Characterization of G Protein-Coupled Receptor Regulation in Antisense mRNA-Expressing Cells with Reduced Arrestin Levels†

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ABSTRACT: Previous studies with overexpressing wild-type or dominant negative nonvisual arrestins have established a role for these proteins in β_2-adrenergic receptor (β_2AR) internalization, desensitization, and resensitization. To validate and extend such findings, we employed an antisense strategy to target the nonvisual arrestins, arrestin-2 and arrestin-3, and determined the associated effects on the regulation of G protein-coupled receptor (GPCR) signaling. HEK293 cells stably expressing antisense constructs targeting arrestin-2 exhibited a selective reduction (∼50%) in arrestin-2 levels, while arrestin-3 antisense constructs resulted in reductions (≥50%) in both arrestin-2 and arrestin-3 levels. Initial analysis of these cells demonstrated that a reduced level of arrestin expression resulted in a significant decrease in the extent of agonist-induced internalization of exogenously expressed β_2ARs, but had no effect on internalization of either m2 or m3 muscarinic acetylcholine receptors. Additional characterization involved assessing the role of arrestins in the regulation of endogenous GPCRs in these cells. Reduced arrestin levels significantly decreased the rate of endogenous β_2AR internalization, desensitization, and resensitization. Further analysis demonstrated that the desensitization of endogenous A_{2b} adenosine and prostaglandin E_2-stimulated receptors was also attenuated in cells with reduced arrestin levels. The effects on the β_2-adrenergic, A_{2b} adenosine, and PGE_2-stimulated receptors were similar among cell lines that exhibited either a selective reduction in arrestin-2 levels or a reduction in both arrestin-2 and -3 levels. These findings establish the utility of antisense approaches in the examination of arrestin-mediated GPCR regulation.

Prolonged exposure of G protein-coupled receptors (GPCRs)[1] to agonist often results in a rapid decrease of receptor responsiveness, a process termed desensitization (*1*). Mechanisms mediating GPCR desensitization include agonist-dependent phosphorylation by G protein-coupled receptor kinases (GRKs), which in turn promotes high-affinity binding of arrestins (*1*). Arrestin binding sterically inhibits G protein interaction with the agonist-activated GPCR (*2*, *3*).

There are currently four cloned arrestin family members. Arrestin-1 or visual arrestin is expressed predominantly in rod cells and acts by preventing light-activated phosphorylated rhodopsin from interacting with transducin (*4*). Arrestin-4 (X-arrestin or arrestin-C) is specifically expressed in cone cells, suggesting a role in regulating cone phototransduction (*5*, *6*). The ubiquitously expressed arrestin-2 (β-arrestin-1) (*7*) and arrestin-3 (β-arrestin-2) (*8*, *9*) likely regulate the interaction of a wide variety of GPCRs with their corresponding G proteins. Indeed, overexpression studies have demonstrated a role for nonvisual arrestins in the desensitization of several GPCRs, including the β_2-adrenergic (*10*), β_1-adrenergic (*11*), and α_{1B}-adrenergic (*12*) receptors.

An additional facet of GPCR regulation involves internalization of the receptor following agonist exposure (*13*). Internalization may play divergent roles for different GPCRs. For example, while internalization plays a key role in the resensitization of the β_2-adrenergic receptor (β_2AR) (*14*−*16*), it appears to prolong desensitization of the m4 muscarinic acetylcholine receptor (m4AchR) (*17*). Inhibition of internalization has also been shown to differentially affect the desensitization and resensitization of A_{2a} adenosine and secretin receptor responsiveness while having no affect on IP-prostanoid responses (*18*). Recent studies have shown that arrestin-2 and arrestin-3 can facilitate internalization of the β_2AR (*19*, *20*) and other GPCRs (*13*). Indeed, the extent of agonist-mediated internalization of the β_2AR in several different cell lines correlates with the endogenous GRK and arrestin levels (*21*). Mechanistic insight into this process has revealed that nonvisual arrestins can bind to both receptors and clathrin and thus can function as adaptor proteins to mediate GPCR uptake into clathrin-coated pits (*20*, *22*).

Studies to date have employed overexpression of either wild-type (*19*, *20*) or dominant negative (*19*, *23*, *24*) arrestins to elucidate many of the functions of these proteins. For

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[1] Abbreviations: mAchR, muscarinic acetylcholine receptor; β_2AR, β_2-adrenergic receptor; BSA, bovine serum albumin; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HEK293 cells, human embryonic kidney cells; Iso, (−)-isoproterenol; PBS, phosphate-buffered saline.

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example, arrestin-2 and arrestin-3 dominant negative mutants that bind well to clathrin but lack GPCR binding activity effectively inhibit agonist-induced internalization of the β_2-AR in HEK293 cells (*23*, *24*). While these studies have provided important insight into arrestin function, one of the inherent drawbacks with this approach is the potential for nonspecific effects associated with protein overexpression in a cell. An alternative and perhaps preferable approach is the use of antisense oligodeoxynucleotide strategies to target specific mRNAs and reduce protein expression. Antisense approaches have been successfully used to selectively decrease a number of cellular signaling proteins, including GPCRs (*25*), G proteins (*26*), and GRKs (*27*). However, an effective arrestin antisense construct has not been reported.

In the study presented here, we systematically characterized the effectiveness of various arrestin-2 and arrestin-3 antisense constructs. Two candidate antisense constructs that reduced the extent of arrestin overexpression in a transient expression system were then stably transfected into HEK293 cells, where their ability to reduce endogenous arrestin levels and effect GPCR regulation was investigated.

EXPERIMENTAL PROCEDURES

Materials. Human embryonic kidney cells transformed with the EBNA vector (HEK293-EBNA) were purchased from Invitrogen. Tissue culture media and fetal bovine serum were obtained from Life Technologies, Inc. Fugene-6 transfection agent, hygromycin, and Geneticin were from Boehringer Mannheim. Anti-mouse immunoglobulin conjugated with alkaline phosphatase and goat anti-mouse and anti-rabbit immunoglobulin conjugated with horseradish peroxidase were purchased from Bio-Rad. [^{125}I]Pindolol, ^{125}I-labeled cyclic AMP, [^{3}H]-*N*-methylscopolamine (NMS), and ^{3}H-labeled cyclic AMP were purchased from NEN Life Science products. All other reagents were from Sigma.

Generation of Antisense Expression Constructs. Full-length and truncated arrestin antisense constructs for transient transfection were generated by PCR amplification followed by subcloning into pcDNA3. A full-length human arrestin-3 antisense construct was amplified using sense (5′ CAA TGG ATC CAT GGG GGA GAA ACC CGG GAC CAG G 3′) and antisense (5′ CAA TGG ATC CTC AGC AGA GTT GAT CAT CAT AGT CGT CAT C 3′) primers that contained *Bam*HI restriction sites at the 5′ end. The resulting product was digested with *Bam*HI, subcloned into *Bam*HI-digested and phosphatase-treated pcDNA3, and then assessed for orientation. Truncated arrestin-3 antisense constructs were generated using the arrestin-3 sense primer and antisense primers that contained a *Hin*dIII site at the 5′ end: base pairs 1−801 (5′ CAA TAA GCT TGG AGC TGG GAG ATA CCT GGT C 3′), 1−402 (5′ CAA TAA GCT TTG GGC CTG GCT GCA GTG TGA C 3′), and 1−102 (5′ CAA TAA GCT TTT TGT CCA GGT GAT CTA CGA A 3′). To generate arrestin-2 antisense constructs, a human arrestin-2 cDNA was first digested with *Hin*dIII and subcloned into pcDNA3. Untranslated sequences were removed by PCR using sense (5′ CAA TTC TAG AAT GGG CGA CAA AGG GAC G 3′) and antisense (5′ CAA TCT CGA GCT ATC TGT CGT TGA GCC GCG G 3′) primers that contained *Xba*I and *Xho*I sites adjacent to the start and stop codons, respectively. Truncated arrestin-2 constructs were generated using the arrestin-2 sense primer and one of the following antisense primers: base pairs 1−801 (5′ CAA TCT CGA GCG TCG AGC TGG GTG CCA CAG T 3′, *Xho*I site at the 5′ end), 1−483 (5′ CCA CAG AAT TCC GCT TGT GGA TCT TCT CCT C 3′, *Eco*RI site at the 5′ end), and 1−134 (5′ CAA TGG ATC CAC CAG GAC CAC ACC ATC CAC A 3′, *Bam*HI site at the 5′ end).

