Selective Regulation of Endogenous G Protein-coupled Receptors by Arrestins in HEK293 Cells*

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Arrestins play an important role in regulating desensitization and trafficking of G protein-coupled receptors (GPCRs). However, limited insight into the specificity of arrestin-mediated regulation of GPCRs is currently available. Recently, we used an antisense strategy to reduce arrestin levels in HEK293 cells and characterize the role of arrestins on endogenous Gα-coupled receptors (Mundell, S. J., Loudon, R. B., and Benovic, J. L. (1999) Biochemistry 38, 8723–8732). Here, we characterized GPCRs coupled to either Gi (M1 muscarinic acetylcholine receptor (M1ACHR) and P2Y1 and P2Y2 purinergic receptors) or Gαi (somatostatin and AT1 angiotensin receptors) in wild type and arrestin antisense HEK293 cells. The agonist-specific desensitization of the M1ACH and somatostatin receptors was significantly attenuated in antisense-expressing cells, whereas desensitization of P2Y1 and P2Y2 purinergic and AT1 angiotensin receptors was unaffected by reduced arrestin levels. To further examine arrestin/GPCR specificity, we studied the effects of endogenous GPCR activation on the redistribution of arrestin-2 epitope tagged with the green fluorescent protein (arrestin-2-GFP). These studies revealed a receptor-specific movement of arrestin-2-GFP that mirrored the arrestin-receptor specificity observed in the antisense cells. Thus, agonist-induced activation of endogenous β2-adrenergic, prostaglandin E2, M1ACH, and somatostatin receptors induced arrestin-2-GFP redistribution to early endosomes, whereas P2Y1 and P2Y2 purinergic and AT1 angiotensin receptor activation did not. Thus, endogenous arrestins mediate the regulation of selective Gαi and Gα-coupled receptors in HEK293 cells.

Arrestins mediate the desensitization and internalization of a number of G protein-coupled receptors (GPCRs)1(1). Agonist-dependent phosphorylation of GPCRs by G protein-coupled receptor kinases promotes the high affinity binding of arrestins (1), which in turn sterically inhibits G protein interaction with the receptor, thereby terminating agonist-mediated signaling (2, 3). Arrestins are recruited to multiple GPCRs after agonist activation (4), highlighting the important role of these proteins in receptor regulation.

Recent evidence indicates that arrestins not only bind to GPCRs but also associate with clathrin heavy chains to promote GPCR internalization via clathrin-coated pits (5). This process has been characterized extensively for the β2-adrenergic receptor (5, 6). Overexpression of arrestin-2 or arrestin-3 augments internalization of β2-adrenergic receptors (5, 6), while dominant negative arrestin mutants that selectively bind to clathrin effectively inhibit agonist-induced β2-adrenergic receptor internalization (7, 8). Whereas many GPCRs undergo agonist-promoted internalization, the functional role of this process may vary between receptors. For example, internalization plays an important role in the resensitization of the β2-adrenergic receptor (9–11) and Lα2, adenosine receptor (12, 13), whereas internalization of the MACH and secretin receptors is involved in receptor desensitization (13–15).

Studies to date have employed overexpression of either wild type (5, 6) or dominant negative (6–8) arrestins with heterologously expressed GPCRs to elucidate many of the functions of these proteins. Although providing important insight into arrestin function and receptor specificity, one of the inherent drawbacks with this approach is the potential for nonspecific effects associated with protein overexpression. In a recent study we demonstrated that an antisense strategy can be successfully employed to reduce endogenous arrestin levels and effect changes in the internalization, desensitization, and resensitization of the Gα-coupled β2-adrenergic receptor (16). Moreover, the regulation of two other endogenous Gα-coupled receptors, the Aα2 adenosine and prostaglandin E2, was also shown to be affected by reductions in arrestin levels (16). These antisense-expressing cells therefore represent a novel system in which to further explore the involvement of arrestins in the desensitization of native receptors in intact cells. Although numerous studies have examined the role of arrestins in regulating the responsiveness of the β2-adrenergic receptor and other Gα-coupled receptors, considerably less attention has been paid to GPCRs coupled to Gαi (which inhibit adenyl cyclase activity) and Gαo (which stimulate inositol phospholipid production and Ca2+ release). Here we utilized HEK293 cells expressing arrestin-2 and arrestin-3 antisense constructs to perform a systematic analysis of arrestin specificity for regulating signaling by endogenous Gαi-coupled P2Y1 and P2Y2 purinergic (17) and M1ACH receptors (18) and Gαi-coupled somatostatin and AT1 angiotensin receptors. In addition, arrestin/GPCR specificity was further characterized by examining changes in arrestin-2 green fluorescent protein (GFP) redistribution induced by endogenous receptor activation.

