Agonist-Mediated Downregulation of $G_{\alpha i}$ via the α_2-Adrenergic Receptor Is Targeted by Receptor-G_i Interaction and Is Independent of Receptor Signaling and Regulation†

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Received May 1, 1998; Revised Manuscript Received August 5, 1998

ABSTRACT: One mechanism of long-term agonist-promoted desensitization of α_2AR function is downregulation of the cellular levels of the α subunit of the inhibitory G protein, G_i. In transfected CHO cells expressing the human α_{2A}AR, a 40.1 ± 3.3% downregulation of $G_{\alpha i2}$ protein occurred after 24 h of exposure of the cells to epinephrine, which was not accompanied by a decrease in $G_{\alpha i2}$ mRNA. The essential step that targets G_i for degradation by agonist occupancy of the receptor was explored using mutated α_{2A}AR lacking specific structural or functional elements. These consisted of $5HT_{1A}$ receptor and β_2AR sequences substituted at residues 113−149 of the second intracellular loop and 218−235 and 355−371 of the N- and C-terminal regions of the third intracellular loop (altered G_i and G_s coupling), deletion of Ser296−299 (absent GRK phosphorylation), and substitution of Cys442 (absent palmitoylation and receptor downregulation). Of these mutants, only those with diminished G_i coupling displayed a loss of agonist-promoted G_i downregulation, thus excluding G_s coupling and receptor downregulation, palmitoylation, and phosphorylation as necessary events. Furthermore, coupling-impaired receptors consisting of mutations in the second *or* third loops ablated G_i downregulation, suggesting that a discreet structural motif of the receptor is unlikely to represent a key element in the process. While pertussis toxin ablated G_i downregulation, blocking downstream intracellular consequences of α_{2A}AR activation or mimicking these pathways by heterologous means failed to implicate cAMP/adenylyl cyclase, phospholipase C, phospholipase D, or MAP kinase pathways in α_{2A}AR-mediated G_i downregulation. Taken together, agonist-promoted G_i downregulation requires physical α_{2A}AR−G_i interaction which targets G_i for degradation in a manner that is independent of α_{2A}AR trafficking, regulation, or second messengers.

Many G protein coupled receptors display the property of desensitization, which is the waning of signaling during continuous activation by agonist (*1*). Early events in this process include receptor phosphorylation by G protein coupled receptor kinases (GRKs) and/or second messenger-dependent kinases such as protein kinase A (PKA) and protein kinase C (PKC) and sequestration of receptors to an intracellular compartment. With agonist exposure on the order of hours, phosphorylation events can be ongoing (*2*), but two additional mechanisms come into play for some receptors, resulting in an even greater degree of depressed cellular responses. These additional mechanisms of long-term agonist-promoted desensitization include a decrease in the total complement of receptors (receptor downregulation) and a decrease in the expression of cellular G proteins (G protein downregulation). Changes in the levels of the α subunits of G_s, G_i, G_q, and G_{11} have been reported (*3*). Little is known, though, about the molecular determinants of agonist-promoted G protein downregulation. Interestingly, in cells expressing different receptors that couple to the same G protein, a G protein downregulation response to the activation of one receptor, but not the other, has been reported (*4*), implying that structural features of these receptors may be an important determinant of this response.

The α_2-adrenergic receptor (α_2AR) subtypes, which primarily couple to the G_i/G_o family of G proteins, undergo extensive long-term agonist-promoted desensitization (*5*), of which a significant component has been ascribed to a downregulation of G_i (*6*, *7*). In this report, we have explored the molecular determinants at the receptor level of agonist-promoted G_i trafficking in CHO cells expressing mutated α_{2A}AR which have specific structural and functional modifications and at the postreceptor level using strategies to alter the cellular consequences of α_{2A}AR activation.

MATERIALS AND METHODS

Construction of Chimeric and Mutated α_{2A}AR cDNAs. Chimeric α_{2A}AR cDNAs were constructed as previously described. Using both site-specific mutagenesis and cassette substitution of annealed oligonucleotides, various regions of either the β_2AR or the $5\text{-}HT_{1A}$ receptor were substituted into analogous regions of the second or third intracellular loops of the α_{2A}AR (*8*, *9*). Construction of the mutants denoted Del(293−304) and α_{2A}(Ala442) have also been described previously. The Del(293−304) receptor contains a small

† Supported by NIH grants HL53436 and HL41496.