Antisense constructs for stable expression were created in the mammalian expression vector pREP4. The pREP4−arrestin-3 antisense construct was made by excising the ∼0.8 kb *Hin*dIII−*Bam*HI insert from pcDNA3−arrestin-3 (base pairs 1−801) and subcloning into *Hin*dIII−*Bam*HI-digested pREP4. Similarly, the 0.8 kb insert from pcDNA3−arrestin-2 (base pairs 1−801) was excised with *Xho*I and *Xba*I, blunted with Klenow, and then subcloned into *Pvu*II-digested pREP4.

Cell Culture and Transfection. HEK293 and COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin G, and 100 μg/mL streptomycin sulfate (complete medium) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. HEK293-EBNA cells were always maintained in the presence of Geneticin (200 μg/mL) to maintain EBNA vector expression.

For transient transfections, cells were grown in 100 mm dishes to 80−90% confluency and transfected with 1−10 μg of DNA using Fugene-6 following the manufacturer's instructions. Briefly, HEK293 cells were incubated with a DNA/Fugene mixture for 24 h; the medium was then replaced, and the cells were analyzed 48 h after transfection. In experiments with COS-1 cells, the cells were transfected with 3 μg of total DNA using Lipofectamine following the manufacturer's instructions.

For stable transfections, 4 μg of DNA was used to transfect HEK293-EBNA cells. Three days after transfection, cells were diluted and replated in complete medium supplemented with 400 μg/mL hygromycin and 200 μg/mL Geneticin. The medium was subsequently replaced every 3 days, and surviving colonies were expanded into individual clonal lines.

Western Blot Analysis. Cells were lysed by addition of 200−500 μL of ice-cold lysis buffer [20 mM HEPES (pH 7.4), 200 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.2 mg/mL benzamidine, 0.1 mg/mL leupeptin, and 0.5 mM phenylmethanesulfonyl fluoride] to cell monolayers. Insoluble material was pelleted by centrifugation in a microcentrifuge at 13 000 rpm for 3 min at 4 °C, and the resulting supernatant was aliquoted, snap-frozen in liquid nitrogen, and stored at −70 °C. Supernatants (40 μg of total protein) were electrophoresed on a 10% SDS−polyacrylamide gel according to the method of Laemmli (*28*). Protein was then transferred to nitrocellulose and incubated with either arrestin-2 or arrestin-3 selective rabbit polyclonal antibodies made against GST fusion proteins containing either the C-terminal 62 amino acids of bovine arrestin-2 (residues 357−418) or 60 amino acids of bovine arrestin-3 (residues 350−409). Blots were then incubated with a goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase and visualized by ECL detection according to the manufacturer's instructions.

β_2AR Binding and Ligand Competition Assays. To determine the β_2AR density in control and transfected cells, whole cells were harvested in ice-cold phosphate-buffered saline

(PBS). Two hundred micrograms of cell protein was then incubated in PBS containing 0.3 nM [^{125}I]iodopindolol with or without 1 μM (−)-alprenolol (to estimate total receptor number) or 100 nM CGP-12177 (to estimate surface receptor number) for 3 h at 14 °C. All binding reactions were terminated by the addition of 5 × 4 mL of ice-cold 25 mM Tris (pH 7.5) and 2 mM $MgCl_2$ followed by rapid filtration through Whatman GF/C filters using a Brandel cell harvester. Protein concentrations were determined using a Bio-Rad protein assay with bovine serum albumin (BSA) as a standard.

Internalization Assays in Cells Overexpressing Flag-Tagged β_2AR. The extent of β_2AR internalization was measured by ELISA as described by Daunt et al. (*29*). Briefly, cells plated at a density of 6 × 10^5 cells per 60 mm dish were transfected with 5 μg of pcDNA3-Flag-β_2AR, and then split after 24 h of transfection into 24-well tissue culture dishes coated with 0.1 mg/mL poly-L-lysine. Twenty-four hours later, cells were incubated with DMEM containing 300 μM ascorbic acid [with or without 0.001−10 μM (−)-isoproterenol (Iso)] for 0−60 min at 37 °C. Reactions were stopped by removing the medium and fixing the cells with 3.7% formaldehyde in TBS [20 mM Tris (pH 7.5), 150 mM NaCl, and 20 mM $CaCl_2$] for 5 min at room temperature. Cells were washed three times with TBS, incubated for 45 min with TBS containing 1% BSA, and then incubated with a primary antibody (anti-Flag monoclonal M1, 1:1000 dilution in TBS/BSA) for 1 h at room temperature. Cells were washed three times with TBS, reblocked with TBS/BSA for 15 min at room temperature, and then incubated with secondary antibody (goat anti-mouse conjugated with alkaline phosphatase, 1:1000 dilution in TBS/BSA) for 1 h at room temperature. Cells were washed three times with TBS, and a colorimetric alkaline phosphatase substrate was added. When adequate color change was achieved, 100 μL of sample was added to 100 μL of 0.4 M NaOH to terminate the reaction, and the samples were read at 405 nm using a microplate reader.

Internalization of Endogenous β_2ARs. Cells on 100 mm dishes (80−90% confluency) were incubated with medium containing 300 μM ascorbic acid with or without 0.01−10 μM Iso at 37 °C for 0−60 min. Cells were then washed three times in ice-cold PBS and surface receptor levels determined in whole cells by ligand binding with [^{125}I]-iodopindolol with or without 100 nM CGP-12177 at 14 °C as described above.

Internalization of m2 and m3 Muscarinic Acetylcholine Receptors. Cells were transiently transfected as described above with 10 μg of pcDNA3 containing either the m2- or m3AchR. One day post-transfection, cells from 100 mm plates were passaged onto 60 mm plates for use the following day. Cells were incubated in the presence or absence of 1 mM carbachol for 0−60 min and then washed with 3 × 10 mL of ice-cold PBS at 4 °C. Two hundred micrograms of cell protein was then incubated with 2 nM [^{3}H]NMS with or without 10 μM atropine for 3 h at 4 °C to assess cell surface mAchR density. Binding reactions were terminated by the addition of 5 × 4 mL of ice-cold 25 mM Tris (pH 7.5) and 2 mM $MgCl_2$ followed by rapid filtration through Whatman GF/C filters using a Brandel cell harvester.