EXPERIMENTAL PROCEDURES

Materials—Human embryonic kidney cells transformed with the EBNA vector (HEK293-EBNA) were purchased from Invitrogen. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were obtained from Life Technologies Inc. Fugene-6 transfection agent, hygromycin, and Geneticin were from Roche Molecular Biochemicals. [3H]cAMP and [3H]inositol were purchased from NEN Life Science.
mM Hepes, pH 7.5, and 20 mM LiCl for 10 min at 37 °C. Cells were then washed in prewarmed DMEM containing 0.5% bovine serum albumin, 20 glucose, without inositol) as described previously (19). After labeling, phosphate levels were measured in vector-transfected (Wt), AS 37, and AS 108 cells after the addition of 1 mM carbachol (A and D), 100 μM ATP (B), 100 μM ADP (C), 100 μM 2-methyl-ADP (2-Me-ADP) (D), or 100 μM UTP (I). In D, cells were stimulated for 30 min. Values represent the mean ± S.E. from four independent experiments and are expressed as fold increase over basal. Carbachol-stimulated inositol phosphate accumulation was significantly greater in AS 37 and 108 cells compared with wild type cells in A (p < 0.05, two-way ANOVA) and D (p < 0.05, Student’s t test).

**Adenylyl Cyclase Assays**—Where required, drugs were added directly to the culture medium for varying times. Cells were harvested in 10 ml of ice-cold phosphate-buffered saline and pelleted by centrifugation at 200 × g for 1 min. The resulting pellets were washed twice in 10 ml of ice-cold phosphate-buffered saline and frozen at −70 °C until use. Adenylyl cyclase activity was measured using a protein binding assay (21). Cell pellets were thawed and homogenized in a glass Dounce homogenizer containing ice-cold homogenization buffer (0.3 M sucrose, 25 mM Tris-HCl, pH 7.4). A 40-μl sample of homogenate was then added to 30 μl of premix buffer (final assay concentration, 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM ATP, 1 mM GTP, 250 μM Ro201724 (4-(3-butoxy-4-methoxybenzyl)imidazolidin-2-one) as phosphodiesterase inhibitor, 20 mM creatine phosphate, and 130 units/ml creatine phosphokinase) and 30 μl of drug at the relevant concentration. The tubes were incubated at 37 °C for 10 min, and the reaction was terminated by the addition of 20 μl of 100% trichloroacetic acid; the tubes were placed on ice for 10 min. Precipitated protein was pelleted by centrifugation at 29000 × g for 20 min at 4 °C, and 50 μl of the resulting supernatant was added to 50 μl...
of 1 M NaOH and 200 μl of TE buffer (50 mM Tris-HCl, 4 mM EDTA, pH 7.4); 50 μl of this solution was then added to fresh tubes containing 100 μl of TE buffer, 100 μl of [3H]cAMP in TE buffer (100 μM), and 100 μl of binding protein in TE buffer (to give a final concentration of -750 μg of protein/ml; prepared from bovine adrenal cortex). Tubes containing 50 μl of standard concentrations of cAMP (0.125–20 pmol) were used to construct a standard curve. After a 2-h incubation at 4 °C, 200 μl of TE buffer containing charcoal (Norit GSX; 50 mg/ml final concentration) and bovine serum albumin (2 mg/ml final concentration) were added, and 15 min later, the tubes were centrifuged at 2900 g for 20 min at 4 °C. The resulting supernatant was transferred into vials for liquid scintillation counting. Standard curve data were fitted to a logistic expression (GraphPAD Software, San Diego, CA), and the unknowns were read off. Protein content of homogenates was determined (22), and adenylyl cyclase activity was expressed as pmol of cAMP/min/mg of protein.

