Receptor desensitization progressively limits responsiveness of cells to chronically applied stimuli. Desensitization in the continuous presence of agonist has been difficult to study with available assay methods. Here, we used a fluorescence resonance energy transfer-based live cell assay for the second messenger diacylglycerol to measure desensitization of a model seven-transmembrane receptor, the G<sub>A</sub>-coupled angiotensin II type 1<sub>A</sub> receptor, expressed in human embryonic kidney 293 cells. In response to angiotensin II, we observed a transient diacylglycerol response reflecting activation and complete desensitization of the receptor within 2–5 min. By utilizing a variety of approaches including graded tetracycline-inducible receptor expression, mutated receptors, and overexpression or short interfering RNA-mediated silencing of putative components of the cellular desensitization machinery, we conclude that the rate and extent of receptor desensitization are critically determined by the following: receptor concentration in the plasma membrane; the presence of phosphorylation sites on the carboxyl terminus of the receptor; kinase activity of G protein–coupled receptor kinase 2, but not of G protein-coupled receptor kinases 3, 5, or 6; and stoichiometric expression of β-arrestin. The findings introduce the use of the biosensor diacylglycerol reporter as a powerful means for studying G<sub>A</sub>-coupled receptor desensitization and document that, at the levels of receptor overexpression commonly used in such studies, the properties of the desensitization process are markedly perturbed and do not reflect normal cellular physiology.

The family of heptahelical receptors, also called seven-transmembrane receptors (7TMRs) or G protein–coupled receptors, regulates myriad physiological and pathological signal transduction pathways. Despite a large diversity of ligands, including catecholamines, chemokines, lipids, peptides, odors, and photons, these receptors share remarkable similarity in their intracellular regulatory mechanisms. Classically, receptor function is initiated by ligand-induced activation of heterotrimeric G proteins. This is followed by receptor inactivation, mediated by receptor phosphorylation by G protein-coupled receptor kinases (GRKs) and other kinases, β-arrestin recruitment, and receptor internalization (1, 2). This inactivation results in desensitization, the loss of agonist efficacy following sustained stimulation.

Until recently our appreciation of the kinetics of 7TMR molecular pharmacology has been limited to extrapolation from either complex in vivo readouts such as changes in blood pressure or from biochemical techniques such as phosphodiester hydrolysis assays (3), which cannot be performed in live, unperturbed cells. Thus, it has not been possible to observe receptor level desensitization in the continuous presence of agonist, the situation that most closely parallels many physiological circumstances. Consequently, despite a vast literature on 7TMR desensitization, uncertainty remains regarding how this process modulates receptor signaling kinetics.

The advent of fluorescent biosensor technology and the increasing number of intracellular signal indicators afford the opportunity to refine our understanding of heptahelical receptor signal kinetics. Indeed, these novel approaches have elucidated the spatiotemporal details of heptahelical receptor signaling at subcellular and sub-second scale (4–8). These powerful new technologies include fluorescent small molecule indicators (9), green fluorescent protein–fused protein localization (10), and most recently resonance energy transfer–based measurements of protein modification, protein conformational change, and protein–protein interactions (11).

Such biosensors have the potential to provide a new appreciation of 7TMR desensitization kinetics. Thus, we set out to develop an assay system that affords sensitive and non-disruptive measurement of 7TMR signal regulation. This would allow us to test the specificity and kinetics of GRK and β-arrestin functions for receptor signal output. Using the angiotensin II type 1A receptor (AT<sub>1A</sub>R) as a model, we designed a system...
that elucidates the kinetics of receptor regulation by real-time detection of the second messenger diacylglycerol (DAG). This has enabled us to determine the rate of receptor desensitization in the continuous presence of a constant ligand concentration and to test how changing concentrations of receptor, GRKs, and β-arrestins alter desensitization kinetics.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—pcDNA3-DAGR and pcDNA3-CYPHER have been described previously (8). pcDNA3.1-HA-AT1AR, pRK5-GRK2 (encoding bovine GRK2), pcDNA3-β-arrestin1-FLAG, and pcDNA3-β-arrestin2-FLAG (encoding rat β-arrestins) have all been described previously (12, 13). pcDNA3.1-HA-AT1AR-13A was produced by site-directed mutagenesis of all 13 serines and threonines in the cytoplasmic tail of the receptor to alanine using appropriately designed primers and two rounds of the QuikChange kit (Stratagene) according to the manufacturer’s instructions. pTetOn and pTRE2-hyg were purchased from Clontech. HA-tagged AT1AR or AT1AR-13A were PCR amplified from respective plasmid templates and with PCR primers tagged with restriction enzymes BamHI and SalI and cloned into pTRE2-hyg to make pTRE2-hyg-HA-AT1AR and pTRE2-hyg-HA-AT1AR-13A.