Adenylyl Cyclase Assays. Vehicle or agonist was added directly to the culture medium for varying times. Cells were harvested by scraping into 10 mL of ice-cold PBS and pelleted by centrifugation at 200*g* for 1 min. The pellet was washed twice in 10 mL of ice-cold PBS and frozen at −70 °C. Adenylyl cyclase activity was measured in cell homogenates using a protein binding assay as previously described (*30*). Standard curve data were fitted to a logistic expression (GraphPad Software, San Diego, CA), and adenylyl cyclase activity was expressed as picomoles of cyclic AMP produced per minute per milligram of total protein (picomoles of cAMP per minute per milligram).

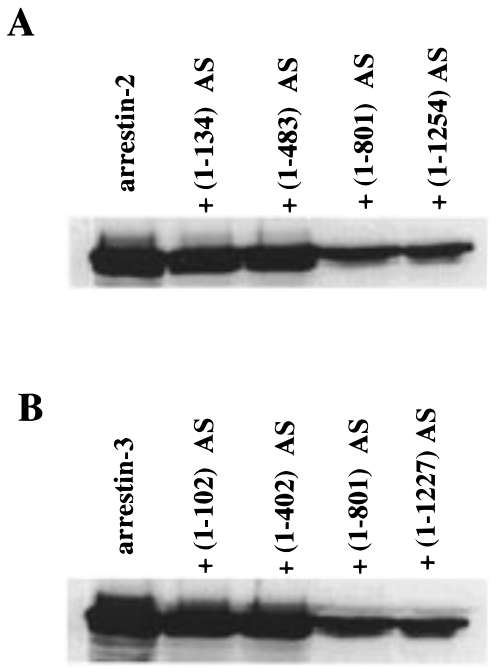

FIGURE 1: Coexpression of different length arrestin antisense mRNAs with wild-type arrestin-2 or arrestin-3 in COS-1 cells. Cells were transiently transfected with (A) 1 μg of pcDNA3−arrestin-2 and either 2 μg of GRK2 (495−689) (control) or 2 μg of different length arrestin-2 antisense constructs in pcDNA3 or (B) 1 μg of pcDNA3−arrestin-3 and either 2 μg of GRK2 (495−689) (control) or 2 μg of different length arrestin-3 antisense constructs in pcDNA3. Whole cell lysates were subjected to SDS−polyacrylamide gel electrophoresis followed by electrophoretic transfer and immunoblotting with rabbit polyclonal antibodies selective for arrestin-2 (A) or arrestin-3 (B). Detection of protein expression was performed by enhanced chemiluminescence (ECL, Amersham).

Whole-Cell cAMP Accumulation. Cells grown to ~90% confluency on 12-well plates were washed with PBS and stimulated at 37 °C with 250 μL of PBS containing 300 μM ascorbic acid, 1 mM isobutylmethylxanthine, and either vehicle (basal), 10 μM Iso, or 100 μM forskolin. Reactions were stopped after 0−45 min by placing the plates on ice, aspirating the medium, and adding 500 μL of ice-cold ethanol. The contents of each well were collected, lyophilized, resuspended, and assayed for cAMP content by radioimmunoassay using [^{125}I]cAMP and anti-cAMP antibody as described previously (*31*).

Experimental Design and Statistics. Concentration−effect curves were analyzed by the iterative fitting program GraphPad Prism (GraphPad Software). Log concentration−effect curves were fitted to logistic expressions for single-site analysis. $t_{0.5}$ values for agonist-induced desensitization were obtained by fitting data to a single-exponential curve. Where appropriate, statistical significance was assessed by

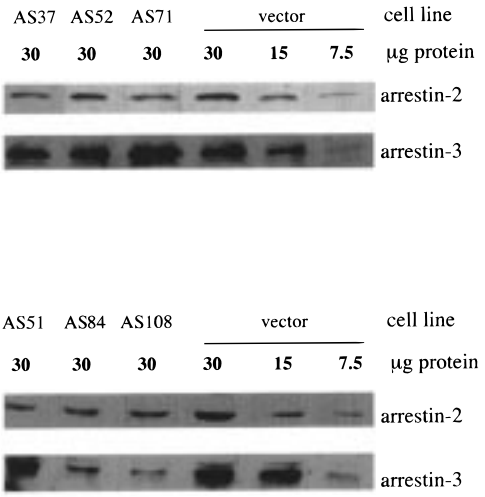

FIGURE 2: Analysis of arrestin levels in arrestin antisense mRNA expressing HEK293 cells. Whole cell lysates (7.5−30 μg) were subjected to SDS−polyacrylamide gel electrophoresis followed by electrophoretic transfer and immunoblotting with rabbit polyclonal antibodies selective for arrestin-2 or arrestin-3. Six antisense mRNA-expressing cell lines (AS 37, 51, 52, 71, 84, and 108) (30 μg of protein) were compared with vector-transfected HEK293-EBNA cells (30, 15, and 7.5 μg of protein).

a Student's *t* test or by a two-way ANOVA using GraphPad Prism.

RESULTS

To test the effectiveness of different length arrestin-2 and arrestin-3 antisense constructs, COS-1 cells were cotransfected with wild-type arrestin-2 or -3 and one of eight different antisense constructs (panels A and B of Figure 1). These studies revealed that only the longer arrestin-2 and arrestin-3 antisense constructs (base pairs 1−801 to full length) caused a significant reduction in the level of arrestin expression.

Our initial attempts to express full-length antisense constructs stably integrated into the genome of HEK293 cells resulted in no reduction in endogenous arrestin levels (data not shown). To circumvent potential problems suggested by these results, we decided to express the antisense constructs in HEK293 cells that express the EBNA vector. The use of this vector helps to maintain plasmids such as pREP4 episomally and inhibits integration of the construct into the host cell genome. It was also decided that because use of the full-length antisense construct had proven to be unsuccessful, the shorter but still effective ~800 bp constructs would be utilized.

HEK293-EBNA cells were transfected with pREP4 vector alone, pREP4 vector containing the 801 bp arrestin-2 or arrestin-3 antisense constructs, or a combination of both antisense-containing constructs. After culture in medium containing hygromycin, surviving clones were isolated and expanded into clonal cell lines. Six clones that exhibited reductions in the arrestin-2 and/or arrestin-3 level were selected for further study. Of these, four had been transfected with pREP4 containing the arrestin-2 antisense construct (AS 37, 51, 52, and 71), one with pREP4 containing the arrestin-3 antisense construct (AS 84), and one that had been transfected with both antisense-containing constructs (AS 108) (Figure 2). The arrestin-2 antisense construct appeared to