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10.1021/bi980999r CCC: $15.00
Published on Web 10/14/1998

Table 1

no.	receptor	mutation	property
1	Del293−304	deletion of βARK phosphorylation sites	absent phosphorylation and short-term desensitization
2	α_{2A}(Ala442)	substitution of intracellular palmitoylated cysteine	absent palmitoylation and receptor downregulation
3	α_{2A}(β_2 2L)	substitution of residues 113−149 with β_2AR sequence	depressed G_i coupling
4	α_{2A}(β_2 CT)	substitution of residues 355−371 with β_2AR sequence	minimally depressed G_i coupling
5	α_{2A}(5-HT CT)	same as 4 but with substitution of 5-HT_{1A} sequence	depressed coupling to G_s
6	α_2(5-HT NT)	substitution of residues 218−235 with 5-HT_{1A} sequence	absent G_s coupling, absent receptor downregulation
7	α_{2A}(5-HT NT+CT)	combination of 5 and 6	depressed G_s coupling, absent receptor downregulation
8	α_{2A}(β_2 CT+2L)	combination of 3 and 4	depressed G_i coupling
9	α_{2A}(β_2 NT+CT)	same as 7 but with β_2AR sequence	absent G_i and G_s coupling, absent receptor downregulation

deletion in the third intracellular loop such that the four serines which serve as βAR kinase (βARK) phosphorylation sites have been removed (*10*). The α_{2A}(Ala442) receptor consists of a substitution of the cytoplasmic tail cysteine 442 with alanine and thus removes the site for palmitoylation in the carboxy-terminus of the α_{2A}AR (*11*). All constructs were verified by sequence analysis, and the coding region was subcloned back into an expression vector (either pBC12BI or pcDNAI/amp). Table 1 summarizes these constructs.

Tissue Culture and Transfection. Calcium phosphate precipitation was utilized to generate CHO cell lines permanently expressing the wild-type, chimeric, or mutated α_{2A}ARs, as previously described, using selection in 1000 μg/mL G418 (12). Transfected cells were then screened for receptor expression using radioligand binding (see below). 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, 5% CO_2.

Radioligand Binding. Membranes were prepared by scraping cells in 5 mM Tris, pH 7.4, and 2 mM EDTA and centrifuging at 40000*g* for 10 min at 4 °C. The pellet was resuspended in 75 mM Tris, pH 7.4, 12 mM $MgCl_2$, and 2 mM EDTA, and receptor expression was determined using saturating concentrations (25 nM) of [^{3}H]yohimbine, with phentolamine (100 μM) to define nonspecific binding. Reactions were carried out for 30 min at 37 °C and were terminated by dilution in cold 10 mM Tris, pH 7.4, followed by filtration over Whatman GF/C glass filters. Filters were counted in a liquid scintillation counter. Specific binding was defined as the difference between total and nonspecific binding normalized to protein concentration. Clonal isolates of α_{2A}ARs analyzed for G_i downregulation in the current study expressed between 1000 and 2000 fmol of receptor/mg of protein. At least two clones of each receptor were studied in order to avoid misinterpretation of results due to clonal variation.

Assessment of G_i Downregulation. CHO cells expressing wild-type or mutant receptors at ~95% confluence were placed in serum-free HAM's F12 medium plus 100 μM ascorbic acid, and G_i downregulation was assessed by exposing the cells to the agonist epinephrine at the indicated concentrations for 24 h, then performing Western blots on cell membranes to determine changes in levels of G_i protein. For studies assessing the effects of various agents on agonist-promoted G_i regulation, cells were pretreated for 2 h with such agents (see text) which remained in the flasks after addition of epinephrine or vehicle for the ensuing 24 h. Cells were washed five times with room-temperature PBS and then detached by scraping in 1 mL of RIPA buffer (1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) and the protease inhibitors, leupeptin (5 μg/mL), benzamidine (10 μg/mL), and soybean trypsin inhibitor (10 μg/mL). The lysate was transferred to a microcentrifuge tube, passed three times through a 21-gauge needle, centrifuged at 20000*g* for 10 min at 4 °C, and the supernatants collected. Protein concentration was then determined and a volume equivalent to 200 μg was added to an equal volume of 2× SDS sample buffer. Samples were electrophoresed through a 10% SDS−polyacrylamide gel at 20 mA constant current for 16 h. Protein was then transferred to nitrocellulose (Protran, Schleicher and Schuell) using a Bio-Rad TransBlot Apparatus for 2 h at 50 V. The filters were blocked for 20 min with 5% nonfat dry milk and incubated with the following antisera for 2 h: 1:500 of AS/7 (Dupont NEN) to detect $G_{\alpha i2}$, 1:1000 of RM/1 (Dupont NEN) to detect $G_{\alpha s}$, and 1:1000 of an anti-$G_{\alpha i3}$ C-terminal antibody (Calbiochem). Membranes were then washed three times in 50 mM Tris, pH 7.4, 200 mM NaCl, and 1% Tween and then reblocked with 5% nonfat dry milk for 20 min. Membranes were then incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody, developed using enhanced chemiluminescence (Dupont NEN) and exposed to Amersham Hyperfilm. Signals were quantitated using ScanAnalysis software (Biosoft, Ferguson, MO).