**Fluorescence Microscopy and Single Cell Imaging**—To assess the distribution of arrestin-2 in living cells, HEK293 cells (60-mm dish) were transfected with 0.25 μg of arrestin-2-GFP. Cells were grown on poly-L-lysine-coated coverslips and mounted on an imaging chamber (Warner Instrument Corp) equipped with an inlet port through which media and drugs could be perfused. For experiments analyzing transferrin distribution, cells were incubated for 15 min with 200 μg/ml rhodamine-conjugated transferrin on glass coverslips. Cells were washed 3 times with PBS before imaging then examined by microscopy on a Nikon Eclipse E800 fluorescence microscope using a Plan-Apo 60 × 1.40 NA oil immersion objective. For experiments analyzing ras-5 distribution, arrestin-2 GFP-transfected cells were incubated with a ras-5-specific antibody (Molecular Probes) for 1 h at 4 °C. The cells were fixed with 3.7% formaldehyde, PBS for 15 min at room temperature, washed with PBS, and permeabilized with 0.05% Triton X-100/PBS/CaCl₂ for 10 min at room temperature. Nonspecific binding was blocked with buffer A (0.05% Triton X-100/PBS/CaCl₂ containing 5% nonfat dry milk) for 30 min at 37 °C. Goat anti-mouse rhodamine-conjugated secondary antibody (Molecular Probes) was then added at a dilution of 1:150 in buffer A for 1 h at 37 °C. The cells were then washed 6 times with permeabilization buffer, with the last wash left at 37 °C for

**FIG. 2.** Dose-response curves for activation of M₁Ach (A), P₂y₁ (B), and P₂y₂ (C) purinergic receptors. Calcium mobilization was measured in response to the indicated concentration of agonist in vector-transfected (Wt), AS 37, AS 83, and AS 108 cells, and dose-response curves were constructed. EC₅₀ values were 4.1 ± 0.4, 3.9 ± 0.7, 3.7 ± 0.8 and 4.3 ± 0.5 μM (A), 0.42 ± 0.02, 0.35 ± 0.05, 0.37 ± 0.08, and 0.32 ± 0.06 μM (B), and 0.36 ± 0.08, 0.33 ± 0.07, 0.44 ± 0.06, and 0.31 ± 0.07 μM (C) for vector-transfected Wt, AS 37, AS 83, and AS 108 cell lines, respectively. Values represent the mean ± S.E. from four independent experiments and are expressed as change in intracellular calcium level (Δ[Ca²⁺]), nM) as assessed by Fura-2 in intact cells.

**FIG. 3.** Agonist-induced desensitization of M₁Ach and P₂y₁ purinergic receptors by carbachol (A) and ATP (B) in vector-transfected control cells. Desensitization was assessed by incubating cells with the indicated concentrations of agonist for 30 min followed by rechallenge with carbachol (1 mM) or ATP (100 μM). The data are expressed as a percentage of the peak calcium response to carbachol (1 mM) or ATP (100 μM) in nonpretreated cells. IC₅₀ values for desensitization: A, M₁Ach by carbachol was 2.9 ± 0.8 μM; B, P₂y₁ purinergic by ATP was 44.5 ± 0.9 nM; B, M₁AchR by ATP was 64.5 ± 1.5 nM. The data represent the mean ± S.E. of four independent experiments.
30 min. Finally, the cells were fixed with 3.7% formaldehyde as described. Coverslips were mounted using Slow-Fade mounting medium (Molecular Probes) and examined by microscopy as described above. All images were collected using QED Camera software and processed with Adobe Photoshop. For confocal microscopy, cells were prepared in the same manner, and images were obtained on a Bio-Rad MRC-Zeiss Axiovert 100 confocal microscope using a Zeiss Plan-Apo 63×1.40 NA oil immersion objective.

Experimental Design and Statistics—Dose-response 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_{50} values for agonist-induced desensitization were obtained by fitting data to a single exponential curve. Where appropriate, statistical significance was assessed by Student’s t test or by two-way ANOVA using GraphPAD Prism.