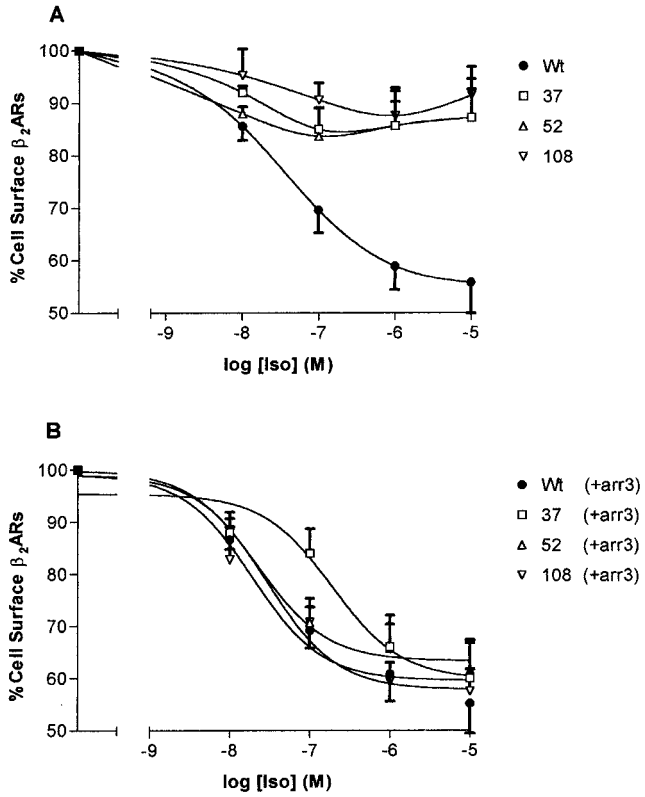

FIGURE 3: Effect of reduced arrestin levels on agonist-induced β_2-AR internalization. Wild-type, AS 37, AS 52, and AS 108 cell lines were transiently transfected with 5 μg of pcDNA3−Flag-β_2AR alone (A) or together with 5 μg of pcDNA3−arrestin-3 (+arr3) (B) and harvested ~48 h after transfection. Cells were incubated with 0−10 μM (−)-isoproterenol at 37 °C for 20 min, washed, and then analyzed for cell surface β_2ARs by ELISA as described in Experimental Procedures. β_2AR expression levels were ~5 pmol/mg in all experiments. The data represent means ± the standard error of four independent experiments.

selectively reduce only arrestin-2 levels, while the arrestin-3 construct reduced both arrestin-2 and -3 levels. Of the cell lines stably transfected with the arrestin-2 construct, all four exhibited a similar reduction (~50%) in arrestin-2 levels (Figure 2). By comparison, AS 84 and 108 exhibited an ~50% decrease in arrestin-2 and an ~75% decrease in arrestin-3 levels (Figure 2). Cells transfected with vector alone exhibited no change in endogenous arrestin levels compared to wild-type HEK293 cells, while endogenous GRK2 levels were not different among any of the clonal lines (data not shown).

To assess the biological effect of reduced arrestin levels, agonist- and time-dependent internalization of a transiently expressed Flag-tagged β_2AR was initially investigated by ELISA analysis in each of the cell lines. In vector-transfected HEK293 cells, a dose-dependent loss of cell surface β_2ARs was observed following a 20 min incubation with Iso (Figure 3A). This loss of cell surface β_2ARs was not affected by overexpression of arrestin-3, confirming that arrestin-3 levels are not limiting in these cells (data not shown). Conversely, in clonal lines expressing various antisense constructs, agonist-induced internalization of the β_2AR was almost completely abolished (Figure 3A). These results were confirmed by ligand binding analysis of cell surface β_2ARs following a 30 min pretreatment with 10 μM Iso (loss of cell surface receptors was 33.7 ± 1.8, 7.3 ± 3.7, and 7.0 ±

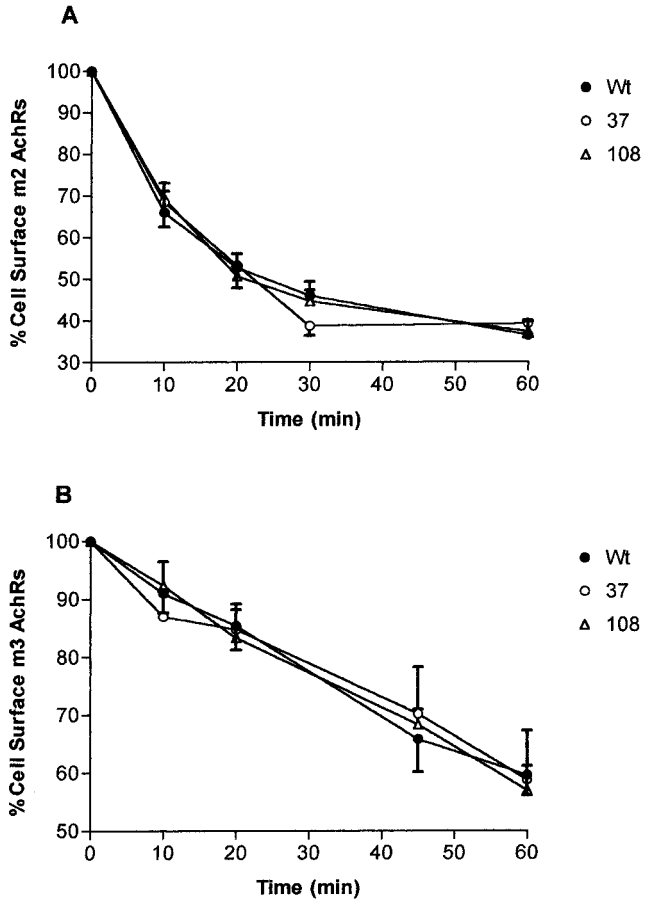

FIGURE 4: Effect of reduced arrestin levels on agonist-induced m2- and m3AchR internalization. HEK293 cells (AS 37, AS 108, and vector-transfected control), transiently transfected with 5 μg of (A) pcDNA3−m2AchR or (B) pcDNA3−m3AchR, were harvested ~48 h after transfection. Cells were incubated with 1 mM carbachol at 37 °C for 0−60 min, washed extensively, resuspended, and then analyzed for cell surface m2- and m3AchRs as described in Experimental Procedures. m2AchR expression levels were ~1 pmol/mg in vector-transfected and antisense cells, while m3AchR levels were ~0.5 pmol/mg. Data represent means ± the standard error of five independent experiments.

5.1% for vector-transfected, AS 37, and AS 108 cells, respectively; $n = 3$). Evidence that this effect was mediated by the selective reduction in cellular arrestin levels was provided by cell lines transiently overexpressing arrestin-3 (>10-fold increase in arrestin-3 levels compared to that in wild-type HEK293 cells) in which the attenuation of agonist-induced receptor internalization was reversed (Figure 3B). Similar results were obtained with AS 51, 71, and 84 cell lines (data not shown). Time course analysis of receptor internalization revealed that none of the lines with reduced arrestin levels demonstrated a significant level of β_2AR internalization throughout a 45 min exposure to 10 μM Iso, while significant internalization was observed in control cells (data not shown).

To demonstrate the selectivity of the arrestin antisense effects, m2- and m3AchRs were transiently overexpressed in antisense and control cells. Both of these receptors have been reported to be internalized by a non-arrestin-dependent mechanism (*32*, *33*). Agonist-induced m2- and m3AchR internalization as assessed by changes in [^{3}H]NMS binding was unaltered in antisense-expressing and control cells (panels A and B of Figure 4). These results confirm that the internalization of the m2- and m3AchRs is arrestin-independent in HEK293 cells.

We next examined the effects of reduced arrestin levels on endogenous β_2ARs in HEK293 cells. These studies utilized the AS 37 and 108 clonal lines, which exhibited reductions in the level of only arrestin-2 or in both arrestin-2 and -3, respectively. The β_2AR density was initially assessed in whole cells using the cell permeable ligand [^{125}I]iodopindolol. Displacement analysis in which the cell impermeable ligand CGP-12177 and cell permeable ligand alprenolol were utilized was used to assess surface and total β_2AR density, respectively. Surface receptor levels were not different among the various lines (6.3 ± 1.2, 6.4 ± 1.1, and 6.8 ± 1.0 fmol of receptor/mg of protein for vector-transfected, AS 37, and AS 108 cells, respectively; $n = 4$). However, the total receptor density did appear to be somewhat higher in the antisense cell lines (8.2 ± 1.1, 11.8 ± 1.9, and 11.9 ± 1.5 fmol of receptor/mg of protein for vector-transfected, AS 37, and AS 108 cells, respectively; $n = 4$). Such results may suggest a role for arrestins in GPCR trafficking to the cell surface. However, since arrestins generally function by binding to agonist-occupied, GRK-phosphorylated GPCRs, we would not expect changes in cellular arrestin levels to directly affect constitutive internalization of the β_2AR. Indeed, previous studies have demonstrated that even high-level overexpression of arrestins does not affect basal β_2AR function (*10*).