Northern Analysis. Total RNA was isolated from confluent tissue culture dishes of CHO cells expressing α_{2A}ARs using Tri-reagent (Molecular Research Center). cDNA probes were labeled with [^{32}P]dCTP by the random primer technique. RNA (10 μg) was denatured, fractionated by electrophoresis in 1% agarose, and transferred to a nylon filter (Nytran, Schleicher and Schuell). The blot was probed first for G_{i2} mRNA using a cDNA probe derived from the entire coding region of human $G_{\alpha i2}$. This hybridization was carried out in 50% formamide, 2× Denhardt's, 5× SSC, 10% SDS, and 200 μg/mL salmon sperm DNA at 42 °C overnight. The blot was then washed once in 2× SSC and 0.1% SDS for 15 min at room temperature, once in 0.5× SSC and 1% SDS for 30 min at 55 °C, and finally twice in 0.1× SSC and 0.1% SDS for 30 min at 55 °C. The filter was blotted dry, and the signal was detected by autoradiography at −70 °C. This blot was then stripped and reprobed with an α-actin probe (Ambion). Actin and G_{i2} mRNA levels were quantitated using a Molecular Dynamics PhosphorImager with Image Quant Software.

Miscellaneous. Protein concentrations were determined using the copper-bicinchoninic acid method (*13*). MAP kinase activity was determined in whole cell lysates by performing Western blots using a phospho-p44/42 MAP kinase antibody (New England Biolabs). Data are presented

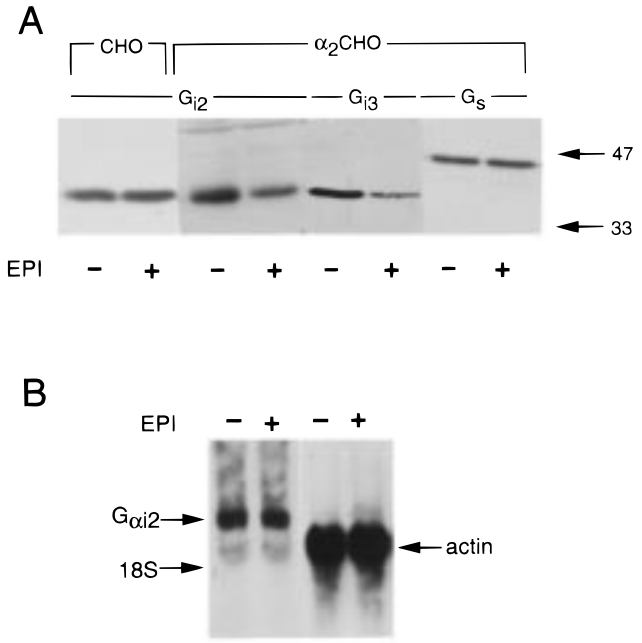

FIGURE 1: Regulation of G proteins by α_{2A}AR activation in CHO cells. Nontransfected CHO cells or those expressing wild-type human α_{2A}AR were exposed to 100 μM epinephrine in culture for 24 h. Western blots (A) or Northern blots (B) were performed as described in the Materials and Methods. Nontransfected cells showed no decrease in G_{i2}, while in α_{2A}AR expressing cells G_{i2} and G_{i3}, but not G_s, underwent downregulation. G_{i2} mRNA levels were not found to be altered. Shown are representative results from four experiments performed.

as mean ± SE or representative experiments as indicated. Comparisons were made by paired or unpaired *t*-tests, as appropriate, with $p < 0.05$ considered significant.

