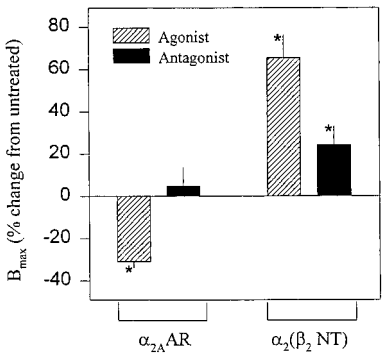

FIGURE 5: Effects of antagonist on receptor number. Cells were treated with and without epinephrine or phentolamine for 24 h prior to harvesting. Maximal binding was then determined as described under Experimental Procedures. Results from these experiments are shown as the percent change in B_{max} relative to untreated cells. While there is no effect on receptor expression for the wild-type α_{2A}AR following treatment with the antagonist phentolamine, there is a significant increase in receptor number for the $\alpha_2(\beta_2$ NT) mutant receptor. Asterisks indicate $p < 0.05$ compared to untreated.

the $\alpha_2(\beta_2$NT) receptor with the antagonist phentolamine indeed resulted in upregulation of the receptor, although not quite to the same extent as agonist. On the other hand, phentolamine had no effect on wild-type receptor expression.

DISCUSSION

The mechanisms and molecular determinants of agonist-promoted downregulation of the α_{2A}AR are not known. Although there is some evidence that elements in the 5′ untranslated region may influence transcription (*19*), studies in transfected and endogenously expressing cells indicate that posttranscriptional mechanisms are at play in the loss of receptor number observed during prolonged agonist exposure. Little is known about the structural requirements for downregulation of the α_{2A}AR. In the current study we have utilized various mutations of the α_{2A}AR that allow for a dissection of the structural and functional requirements for downregulation. We considered that it was not unreasonable to expect that some of the paradigms established for downregulation of β_2AR or m1 or m3 muscarinic receptors would be relevant to the α_{2A}AR. This did not turn out to be the case. Given that phosphorylation by GRKs is the most rapid regulatory event that occurs after agonist exposure, and the sites in the α_{2A}AR for such phosphorylation are known (*12*), downregulation was assessed in the α_2(Del 293−304) mutant, which lacks all four serines phosphorylated in the third intracellular loop. The downregulation process, however, was not impaired. Thus, while downregulation can be imparted to the β_3AR by substitution of the serine/threonine-rich β_2AR cytoplasmic tail (*5*), and βARK-mediated phosphorylation/β-arrestin binding play important roles in agonist trafficking of the β_2AR (*7*), and potential phosphorylation sites in the m1 and m3 muscarinic receptors are required for downregulation (*8*−*10*), a phosphorylation-dependent pathway does not appear to be present in agonist-promoted downregulation of the α_{2A}AR.

Nor does it appear that functional coupling is necessary for α_{2A}AR downregulation. Mutants that lack G_i coupling, such as $\alpha_2(\beta_2$ 2L) and $\alpha_2(\beta_2$ CT + 2L), nevertheless displayed a wild-type downregulation phenotype. In contrast, the $\alpha_2(\beta_2$ NT + CT) mutant, which is also deficient in G_i coupling, lacked downregulation. Two discrete regions that

are necessary for G_S coupling, the second intracellular loop and the amino terminus of the third intracellular loop, have been identified (*13*). α_{2A}AR chimeras individually substituted with $5HT_{1A}$ sequence in these two regions show markedly impaired G_S coupling, but only the third loop mutant lacks downregulation. Similar observations with the other mutations indicate that while G protein coupling is not necessary, an intact amino-terminal portion of the third intracellular loop is an absolute requirement. Again these results differ from those found with the β_2AR, where a blunted downregulation is observed in CYC− cells lacking G_S (*20*), and in transfected cell lines expressing receptors with decreased G_S coupling (*3*).

Not only was downregulation lost in mutants with substituted amino-terminal portions of the third loop, but agonist exposure resulted in increases in cell surface receptors. This suggested that this region of the native α_{2A}AR serves to destabilize the receptor when the receptor is bound by agonist. If so, we expected to find that α_{2A}AR downregulation is primarily due to enhanced receptor degradation rather than depressed synthesis. In studies where protein synthesis was inhibited, levels of receptor decreased, confirming that under steady-state conditions both synthesis and degradation are underway. In the face of protein synthesis inhibition and agonist exposure, downregulation was indeed observed, pointing toward enhanced degradation as the principal mechanism of α_{2A}AR downregulation. In contrast, in mutant receptors lacking this destabilization sequence, agonist binding failed to induce degradation. Consistent with this notion, antagonist binding also stabilized these mutant receptors and led to upregulation. Alternatively, as with any study of this kind, these mutations may have affected other, yet uncharacterized, properties of the receptor that contribute to the downregulation process. Interestingly, recent studies with β_2AR mutated to mimic the agonist bound state also displayed marked instability and resulted in low expression (*21*, *22*). On the basis of these studies and the current work, it may well be that some G protein-coupled receptors that undergo downregulation possess specific destabilizing motifs which are activated by ligand binding. Further studies will be necessary to test the generality of these findings to other receptors. As discussed above, however, mutagenesis studies with several other receptors suggest the evolution of unique, receptor-specific, structural requirements for downregulation.