RESULTS

Previously, we used stable expression of arrestin antisense constructs to successfully reduce endogenous arrestin levels in HEK293 cells (16). These cells revealed an important role for arrestins in the desensitization of endogenous Gs-coupled receptors such as the β_{2}-adrenergic, adenosine A_{2b}, and prostaglandin E_{2} (16). Three of these previously characterized cell lines with demonstrated reductions in arrestin-2 and/or arrestin-3 were used in the present study. The line AS 37, stably transfected with an arrestin-2 antisense construct, exhibits an ~50% reduction in arrestin-2 levels, whereas the lines AS 83, transfected with an arrestin-3 antisense construct, and AS 108, transfected with both antisense constructs, exhibit an ~50% decrease in arrestin-2 and ~75% decrease in arrestin-3 levels. Endogenous arrestin levels in vector-transfected cells were comparable with those in wild type HEK293 cells.

FIG. 4. Agonist-induced desensitization of M_{1}Ach and P2γ1, purinergic receptors in vector-transfected (Wt), AS 37, AS 83, and AS 108 cells. Desensitization was assessed by incubating cells with (A) carbachol (1 mM) or (B) ATP (100 μM) for 30 min followed by rechallenge with carbachol (1 mM) or ATP (100 μM). The data presented are expressed as a percentage of the peak calcium response to carbachol or ATP in nonpretreated cells. In A, desensitization of M_{1}Ach receptors was significantly less in AS 37, AS 83, and AS 108 cells versus vector-transfected control HEK-293 cells (p < 0.05, Student’s t test). The data represent the mean ± S.E. of four independent experiments.

FIG. 5. Effect of reduced arrestin expression on agonist-induced desensitization of endogenous M_{1}Ach (A), P2γ2 (B), and P2γ1 purinergic receptors (C). Cells were pretreated (0–30 min) with carbachol (1 mM) (A), ADP (100 μM) (B) or ATP (100 μM) (C) for 0–30 min and then rechallenged with the same concentration of agonist. The data presented are expressed as a percentage of the peak calcium response to carbachol (1 mM) (A), ADP (100 μM) (B), or ATP (100 μM) (C) in cells that were not pretreated with agonist. In A, desensitization of M_{1}AchR was significantly less in AS 37, AS 83, and AS 108 cells versus vector-transfected control HEK-293 cells (p < 0.05, two-way ANOVA). The data represent the mean ± S.E. of four independent experiments.
To further define the arrestin specificity in regulating GPCRs, we next focused on endogenous Gq-coupled receptors. HEK293 cells have been reported to contain a number of Gq-coupled receptors including muscarinic (18), thrombin, lysophosphatidic acid, sphingolipid (23), endothelin, bradykinin (24), and P2y1 and P2y2 purinergic (17). The addition of carbachol (M1Ach), ADP (P2y1), and ATP (P2y2) to HEK293 cells resulted in significant stimulation of inositol phosphate accumulation (Fig. 1). In contrast, incubation with bombesin, bradykinin, somatostatin, or angiotensin II (100 nM; AT1 angiotensin) resulted in significant stimulation of inositol phosphate accumulation (Fig. 1). To further investigate arrestin/receptor specificity, we characterized Ca2⁺ mobilization induced by activation of each of these Gq-coupled GPCRs. In agreement with previous studies we found that ADP, ATP, and carbachol all triggered a rapid and transient intracellular mobilization of Ca2⁺ (17, 18). Complete concentration-effect curves were subsequently generated for the M1Ach, P2y1, and P2y2 purinergic receptors in antisense and vector transfected control cells (Fig. 2). The magnitude of the calcium response and agonist potency was unchanged in antisense-expressing cells compared with vector-transfected controls. In addition, the duration of the transient Ca2⁺ responses was similar in antisense and control cell lines for each of the agonists tested (data not shown). These findings appear to contrast with our M1AchR-mediated inositol phosphate analysis, where increased inositol phosphate accumulation was observed in the antisense lines (Fig. 1). However, these differences are likely due to the transient nature of the Ca2⁺ responses (measured ~30 s after agonist addition) versus the longer incubations used for inositol phosphate accumulation (>10 min).