RESULTS

As shown in Figure 1, G_{i2} in CHO cells expressing the human wild-type α_{2A}AR undergoes a 40.1 ± 3.3% downregulation after 24 h of exposure of the cells to 100 μM epinephrine ($n = 15$, $P < 0.001$ compared to untreated). The response plateaued at 18−24 h of exposure and was maximal at concentrations of 10−100 μM epinephrine (data not shown). As indicated, this response is α_{2A}AR specific, in that there is no change in G_i levels in nontransfected CHO cells with epinephrine exposure. G_{i2} and G_{i3} are expressed in CHO cells, and both were found to undergo agonist-promoted downregulation to about the same extent; no change in G_s levels were observed (Figure 1a). Subsequent studies were carried out primarily to examine changes in G_{i2}. The downregulation of G_{i2} protein does not appear to be regulated by transcriptional events, as $G_{\alpha i2}$ mRNA levels were not changed by agonist exposure (Figure 1b). Exposure of α_{2A}AR expressing CHO cells to the protein synthesis inhibitor cyclohexamide (10 μg/mL) for 24 h resulted in a 44.8 ± 4.3% ($n = 4$) loss of G_i protein. Concomitant exposure to the agonist epinephrine resulted in an additional downregulation amounting to a total of 69.5 ± 2.5%. Taken together, these studies indicate that, like agonist-promoted G protein downregulation evoked by other receptors, the downregulation of G_i by α_{2A}AR activation appears to be due to protein degradation.

To further explore the determinants at the receptor level of agonist-promoted G_i downregulation, a number of mutated

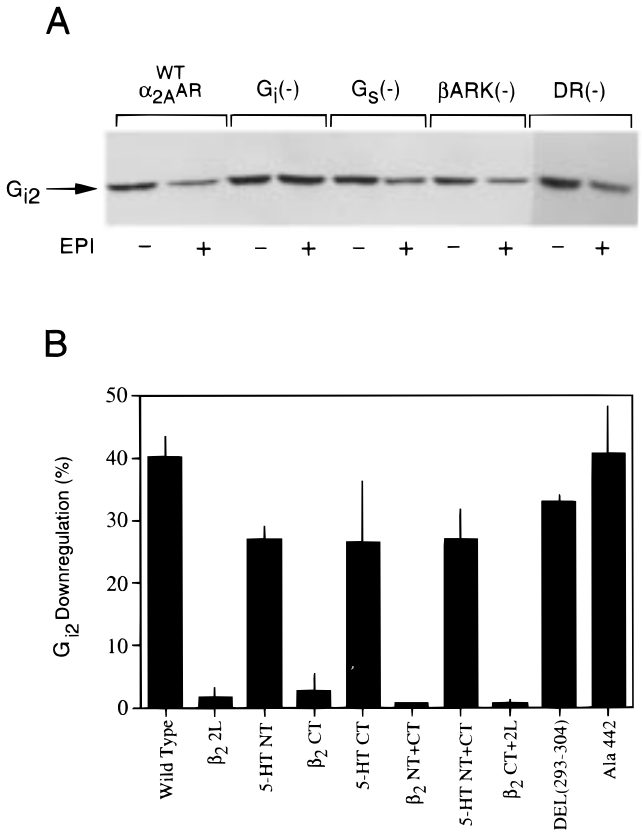

FIGURE 2: Mutations of the α_{2A}AR alter agonist-promoted downregulation of G_{i2}. CHO cells expressing mutated α_{2A}AR were exposed to 100 μM epinephrine in culture for 24 h. Western blots were performed on cell membranes as described in Methods. The specific mutations and their consequences on receptor function are outlined in Table 1. In panel A, a representative blot indicates that G_i downregulation is not altered by mutations that ablate G_s coupling (5-HT NT mutant), βARK phosphorylation (Del293−304 mutant) or receptor downregulation (Ala442 mutant). However, as typified by the β_2 NT + CT receptor, mutations that resulted in a loss of G_i coupling failed to undergo agonist-promoted G_i downregulation. In panel B, mean results ($n = 4$−6 experiments) with all mutant receptors are shown. The indicated depressed downregulation observed was significantly ($p < 0.01$) less than wild-type α_{2A}AR.