Experiments were subsequently performed to identify potential differences in agonist-induced β_2AR internalization among the clonal lines. Both AS 37 and AS 108 exhibited greatly reduced levels of agonist-dependent receptor internalization compared to control cells, although both lines exhibited significant surface receptor loss at higher agonist concentrations (panels A and B of Figure 5). However, the agonist concentration dependence of receptor internalization was not significantly altered in antisense cells (EC_{50} of 101 ± 9 nM for AS 37, 89 ± 8 nM for AS 108, 88 ± 8 nM for wild-type HEK293, and 86 ± 7 nM for vector-transfected cells). The time course of receptor internalization was also significantly slowed in antisense cells compared to control cells (panels C and D of Figure 5). Interestingly, although the $t_{0.5}$ of internalization was shorter in each antisense line ($t_{0.5}$ of 22.2 ± 4.8 and 13.8 ± 3.7 min for AS 37 and 108, respectively, compared to 6.7 ± 1.9 and 5.3 ± 2.1 min for wild-type and vector-transfected HEK293 cells, respectively), both antisense lines exhibited significant levels of receptor internalization at later time points.

Since arrestins have also been implicated in the uncoupling of receptor and G protein (*2*, *3*, *8*, *10*−*12*), we next examined the effect of reduced arrestin levels on the desensitization of endogenous β_2ARs. Agonist-stimulated adenylyl cyclase activity was first assessed in homogenates from wild-type, vector-transfected, and antisense-expressing HEK293 cells. There were no significant differences among the various cell lines in basal (untreated) or Iso (0.01−10 μM)-, NaF (10 mM)-, or forskolin (10 μM)-stimulated adenylyl cyclase activities (data not shown). The time course and concentration dependence of agonist-mediated desensitization were then examined by pretreating cells with Iso (0.1−10 μM) for 0−45 min prior to homogenization of the cells and subsequent determination of adenylyl cyclase activity (Figure 6). The rate of β_2AR desensitization was significantly slower

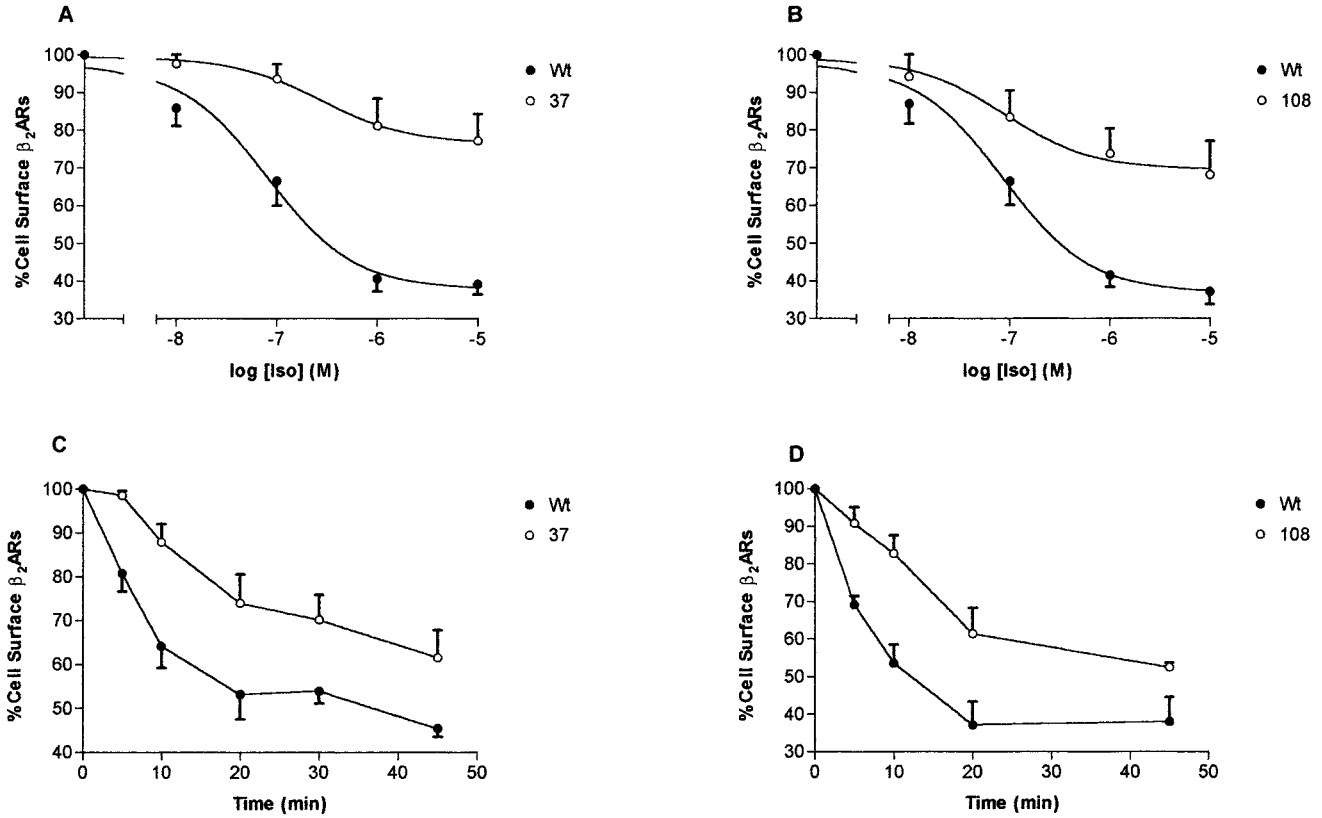

FIGURE 5: Effect of a reduced level of arrestin expression on endogenous β_2AR internalization. (A and B) Cells were treated with a range of concentrations (0.01−10 μM) of (−)-isoproterenol for 20 min and washed extensively in PBS, and the cell surface β_2AR level was analyzed as described in Experimental Procedures. (C and D) Cells were treated with 10 μM (−)-isoproterenol for 0−45 min and washed extensively in PBS, and the surface β_2AR level was analyzed. Data represent means ± the standard error of five independent experiments. Note that the total number of receptors was unaffected by agonist pretreatment (8.4 ± 1.6, 12.5 ± 1.9, and 12.7 ± 1.5 fmol of receptor/mg of protein prior to agonist treatment vs 8.0 ± 1.5, 11.9 ± 1.3, and 11.8 ± 1.3 fmol/mg following a 45 min treatment with 10 μM Iso for vector-transfected, AS 37, and AS 108 cells, respectively).

in both antisense cell lines ($t_{0.5}$ of 5.0 ± 0.7 and 8.4 ± 0.8 min for AS 37 and 108, respectively, compared to 2.6 ± 0.4 and 1.3 ± 0.5 min for wild-type and vector-transfected cells, respectively) (panels A and B of Figure 6). Additional analysis of these curves revealed that the initial rapid loss of adenylyl cyclase activity (during the first 10 min) was significantly faster in control cells than in antisense-expressing cells (3.2 ± 0.5 and 4.3 ± 0.4% loss of activity per minute for wild-type and vector-transfected cells, respectively vs 1.9 ± 0.3 and 1.4 ± 0.3% for AS 37 and 108 cell lines, respectively). In contrast, the loss of adenylyl cyclase activity during the latter phase (10−45 min) was somewhat greater in antisense-expressing cells (0.11 ± 0.06 and 0.10 ± 0.07% loss per minute for wild-type and vector-transfected cells, respectively, vs 0.24 ± 0.10 and 0.29 ± 0.09% for AS 37 and 108 cell lines, respectively). This is likely due to the increased number of non-desensitized receptors avaliable in antisense cells during this latter period that can undergo agonist-induced desensitization. The extent of receptor desensitization was also greatly reduced over a range of agonist concentrations in antisense versus control cells, although the EC_{50} of desensitization was not significantly changed (EC_{50} values of 10.8 ± 4.2 and 21.0 ± 7.8 nM for AS 37 and 108, respectively, compared to values of 18.2 ± 4.2 and 11.4 ± 5.2 nM for wild-type and vector-transfected cells, respectively) (panels C and D of Figure 6).