Our studies next focused on the desensitization of P2y2 and M1AchR-mediated calcium responses in vector-transfected cells. The concentration dependence of agonist-mediated desensitization was examined by pretreating cells with ATP (0.01 nM–100 nM) or carbachol (0.1 nM–1 mM) for 30 min before subsequent determination of intracellular calcium mobilization by a maximally activating concentration of each of these agonists (Fig. 3, A and B). Carbachol pretreatment produced a homologous, concentration-dependent desensitization of the subsequent carbachol response, whereas the response to ATP remained largely unaffected. In contrast, ATP pretreatment...
led to homologous desensitization of the P2Y2 receptor but also resulted in heterologous desensitization of the M1 AChR. Note that the duration of the transient Ca\(^{2+}\) response was unaffected by agonist pretreatment, with only the magnitude of response affected.

To investigate the involvement of arrestins in the desensitization of these responses, antisense and control cells were pretreated with a fixed concentration of agonist for 30 min, and then intracellular Ca\(^{2+}\) mobilization was measured in response to ATP or carbachol. As previously shown, carbachol pretreatment caused homologous desensitization of the M1 AChR, whereas the response to ATP was not altered in wild type cells (Fig. 4A). The extent of M1 AChR desensitization was significantly lower in the various antisense cell lines, further confirming a role for arrestins in M1 AChR desensitization (Fig. 4A).

Conversely, the homologous desensitization of the P2Y2 receptor by ATP was unaffected by reductions in arrestin levels, again suggesting that arrestins do not play a role in desensitization of this receptor (Fig. 4B). Interestingly, the heterologous desensitization of the M1 AChR by ATP pretreatment was also unchanged in antisense cells, indicating that arrestins are involved only in the homologous desensitization of the M1 AChR (Fig. 4B).

Time courses of agonist-mediated P2Y1, P2Y2, and M1 AChR desensitization were next constructed by pretreating cells with agonist for 0 to 45 min before rechallenge with a maximally activating concentration of agonist (Fig. 5). The rate and extent of M1 AChR desensitization was significantly diminished in antisense cell lines (\(t_{1/2} = 4.3 \pm 0.6, 4.4 \pm 0.9, \) and \(6.0 \pm 0.8\) min for AS 37, 83, and 108, respectively, compared with \(1.8 \pm 0.4\) for vector-transfected cells) (Fig. 5A). In contrast, the time courses of P2Y1 and P2Y2 receptor desensitization were unaffected by reductions in arrestin levels (Fig. 5, B and C).

Since the endogenous arrestins appeared to differentially regulate G\(_s\)-coupled receptors in HEK293 cells, we were also interested in characterizing G\(_s\)-coupled receptors in these cells. Screening for multiple G\(_s\)-coupled receptors (somatostatin, angiotensin, purinergic, \(\alpha_{1}\)-adrenergic, lysophosphatidic acid, and muscarinic) revealed that the AT1 angiotensin and somatostatin receptors are functionally expressed in HEK293 cells. SRIF-14 (Fig. 6A) and angiotensin II (Fig. 6B) both produced a concentration-dependent inhibition of forskolin-stimulated adenylyl cyclase activity in whole cell homogenates. Both the IC\(_{50}\) and the magnitude of inhibition of forskolin-stimulated adenylyl cyclase activity by each agonist was similar in vector-transfected and antisense-expressing cells.

We next constructed time courses of AT1 angiotensin and somatostatin receptor desensitization in control and antisense cells. In vector-transfected cells, pretreatment with SRIF-14 and angiotensin II resulted in rapid desensitization of receptor activity (Fig. 6, C and D). Interestingly, the SRIF-14-promoted desensitization was significantly attenuated in AS 37 and AS 108 lines (Fig. 6C). The rate of somatostatin receptor desensitization was significantly slower in both antisense cell lines (\(t_{1/2} = 4.1 \pm 0.6\) and \(5.4 \pm 0.6\) min for AS 37 and 108, respectively, compared with \(2.3 \pm 0.4\) for vector-transfected cells) (Fig. 6C). Interestingly, although the \(t_{1/2}\) of desensitization was slower in both antisense cell lines, both lines exhibited significant levels of receptor desensitization at later time points. Conversely, angiotensin II-promoted desensitization was no different in vector-transfected control and antisense cell lines.