In summary, we have explored the molecular basis of agonist-promoted downregulation of the α_{2A}AR using receptors mutated in the second intracellular loop, the amino- and carboxy-terminal portions of the third intracellular loop, and the βARK phosphorylation sites. Downregulation is not dependent on functional coupling of the receptor to G proteins or on phosphorylation. Rather, a critical sequence within the amino terminus of the third intracellular loop was found to serve as a destabilization domain that is evoked by agonist occupancy of the receptor.

ACKNOWLEDGMENT

We thank Cheryl Theiss for technical assistance and Esther Getz for manuscript preparation.

REFERENCES

1. Liggett, S. B., and Lefkowitz, R. J. (1993) in *Regulation of cellular signal transduction pathways by desensitization and amplification* (Sibley, D., and Houslay, M., Eds.) pp 71−97, John Wiley & Sons, London.
2. Liggett, S. B. (1996) in *The Lung: Scientific Foundations* (Crystal, R., West, J. B., Weibel, E. R., and Barnes, P. J., Eds.) Raven Press, New York.
3. Campbell, P. T., Hnatowich, M., O'Dowd, B. F., Caron, M. G., Lefkowitz, R. J., and Hausdorff, W. P. (1990) *Mol. Pharmacol. 39*, 192−198.
4. Bouvier, M., Collins, S., O'Dowd, B. F., Campbell, P. T., Deblasi, A., Kobilka, B. K., MacGregor, C., Irons, G. P., Caron, M. G., and Lefkowitz, R. J. (1989) *J. Biol. Chem. 264*, 16786−16792.
5. Liggett, S. B., Freedman, N. J., Schwinn, D. A., and Lefkowitz, R. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3665−3669.
6. Valiquette, M., Bonin, H., Hnatowich, M., Caron, M. G., Lefkowitz, R. J., and Bouvier, M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5089−5093.
7. Menard, L., Ferguson, S., Barak, L., Bertrand, L., Premont, R., Colapietro, A., Lefkowitz, R., and Caron, M. (1996) *Biochemistry 35*, 4155−4160.
8. Yang, J., Logsdon, C., Johansen, T., and Williams, J. (1993) *Mol. Pharmacol. 44*, 1158−1164.
9. Lee, N., and Fraser, C. (1993) *J. Biol. Chem. 268*, 7949−7957.
10. Lameh, J., Philip, M., Sharma, Y., Moro, O., Ramachandran, J., and Sadee, W. (1992) *J. Biol. Chem. 267*, 13406−13412.
11. Eason, M. G., Jacinto, M. T., Theiss, C. T., and Liggett, S. B. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11178−11182.
12. Eason, M. G., Moreira, S. P., and Liggett, S. B. (1995) *J. Biol. Chem. 270*, 4681−4688.
13. Eason, M. G., and Liggett, S. B. (1996) *J. Biol. Chem. 271*, 12826−12832.
14. Eason, M. G., and Liggett, S. B. (1992) *J. Biol. Chem. 267*, 25473−25479.
15. Jewell-Motz, E. A., and Liggett, S. B. (1996) *J. Biol. Chem. 271*, 18082−18087.
16. Eason, M. G., and Liggett, S. B. (1995) *J. Biol. Chem. 270*, 24753−24760.
17. Eason, M. G., Kurose, H., Holt, B. D., Raymond, J. R., and Liggett, S. B. (1992) *J. Biol. Chem. 267*, 15795−15801.
18. von Zastrow, M., Link, R., Daunt, D., Barsh, G., and Kobilka, B. (1993) *J. Biol. Chem. 268*, 763−766.
19. Shilo, L., Sakaue, M., Thomas, J., Philip, M., and Hoffman, B. (1994) *Cell. Signalling 6*, 73−82.
20. Mahan, L., Koachman, A., and Insel, P. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 129−133.
21. Samama, P., Bond, R., Rockman, H., Milano, C., and Lefkowitz, R. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 137−141.
22. Gethert, U., Ballesteros, J. A., Seifert, R., Sanders-Bush, E., Weinstein, H., and Kobilka, B. K. (1997) *J. Biol. Chem. 272*, 2587−2590.

BI970487X