β_2AR desensitization was also assessed in intact cells by measuring a time course of cAMP production following addition of agonist. The level of β_2AR-stimulated cAMP accumulation was significantly greater in antisense than in control cells, with a 2−3-fold increase in the level of cAMP production following a 45 min incubation with Iso (Figure 7A). In contrast, stimulation with 100 μM forskolin produced equivalent increases in the cAMP level among the various cell lines (Figure 7B).

Previous studies have shown that β_2AR internalization (which is mediated by arrestins) is critical to the process of receptor resensitization (*14*−*16*). To test this possibility, vector-transfected, AS 37, and AS 108 cell lines were treated with Iso for 30 min, then washed, and allowed to resensitize in fresh medium. As shown in Figure 8, β_2AR resensitization was greatly impaired in cells with reduced arrestin levels compared to that exhibited in control cells.

The ability of reduced arrestin levels to effectively attenuate β_2AR regulation prompted us to also characterize the regulation of additional endogenous GPCRs in these cells. In this regard, we studied the desensitizations of two GPCRs that are also coupled to activation of adenylyl cyclase in these cells, the A_{2b} adenosine receptor (*34*) and a prostaglandin E_2 (PGE_2)-stimulated receptor. In wild-type cells (and vector-transfected cells), pretreatment with the adenosine receptor agonist NECA resulted in rapid desensitization that reached a level of ∼75% after 30 min (Figure 9A). Similarly, the PGE_2 response was also subject to rapid desensitization reaching ∼75% following a 20 min pretreatment with agonist (Figure 9B). Interestingly, both the NECA- and PGE_2-

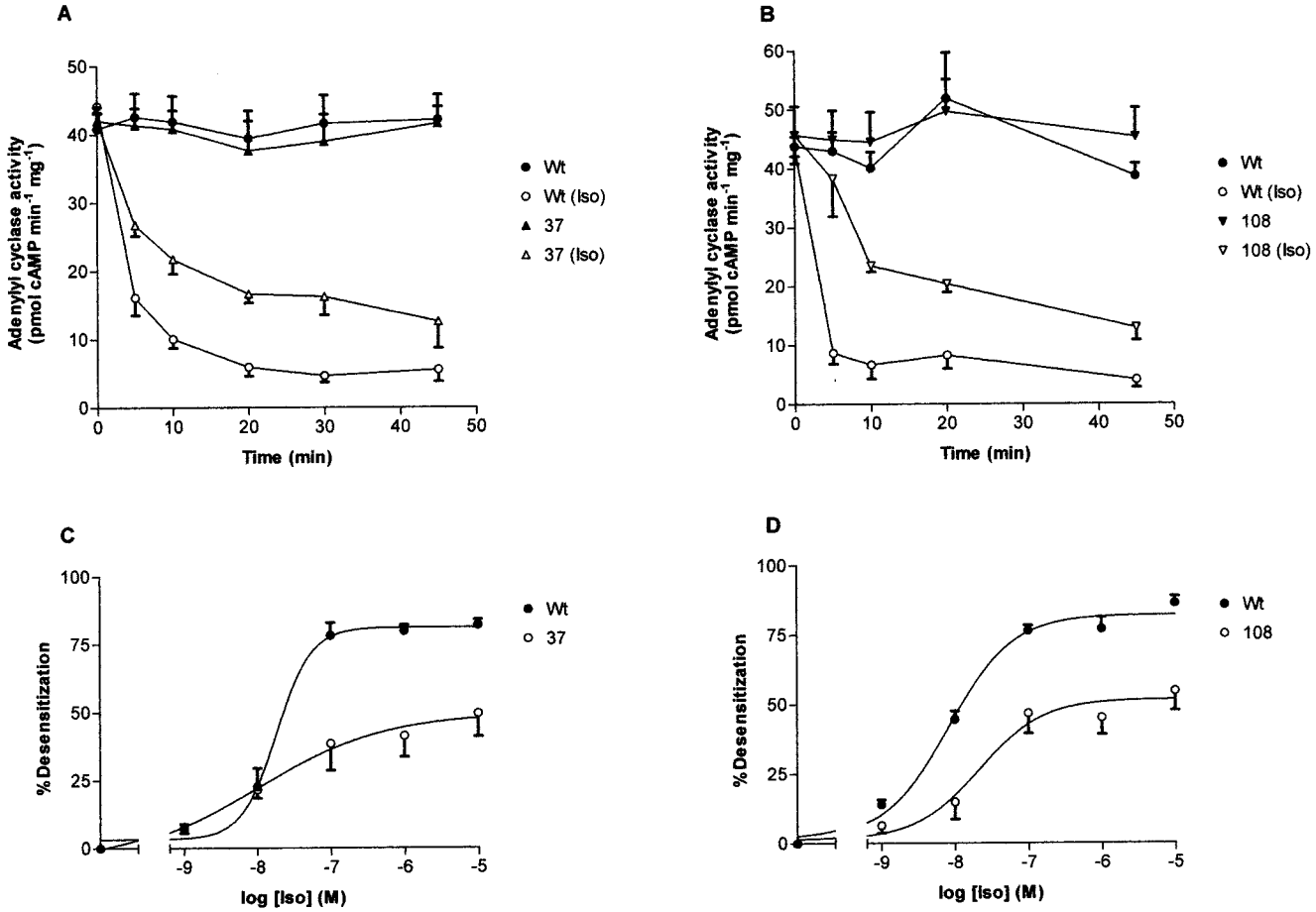

FIGURE 6: Effect of a reduced level of arrestin expression on agonist-induced desensitization of endogenous β_2ARs. (A and B) Cells were pretreated with either vehicle or (−)-isoproterenol (10 μM) for 0−45 min. Cells were harvested, and isoproterenol (10 μM)-stimulated adenylyl cyclase activity was measured in homogenates as described in Experimental Procedures. (C and D) Cells were pretreated with either vehicle or (−)-isoproterenol (0.01−10 μM) for 20 min. Cells were harvested, and isoproterenol (10 μM)-stimulated adenylyl cyclase activity was measured in homogenates as described above. Data represent means ± the standard error of five independent experiments. The level of desensitization was significantly lower for β_2AR in AS 37 than in wild-type HEK293 cells and in AS 108 than in vector-transfected control HEK293 cells ($p < 0.05$, two-way ANOVA).

promoted desensitization were significantly attenuated in the AS 37 and AS 108 lines (Figure 9). Thus, endogenous arrestins appear to play an important role in regulating the rapid agonist-promoted desensitization of the A_{2b} adenosine receptor and a PGE_2-stimulated receptor in HEK293 cells.