The AT1 angiotensin receptor can also couple to activation of G\(_s\) and phospholipase C in a number of cell lines. Unfortunately, when we measured inositol phosphate accumulation in vector-transfected and antisense cell lines in response to angiotensin II, we found a very weak response with <2-fold stimulation of inositol phosphate accumulation over basal (data not shown). This poor response precludes an accurate assessment of the effects of reduced arrestin levels on AT1 angiotensin receptor coupling to G\(_s\).

In addition to their role in regulating GPCR desensitization, arrestins have also been implicated in mediating receptor endocytosis (5–8). Since our antisense studies suggested a role for arrestins in desensitization of selectiveGPCRs in HEK293 cells, we also wanted to address the potential role of arrestins in internalization of these receptors. Indeed, our previous studies revealed that arrestins mediate both desensitization and internalization of endogenous \(\beta_2\)-adrenergic receptors in HEK293 cells (16). Unfortunately, adequate reagents to study the trafficking of most endogenous GPCRs are unavailable. Thus, to further investigate the potential role of arrestins in GPCR internalization, we transfected HEK293 cells with an arrestin-2-GFP chimera and visualized arrestin trafficking in living cells. Arrestin-2-GFP has been demonstrated to redistribute from the cytosol to the plasma membrane upon receptor stimulation (24) and colocalize with selective internalized GPCRs (25, 26).

Transfected HEK293 cells expressing arrestin-2-GFP were initially stimulated with G\(_s\)-coupled receptor agonists. Before agonist stimulation, arrestin-2-GFP displayed a diffuse cytoplasmic distribution (Fig. 7, A and B). Initial attempts to visu-
alize arrestin translocation from the cytosol to the membrane following endogenous receptor stimulation (0–10 min) proved unsuccessful. However, it was noted that at later time points (>15 min), stimulation with the β-agonist isoproterenol (Fig. 7, C and E) or prostaglandin E2 (Fig. 7, D and F) resulted in significant redistribution of arrestin-2-GFP into a distinct punctate pattern. A similar series of studies revealed that carbachol (Fig. 8, B and C) and SRIF-14 (Fig. 8, E and F) treatment also promoted a punctate distribution of arrestin-2-GFP. In contrast, stimulation with angiotensin II (Fig. 8H), ADP (data not shown), or ATP (data not shown) for up to 60 min had no effect on arrestin-2-GFP localization. Thus, these results reveal a receptor-specific movement of arrestin-2-GFP that mirrored the arrestin-receptor specificity observed in the antisense cells.

To further characterize the localization of these punctate vesicles containing arrestin-2-GFP, lysosomal tracker red, which is a marker for lysosomes (27), and rhodamine-labeled transferrin, which is a marker for early endosomes and the recycling centriolar compartment (28), were utilized. Before agonist stimulation, arrestin-2 displayed a diffuse cytoplasmic distribution (Fig. 9A), whereas transferrin displayed its typical punctate endosomal pattern (Fig. 9B). After carbachol stimulation for 30 min, arrestin-2-GFP redistributed into a distinct punctate pattern (Fig. 9E), exhibiting a significant degree of colocalization with labeled rab-5 (note arrows in Fig. 9, E and F).

**DISCUSSION**

In this study, two separate approaches were used to investigate arrestin/GPCR specificity in HEK293 cells. First, using previously characterized antisense arrestin-expressing cells (16), we investigated the effect of reduced arrestin levels on the regulation of endogenous Gα signaling (P2y1 and P2y2 purinergic, M1AchR) and Gβγ-coupled (somatostatin and AT1 angiotensin) GPCRs. Studies on the Gα-coupled GPCRs (examining both total inositol phosphate production and intracellular Ca2⁺ mobilization) revealed that the agonist-specific desensitization of the M1AchR was significantly attenuated in cells with reduced arrestin levels, whereas the desensitization of the P2y1 and P2y2 purinergic receptors was unaffected. In the intracellular calcium mobilization experiments, heterologous desensitization of the M1AchR by ATP was unaltered by reductions in arrestins, providing further evidence that arrestins are mainly involved in the homologous desensitization of agonist-occupied GPCRs.