α_{2A}AR were utilized (Table 1). These receptors lack specific structural or functional features that represent candidate determinants. Seven mutants have substitutions of analogous 5-HT_{1A} receptor or β_2AR sequences in the second intracellular loop (2L), the amino-terminal (NT), or the carboxy-terminal (CT) regions of the third intracellular loop, thus altering regions necessary for G_i or G_s coupling (*9*). These receptors are referred to as α_{2A}(β_2 2L), α_{2A}(β_2 CT), α_{2A}(β_2 CT+2L), α_{2A}(β_2 NT+CT), α_{2A}(5-HT CT), α_{2A}(5-HT CT+2L), α_{2A}(5-HT NT+CT), and α_{2A}(5-HT NT). The coupling properties of these receptors are summarized in Table 1. The α_{2A}(Ala442) mutant lacks the palmitoylated C-terminal tail cysteine and fails to undergo agonist-promoted receptor downregulation (*11*). The Del293−304 mutant lacks the four serines known to be phosphorylated by βARK and fails to undergo agonist-promoted short-term desensitization (*10*). The results of studies with these mutants are shown in Figure 2. Cells expressing mutant α_{2A}AR that lack (or have markedly depressed) functional coupling to G_i failed to display agonist-promoted downregulation of G_i. These four receptors each consisted of β_2AR sequence substituted into

the second intracellular loop, amino-terminus, or carboxy-terminus of the third intracellular loop of the receptor. Thus, this does not appear to be due to the loss of a specific structural motif, since alterations in different areas of the receptor ablated agonist-promoted G_i downregulation, with the common feature of each mutation being its depressed G_i coupling. In contrast, substitutions with 5-HT_{1A} receptor sequence in these same regions failed to alter agonist-promoted G protein downregulation. Each of these mutants retains functional G_i coupling, thus confirming the notion that coupling is necessary rather than a specific intracellular domain for agonist-promoted G_i downregulation. Of note, the α_{2A}(5-HT NT+CT) mutant has wild-type coupling to G_i, but does not couple to G_s. Thus, these results point toward coupling specifically to G_i as a requisite for agonist-promoted G_i downregulation.

Neither agonist-promoted phosphorylation by GRKs nor short-term agonist-promoted functional desensitization appears to be necessary for G_i downregulation, as the Del293−304 mutant displayed a wild-type phenotype (33 ± 1% downregulation). In addition, neither palmitoylation of the receptor nor agonist-promoted downregulation of the receptor appears to be necessary, since the α_{2A}(Ala442) receptor underwent agonist-promoted downregulation of G_i to the same extent (40.8 ± 7.6%) as wild-type α_{2A}AR (Figure 2). In addition, we have previously reported that substitution of nonnative sequence into the amino-terminus of the third intracellular loop of the α_{2A}AR results in a receptor that does not undergo agonist-promoted receptor downregulation, which is independent of the coupling status to G proteins (*14*). Thus, the α_{2A}(5-HT NT) and α_{2A}(β_2 NT) receptors, which couple with wild-type efficacy to G_i, do not undergo agonist-promoted receptor downregulation. However, cells expressing these mutated receptors do display agonist-promoted downregulation of G_i, confirming that this process is independent of downregulation of the receptor (Figure 2).

The above results indicated that functional coupling of the receptor to G_i is necessary for agonist-promoted G_i downregulation. If so, we expected that pertussis toxin pretreatment, which ablates G_i coupling due to α subunit ADP ribosylation, would inhibit agonist-promoted G_i downregulation. As shown in Figure 3, this turned out to be the case, with no G_i downregulation (3.6 ± 2.0%) observed when cells were concomitantly treated with agonist and 500 ng/mL pertussis toxin. Thus, it appeared that agonist occupancy alone was insufficient to evoke the process but that receptor-G_i binding or functional activation was necessary. We reasoned that agonist-promoted G_i downregulation could thus be evoked by the physical interaction of the receptor and the G protein or by downstream events/second messengers which are consequences of receptor activation. To address this issue, cells expressing wild-type α_{2A}AR were exposed to conditions that either mimicked or blocked the intracellular consequences of receptor activation and agonist-promoted downregulation of G_i assessed (Figure 3).