DISCUSSION

This study is the first to demonstrate that expression of antisense mRNAs can effectively reduce endogenous cellular arrestin levels. Using this technique, we have characterized the effects on the regulation of both exogenously expressed (β_2AR, m2AchR, and m3AchR) and endogenous (β_2AR, A_{2b} adenosine, and PGE_2-stimulated) GPCRs induced by significant reductions in arrestin levels.

Initial characterization of the effectiveness of different length antisense mRNAs in reducing the level of arrestin overexpression in COS-1 cells yielded candidate constructs for arrestin-2 and arrestin-3. HEK293-EBNA cells were then utilized to stably transfect and maintain the transfection vector (pREP4) and incorporated antisense construct episomally. This approach seemed to optimize the effectiveness of the antisense signal, since our initial studies in which the antisense construct was integrated into the host cell genome proved to be unsuccessful. Interestingly, although the arrestin-2 construct selectively reduced arrestin-2 levels, the arrestin-3 antisense construct reduced the level of expression of both arrestin-2 and -3. The lack of specificity of our arrestin-3 antisense construct might be attributed to regions of high homology between the corresponding human arrestin-2 and arrestin-3 mRNAs (*35*, *36*). However, in this regard it is unclear why the arrestin-2 antisense mRNA did not alter arrestin-3 expression. Future studies using regions of lower homology between arrestin-2 and -3 may help identify antisense sequences with greater specificity, although our ∼100−500 bp constructs proved to be ineffective.

Our initial studies focused on the internalization of an overexpressed Flag-tagged β_2AR. These studies showed that reductions in the level of endogenous arrestin expression in antisense-expressing cells effectively reduced the level of receptor internalization whether assessed by cell surface immunoreactivity or ligand binding. Interestingly, this abolition of receptor internalization was evident in all antisense cell lines, even those with only a 50% reduction in the arrestin-2 level alone (AS 37, 51, 52, and 71). To confirm that reduced arrestin expression and not nonspecific effects were mediating the lack of receptor internalization, receptors were transiently coexpressed with arrestin-3 in an attempt

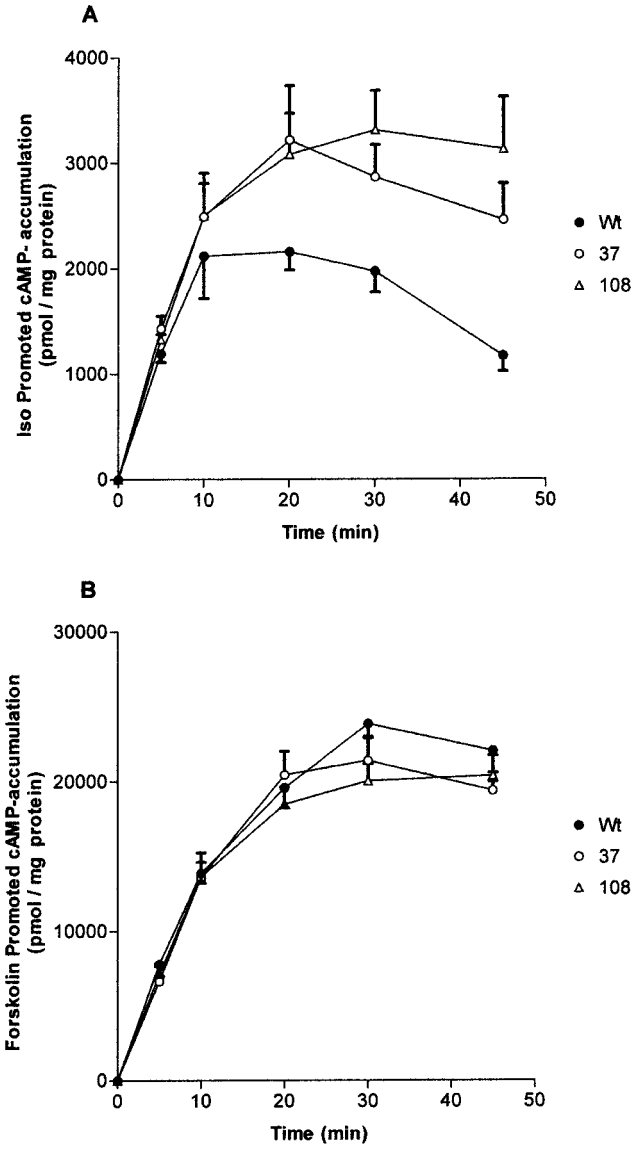

FIGURE 7: Cyclic AMP accumulation in intact wild-type and antisense-expressing HEK293 cells. Cyclic AMP levels were measured in vector-transfected (Wt), AS 37, and AS 108 cells after the addition of 10 μM (−)-isoproterenol (A) or 100 μM forskolin (B). Values represent means ± the standard error from four independent experiments. The level of Iso-stimulated cAMP accumulation was significantly greater in AS 37 and 108 cells than in wild-type cells ($p < 0.05$, two-way ANOVA).

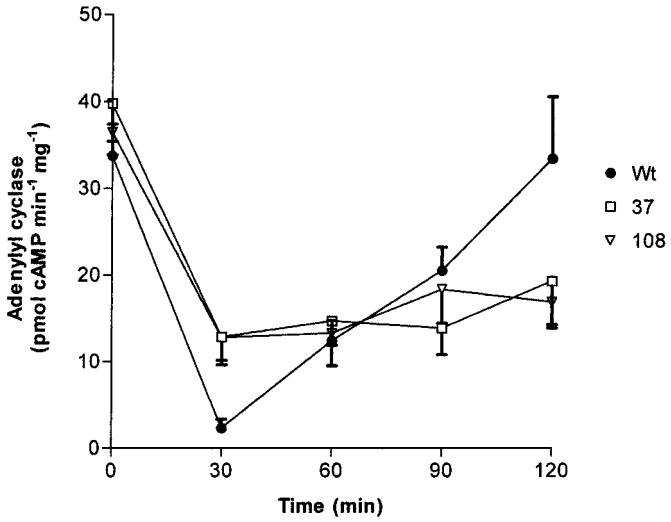

FIGURE 8: Effect of a reduced level of arrestin expression on β_2-AR resensitization. Vector-transfected (Wt) and antisense cells (AS 37 and 108) were pretreated with 10 μM (−)-isoproterenol for 30 min, washed, and then reincubated with fresh medium for an additional 90 min. Cells were harvested before treatment and 0, 30, 60, and 90 min after agonist washout and assayed for isoproterenol-stimulated adenylyl cyclase activity as described in Experimental Procedures. Values represent means ± the standard error from four independent experiments.

to restore internalization. Overexpression of arrestin-3 (~10-fold over basal) had a minimal effect on β_2AR internalization in wild-type and vector control cells, indicating that endogenous arrestin levels are sufficient to mediate receptor internalization even when the level of receptor expression is increased, as previously reported (*18*, *21*). In antisense-expressing cells, overexpression of arrestin-3 was sufficient to restore receptor internalization to control levels. This finding illustrates that the selective reduction in arrestin levels mediated the attenuation of receptor internalization. To further explore the specificity of the arrestin antisense effects, we examined internalization of the m2- and m3AchR in the AS 37 and 108 lines. The agonist-dependent internalization of the m2- and m3AchRs was unaffected by reductions in arrestin levels, confirming previous studies that demonstrated that internalization of these receptors in HEK293 cells was arrestin-independent (*32*, *33*). These results indicate that antisense arrestin mRNA expression specifically affected those mechanisms mediating β_2AR internalization while not affecting arrestin-independent mechanisms mediating internalization of m2- and m3AchR.