There are conflicting reports regarding the involvement of arrestins in MACHR regulation. A recent report suggested that overexpressed M1, M3, and M4AchRs undergo arrestin-2 and dynamin-dependent sequestration in HEK-293 tsA201 cells (30), whereas a previous report indicated that sequestration of these receptors is largely arrestin-independent (31). The disparity between these studies may reflect the potentially problematic nature of receptor overexpression in nonnative cell lines. In addition, neither of these studies investigated the consequence of arrestin co-expression on receptor signaling. Although the low endogenous levels of M1AchR expression precludes assessment of receptor internalization in HEK293...
rab-5 in HEK293 cells. HEK293 cells were transfected with 0.25 M1AchR. The involvement of arrestins in the regulation of endogenous G_i-coupled receptors in HEK293 cells. Agonist-stimulation of endogenous GPCRs results in rapid homologous desensitization (32), receptor desensitization and sequestration (33). Although these responses are largely unexplored. Previous studies have demonstrated that somatostatin receptor desensitization is dependent on receptor internalization, although the mechanism underlying this phenomenon is still unknown (35, 36). A recent study suggested that somatostatin receptor desensitization may be dependent upon receptor internalization, although this process was shown to be G protein-coupled receptor kinase-independent (36). As is the case with the M1AchR, low levels of endogenous sst receptors limit our ability to assess agonist-induced changes in receptor distribution. Our study does, however, provide the first evidence of arrestin involvement in the desensitization of somatostatin receptors.

Previous studies have shown that AT1 angiotensin receptor internalization is independent of dynamin and arrestin-2, although arrestin overexpression can augment internalization (37). An association between protein kinase C-induced receptor phosphorylation and desensitization by angiotensin II has been demonstrated (38). In agreement with these studies, our results suggest that arrestins are not involved in the desensitization of endogenously expressed AT1 angiotensin receptor responses.

To further investigate arrestin-receptor specificity we used an arrestin-2-GFP conjugate. Several studies have utilized this approach to provide real-time analysis of receptor-arrestin interactions in single cells overexpressing various GPCRs (24–26). Unlike these studies, however, we failed to see a rapid translocation (0–120 s) of arrestins from the cytosol to the membrane upon activation of any of the endogenous GPCRs. Under investigation in this study (24). We attribute this to the low levels of endogenous receptor expression, which may limit the ability to recruit a sufficient amount of arrestin that is visible by conventional immunofluorescent microscopy. We did, however, observe an agonist-specific redistribution of arrestins into a punctate pattern following more prolonged activation of endogenous GPCRs. The pattern of GPCR-activated arrestin-2-GFP redistribution mirrored the arrestin-receptor specificity identified above. Only agonist-induced activation of β2-adrenergic, prostaglandin E2, M1Ach, and somatostatin receptors induced arrestin-2-GFP redistribution, whereas the P2y1 and P2y2 purinergic and AT1 angiotensin receptor did not. Of note, activation of overexpressed AT1 angiotensin receptors has recently been shown to trigger a clear time-dependent redistribution of arrestins to an intracellular vesicular compartment where they colocalize with internalized receptors (26). The discrepancy between these results and the present study may be explained by differences in receptor expression levels. However, since our results suggest that endogenously expressed AT1 angiotensin receptors are not regulated by arrestins in HEK293 cells, one needs to be cautious when interpreting results using overexpressed receptors and arrestins.

Previous studies have indicated that for some GPCRs, arrestins can traffic with internalized receptors into early endosomes (25, 26). Unfortunately, we were unable to visualize the trafficking of endogenous receptors, but studies using rhodamine-labeled transferrin, rab-5, and lysosomal tracker red indicate that arrestin-2-GFP-containing vesicles colocalize with transferrin receptors and rab-5 in early endosomes (28, 29). The function of this prolonged association of arrestin-2 in an endosomal compartment is still unclear, although a recent study using heterologously overexpressed arrestin and GPCR, suggested that this may dictate the rate of receptor dephosphorylation, recycling, and resensitization (39).

In summary, this study demonstrates that arrestins selec-
tively regulate endogenously expressed GPCRs from the Gq (M₄Ach)- and Gi (somatostatin)-coupled families in HEK293 cells. Future studies will investigate the structural determinants of GPCRs that contribute to arrestin selectivity.

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