Upon activation of α_{2A}AR in CHO cells, the G_i-associated $\beta\gamma$ subunits activate phospholipase C (PLC), resulting in increases in IP_3 and diacylglycerol with subsequent activation of PKC (*15*). We thus tested whether the PKC activator phorbol 12-myristate 13-acetate (PMA, 100 nM) alone could evoke G_i downregulation or whether the PKC inhibitors staurosporine (1.0 μM) or bisindolylmaleimide 1 (Bis 1, 1.0 μM) could inhibit agonist-promoted G_i downregulation. As shown in Figure 3, PKC activation does not appear to play a role in this process, as PMA had no effect alone (7.4 ± 8% downregulation) nor did the inhibitors alter agonist-promoted downregulation of G_i. Within this pathway, we also considered that IP_3, or increased intracellular calcium, might act as triggers for G_i downregulation; however, PLC blockade by 2 nM U73122, which we have previously shown to block the IP_3 and calcium responses to α_2AR activation in these cells (*15*), had no effect on the process. Since α_{2A}AR primarily acts to inhibit adenylyl cyclase, we reasoned that if a decrease in the levels of intracellular cAMP is a pertinent signal for the process, incubation of the cells with the permeable cAMP analogue dibutyryl cAMP (dbcAMP, 100 nM) would block agonist-promoted downregulation of G_i. As shown in Figure 3, epinephrine-mediated downregulation was not perturbed by dbcAMP. Recent studies have shown that α_2AR couples to phospholipase D (PLD) (*16*, *17*) and the mitogen activated protein (MAP) kinase pathways (*18*). In the presence of the PLD inhibitor 2,3-DPG (10 mM), epinephrine treatment resulted in a 23.6 ± 4.0% (n = 4) downregulation of G_i. While this tended toward being less downregulation than in the absence of the inhibitor (p = 0.08), it should be noted that 2,3-DPG exposure alone caused a 12.5% *increase* in G_i. We thus conclude that activation of PLD is unlikely to be required for agonist-promoted downregulation of G_i. In additional studies, incubations of CHO cells expressing the wild-type α_{2A}AR with the MAP kinase inhibitor PD98059 (50 μM) or the tyrosine kinase inhibitor genistein (50 μM) were found to block >90% of epinephrine induced phosphorylation of $ERK^{1}/_{2}$ (data not shown). However, these agents failed to antagonize agonist-promoted downregulation of G_i (Figure 3), indicating that activation of the MAP kinase pathway is not required for G_i downregulation. Receptor sequestration to a subcellular compartment(s) is another event that is triggered by agonist occupancy of the α_{2A}AR. It does not, though, appear to require functional coupling to G_i. We nevertheless wondered whether this process might involve cointernalization of receptor with its bound G protein, thus leading to G_i downregulation. However, cells exposed to

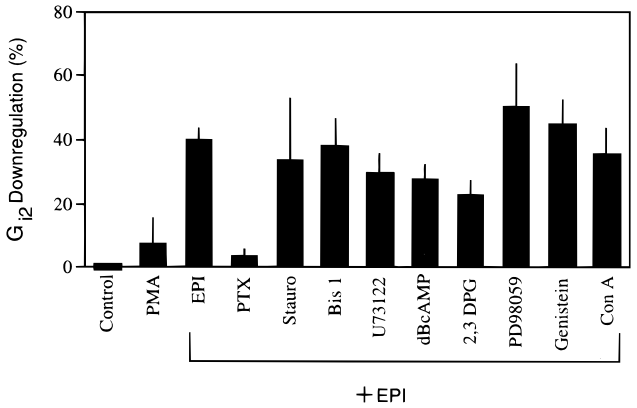

FIGURE 3: Characteristics of α_{2A}AR mediated downregulation of G_i. CHO cells expressing wild-type α_{2A}AR were exposed to media alone (control) or the indicated conditions for 24 h. The concentrations of agents are provided in the text. As shown, PMA failed to induce G_i downregulation, while pertussis toxin co-incubation blocked epinephrine promoted G_i downregulation. None of the other conditions had any significant effect on the process. See Results for details. Shown are mean ± SE from four to eight experiments.

250 μg/mL concanavalin A, which we have previously shown blocks sequestration of the α_{2A}AR (*7*), displayed the same extent of agonist-promoted G_i downregulation as did control cells (Figure 3).