Subsequent studies focused on the regulation of endogenous, natively expressed β_2ARs. The level of agonist-stimulated receptor internalization was reduced in both AS 37 and 108 cell lines. The time course of Iso-stimulated internalization revealed that reduced arrestin levels inhibited rather than stopped internalization. This suggests either that the remaining endogenous arrestins mediated receptor internalization or that mechanisms other than arrestin-mediated internalization are involved. For example, β_2ARs have been shown to colocalize with cavaolae (*37*), and this may represent an arrestin-independent mechanism of internalization similar to that suggested for the bradykinin receptor (*38*).

The reduction in the extent of receptor internalization caused by arrestin antisense expression was more dramatic with overexpressed than with endogenous β_2AR. This may indicate that a threshold for measurable receptor internalization levels exists that is dependent on both receptor and arrestin expression. For example, in a system with a greatly increased level of receptor expression and a reduced level of arrestin expression, receptor internalization may no longer be evident. Conversely, when the level of receptor expression is low, extant endogenous arrestins in the antisense-expressing cells can still mediate a measurable level of receptor internalization, albeit at a slower rate.

We observed little difference in endogenous β_2AR internalization between AS 37 and 108 lines even though the latter exhibits a much greater reduction in arrestin-3 levels. This finding suggests that arrestin-3 plays a relatively lesser role in endogenous β_2AR internalization or that the remaining endogenous arrestins are sufficient to induce receptor internalization. No clear functional differences have yet to

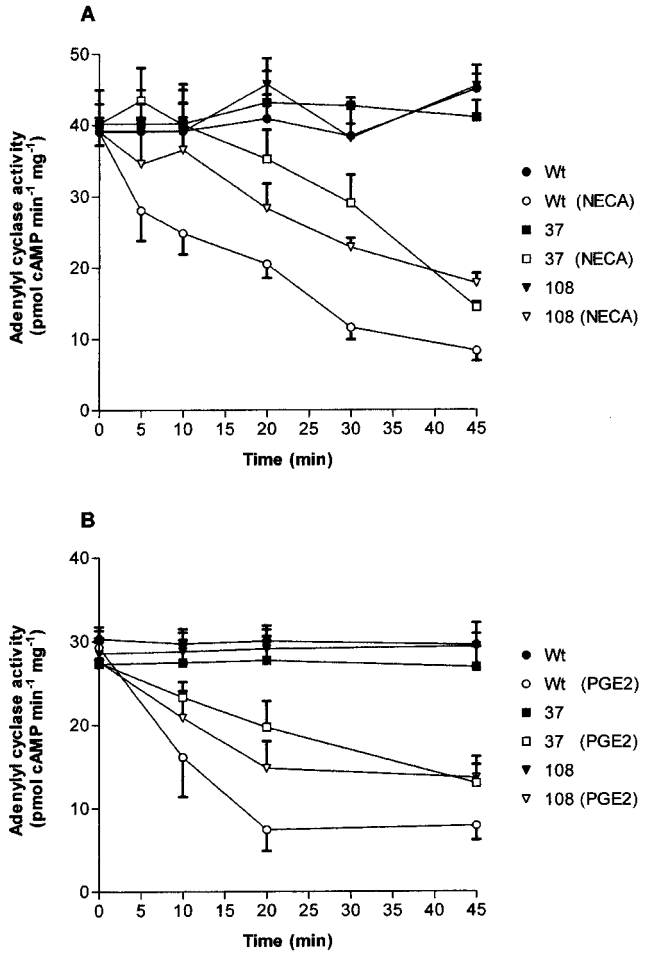

FIGURE 9: Effect of a reduced level of arrestin expression on agonist-induced desensitization of endogenous A_{2b} adenosine receptor (A) and PGE_2-stimulated adenylyl cyclase activity (B). Cells were pretreated with either vehicle or (A) NECA (100 μM) or (B) PGE_2 (1 μM) for 0−45 min. Cells were harvested, and (A) NECA (100 μM)- or (B) PGE_2 (1 μM)-stimulated adenylyl cyclase activity was measured in homogenates as described in Experimental Procedures. Data represent means ± the standard error of five independent experiments. The level of desensitization was significantly lower for A_{2b} adenosine receptor and PGE_2-stimulated adenylyl cyclase activity in AS 37 and AS 108 than in vector-transfected control HEK293 cells ($p < 0.05$, two-way ANOVA).

be identified between arrestin-2 and arrestin-3 (*13*). Identification of an arrestin-3 antisense construct that specifically reduces endogenous arrestin-3 protein may help to resolve such differences, if any exist. It will also be interesting to investigate if other methods of reducing arrestin function used in conjunction with reduced arrestin expression can further alter receptor signaling.

The role of arrestins in the agonist-induced desensitization of a number of GPCRs has previously been reported (*13*). In intact cells, agonist-specific desensitization of the β_2AR was significantly attenuated by expression of antisense constructs. This indicated, as previously suggested, that arrestins are involved in the desensitization of β_2AR responses (*10*). Importantly, cAMP accumulation as a result of forskolin stimulation was identical in antisense and control cells, suggesting the lack of involvement of arrestins in this response. Similarly, β_2AR desensitization assessed in cell homogenates was also attenuated by arrestin antisense constructs. Arrestin antisense expression increased the $t_{0.5}$ of β_2AR desensitization, although a similar level of desensitization was ultimately achieved at later time points. This could indicate that remaining endogenous arrestins interact with and uncouple phosphorylated receptors from G protein or that mechanisms other than arrestin-mediated uncoupling are involved.

Arrestin-2 has been shown to play an integral role in regulating not only desensitization and intracellular trafficking of GPCRs but also the ability of the β_2AR to resensitize (*39*). These findings were further confirmed in our study. In antisense-expressing cells, no significant resensitization of response was observed, indicating that arrestins play a major role in receptor resensitization.

Since reduced arrestin levels effectively attenuated β_2AR regulation, we next used the antisense lines to characterize the involvement of arrestins in desensitization of the endogenously expressed A_{2b} adenosine receptor (*34*). While GRK2 has previously been implicated in the desensitization of A_{2b} adenosine receptors in NG108-15 cells (*30*), a role for arrestins in this process has not been characterized. Agonist-mediated desensitization of the A_{2b} adenosine receptor was significantly attenuated in cells containing reduced arrestin levels, implicating arrestin involvement in the desensitization of A_{2b} adenosine receptors in HEK293 cells.

We also used the antisense lines to characterize desensitization of a PGE_2-stimulated receptor in these cells. At present, it is unclear which prostanoid receptors are endogenously expressed in HEK293 cells, although the rapid desensitization of the PGE_2 response we observed mirrors that of the prostaglandin EP_4 receptor (*40*). Interestingly, desensitization of the PGE_2-stimulated adenylyl response was also significantly attenuated in antisense-expressing cells. This provides the first evidence of arrestin involvement in the desensitization of prostanoid receptors.

In summary, this study shows that an antisense strategy can successfully be employed to reduce endogenous arrestin levels and effect changes in β_2AR internalization, desensitization, and resensitization. Further, our results demonstrate that the antisense cells can also be effectively used to examine arrestin involvement in the regulation of other GPCRs. Future studies will attempt to investigate the regulation of additional endogenous and transfected GPCRs in these cells.

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