DISCUSSION

Regulation of cellular G protein levels by long-term receptor activation occurs with a diverse number of G protein coupled receptors including β_2AR (*19*), β_3AR (*20*), α_{1A}, α_{1B}, and α_{1C}AR (*21*), α_{2A}, α_{2B}, and α_{2C}AR (*7*), IP and EP prostanoid receptors (*4*), the M1 (22) and M3 (23) muscarinic receptors, the TRH receptor (*24*), the GnRH receptor (*24*), and the adenosine A1 receptor (*25*). This agonist-promoted downregulation of G proteins occurs in transfected cells as well as cells endogenously expressing receptors. When examined, G proteins that are not involved in the signaling pathway of a given receptor have not been found to undergo downregulation. Interestingly, in the NG108−15 neuroblastoma X glioma hybrid cell line, expressing an IP prostanoid, secretin, and adenosine (A2) receptor (which are all coupled to G_s), only prolonged activation of the IP prostanoid receptor results in downregulation of G_s (*4*). This implies that there may be some property of the receptor itself that is necessary to evoke the process. Agonist-promoted downregulation of G proteins has been found to be due to enhanced cellular degradation of the G protein, rather than alterations in G protein production (*21*). However, very little is known about the structural requirements of the receptor necessary for agonist-promoted downregulation of G proteins. Agonist-promoted downregulation of G_i has been reported in transfected Chinese hamster fibroblasts expressing the α_{2A}AR (*6*), in transfected CHO cells expressing the α_{2A}, α_{2B}, and α_{2C} subtypes (*7*), and in rat adipocytes endogenously expressing the α_{2A}AR (*26*). Also, 3T3F442A adipocytes transfected to express a constitutively active α_{2A}AR undergo a downregulation of G_i in the absence of agonist (*27*).

We considered a number of potential mechanisms by which G protein downregulation could be evoked by activation of the α_{2A}AR. One possibility was that G_i was downregulated concomitantly with receptor trafficking. This was evaluated with mutant α_{2A}ARs that do not undergo agonist-promoted receptor downregulation. We have previously shown that the palmitoylated intracellular cysteine of the α_{2A}AR is necessary for receptor downregulation (*11*), as is a small region within the amino-terminal portion of the third intracellular loop of the receptor (*14*). Mutant α_{2A}ARs lacking these structural features [α_{2A}(Ala442), α_{2A}(5-HT NT), and α_{2A}(β_2 NT)] nevertheless exhibited agonist-promoted downregulation of G_i. Similarly, blocking agonist-promoted internalization of the receptor by concanavalin A had no effect on G_i downregulation. Finally, recent studies have indicated the importance of receptor phosphorylation by GRKs in agonist-promoted receptor trafficking (*28*). Cells expressing the Del293−304 mutant, though, which lacks all the serines phosphorylated by the βAR kinase, underwent wild-type agonist-promoted G_i downregulation. These results are in agreement with what has been described for M3 muscarinic receptor-mediated downregulation of G_q and G_{11}. A mutated M3 receptor, lacking potential phosphorylation sites in the cytoplasmic tail, fails to undergo agonist-promoted receptor sequestration and downregulation, but agonist-promoted downregulation of the aforementioned G proteins is maintained (*23*). Our current results are also consistent with previous observations in transfected Chinese hamster fibroblasts, where agonist-promoted α_{2A}AR downregulation of receptor number does not occur, but G_i downregulation is nevertheless observed (*6*). Thus, α_{2A}AR-mediated downregulation of receptor number appears to be fundamentally different than downregulation of G_i.

Additional mutated receptors were utilized to discern whether physical coupling of the receptor to G_i, or a specific structural region, was necessary for G_i downregulation. These chimeric receptors consisted of the α_{2A}AR with small substitutions of 5-HT_{1A} receptor (which is G_i coupled) or β_2AR (which is G_s coupled) sequence in the second intracellular loop, or the amino- or carboxy-terminal portions of the third intracellular loop. These mutated receptors thus retain either G_i or G_s coupling, as opposed to coupling to both G proteins which is observed with the wild-type α_{2A}AR (*9*, *29*). We found that any mutant which failed to couple to G_i also failed to display agonist-promoted downregulation of G_i. This was so regardless of the location of the mutation. This implies that the agonist-promoted interaction of the receptor with G_i, or the intracellular signaling that results, evokes the response. Interestingly, this is different than what has been found for agonist-promoted downregulation of receptor number. For this process, coupling of the receptor to G_i does not appear to be necessary (*14*). However, the native α_{2A}AR sequence in the amino-terminus of the third intracellular loop, which acts as a destabilization motif, is required. Consistent with the notion that agonist-promoted G_i downregulation requires coupling between the α_{2A}AR and G_i, pertussis toxin pretreatment abolished G_i downregulation.

Because the above experiments do not discriminate between physical receptor-G_i coupling versus a downstream (second messenger)-dependent process, we further explored the latter possibility using agents that perturb intracellular signaling of α_{2A}AR. Enhancement and/or blockade at multiple levels of the adenylyl cyclase, PLC, PLD, and MAP kinase pathways had no effect on α_{2A}AR-promoted downregulation of G_i. This is similar to what is known about β_2AR-induced downregulation of G_s, which has been found to be independent of the second messenger cAMP (*30*), and downregulation via the GnRH receptor of G_q, which is not blocked by PKC inhibitors or mimicked by PMA (*24*). While it is recognized that the α_{2A}AR is highly promiscuous and may couple to as yet unidentified pathways in CHO cells, the data at present do not support the notion that second messengers or postreceptor/G_i coupling events are sufficient to evoke α_{2A}AR-mediated downregulation of G_i. Of note, the mutant α_{2A}ARs that display decreased functional coupling to G_i (Table 1), also have decreased high-affinity agonist binding as determined in competition studies performed in the absence of guanine nucleotide (*9*). Indeed, the mutant α_{2A}AR with K_L/K_H values of <100 as determined in the aforementioned experiments were the same receptors that evoked very little G_i downregulation in the current study (Figure 4). In contrast, those with K_L/K_H values similar to wild-type α_{2A}AR displayed a wild-type G_i downregulation phenotype. Since K_L/K_H is a measure of coupling that is independent of downstream effectors, these data are consistent with the notion that physical interaction of receptor and G_i appears to be the critical event that targets G_i for degradation. Finally, it is interesting to note that when early

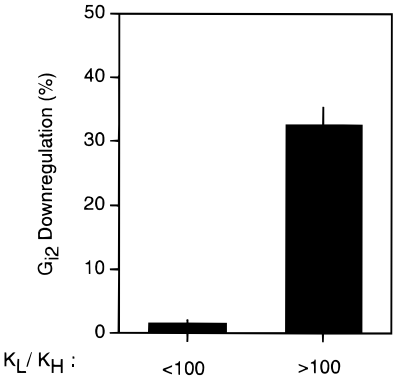

FIGURE 4: Relationship between agonist-promoted physical coupling of α_{2A}AR to G_i and agonist-promoted downregulation of G_i. The ratios of low affinity to high affinity binding constants were derived from agonist competition curves carried out in the absence of guanine nucleotide (taken from ref 9). K_L/K_H values of <100 were found with mutants 3, 4, 8, and 9 of Table 1; those with K_L/K_H values of >100 were from the remaining receptors. Downregulation data were obtained from experiments described in Figure 3.

desensitization events (βARK phosphorylation and sequestration) were ablated, agonist-promoted downregulation of G_i was not *augmented*. This might be expected since receptor-G_i coupling remained less perturbed under these circumstances compared to wild-type receptor. It may be, though, that there is an upper limit to the extent of G_i downregulation in these cells imposed by a limiting factor in the degradation process.

In conclusion, we have delineated a mechanism of agonist-promoted downregulation of the α subunit of G_i in cells expressing the α_{2A}AR. Physical coupling of the receptor to G_i appears to be the key requirement. The intracellular signaling consequences of such coupling of the receptor to G_i do not appear to be necessary, as we were unable to identify a second messenger-dependent component. Also, the events that also occur concomitantly with agonist binding, such as phosphorylation by GRKs, sequestration, or receptor downregulation, are not necessary. Taken together, the data suggest that repetitive receptor G_i high-affinity agonist-binding events that occur with long-term agonist exposure target $G_{\alpha i}$ to a degradation pathway. Thus, this mechanism of long-term agonist-promoted desensitization is not only receptor and G protein specific, it cannot be heterologously evoked. Its apparent independence from downstream second messengers and other forms of desensitization and receptor trafficking suggests that it may be a mechanism that persists (as long as agonist is present) even when receptor function has been otherwise desensitized.

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BI980999R