**siRNA and Plasmid Transfections**—GRK2, 3, 5, and 6 siRNA were all previously described (12). Transfection of GRK2 siRNA plus 100 ng of pcDNA3-DAGR into HEK-TetOn-AT1AR cells was performed using Gene Silencer (Gene Therapy Systems) as previously described (12, 14). To compensate for GRK2 siRNA-mediated receptor up-regulation, 50 ng/ml doxycycline was added to control samples and no doxycycline was added to samples with siRNA and plasmid transfections. For β-arrestin1/2 silencing via siRNA, control siRNA or siRNA targeting both β-arrestins1 and 2 (15) plus 100 ng of pcDNA3-DAGR were transfected into HEK-TetOn-AT1AR cells using Gene Silencer. 24 h after transfection, cells were treated with 100 ng/ml doxycycline to induce a modest level (170 fmol/mg) of angiotensin receptor expression, which was not different between the two siRNA conditions. For β-arrestin and GRK2 overexpression experiments, the 1 μg of the appropriate β-arrestin plasmid or 0.5 μg of pRK5-bGRK2 was co-transfected with 100 ng of pcDNA3-DAGR into a 10-cm plate of AT1AR-293 cells using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions.

**Cell Culture**—HEK-293 cells were maintained in minimum essential Eagle’s medium (M2279; Sigma) plus 10% fetal bovine serum and penicillin/streptomycin. Where applicable, G418 was used at 500 μg/ml for selection and 150 μg/ml for maintenance and hygromycin was used at 250 μg/ml for selection and 150 μg/ml for maintenance. AT1AR-stable HEK-293 cells (AT1AR-293) have been described previously (14) and were cultured in the presence of 100 μg/ml zeocin (Invitrogen).

**Creation of Tet-inducible HEK-TetOn-AT1AR Cells**—HEK-293 cells were transfected with pTET-On and subjected to G418 (500 μg/ml) selection for 3 weeks. Stable clones were selected based on their ability to inducibly express the AT1AR from transient transfection of pTRE2-hyg-HA-AT1AR and subsequent treatment with 1 μg/ml doxycycline or vehicle for 48 h. A second round of transfection was performed with pTRE2-hyg-HA-AT1AR, and cells were subjected to hygromycin selection (200 μg/ml) for 3 weeks. Positive clones were selected based on low background and ability to induce AT1AR expression in response to doxycycline. Receptor expression was determined in all experiments using previously described standard protocols for binding assays using fractional occupancy (16). Protein concentrations were determined by Bradford assay (Pierce). Constitutive AT1AR expression in these cell lines was very low (10–150 fmol/mg total protein) but up-regulated in a dose-dependent fashion upon addition of the tetracycline analogue doxycycline to 500–1500 fmol/mg total protein. Careful titration experiments revealed that in several clonal transfectant cell lines we could reliably select for specific receptor density across a range of 10–fold. Receptor concentrations reported as fmol/mg protein convert to number of receptors/cell as follows: 100 fmol/mg protein equals ~90,000 receptor/cell, based on our measurement of 1.5 ng of protein/HEK-293 cell.

**Immunoblotting**—To confirm either protein overexpression or silencing, a portion of the cells used for imaging were lysed and run on SDS-PAGE gels according to standard protocols. Western blots were performed using the following antibodies, endogenous β-arrestins with A1CT at 1:3000, GRK2 with GRK2-C15 at 1:1000 (S-562; Santa Cruz Biotechnology), and transiently expressed β-arrestins with FLAG M2 at 1:1000 (F1804; Sigma).

Chemiluminescent detection was performed with horseradish peroxidase-coupled secondary antibody (Amersham Biosciences) and SuperSignal West Pico reagent (Pierce). Chemiluminescence was quantified by a charge-coupled device camera (Syngene ChemiGenius2); representative images are shown as inverted grayscale.

**Reagents**—FURA2-AM was purchased from Molecular Probes (F1221) and was used with 15 min of preincubation at 1 μg/ml. Valsartan was obtained from Novartis and was used at a concentration of 1 μM. Angiotensin II was purchased from Sigma (A9525) and was used at 100 nM concentration in all experiments. Phorbol 12-myristate 13-acetate was purchased from Sigma (P1585) and used at a concentration of 1 μM. Doxycycline hyclate was purchased from Sigma (D9891) and used at concentrations ranging from 50 ng/ml to 1 μg/ml as indicated. Calphostin C was purchased from Calbiochem and used at 2 μM.

**Imaging**—For all imaging experiments, 24 h prior to assay cells were split and plated to imaging dishes precoated with fibronectin. Cells were washed once and placed in imaging buffer (125 mM NaCl, 5 mM KCl, 1.5 mM MgCl₂, 1.5 mM CaCl₂, 10 mM glucose, 0.2% bovine serum albumin, 10 mM HEPES, pH 7.4) and imaged in the dark on a stage heated to 37 °C. Images were acquired on a Zeiss Axiovert 200 M microscope (Carl Zeiss MicroImaging, Inc.) with a Roper Micromax cooled charge-coupled device camera (Photometrics) controlled by SlideBook 4.0 (Intelligent Imaging Innovations). Cyan fluorescent protein (CFP) and fluorescence resonance energy transfer (FRET) images were obtained through a 436/20 excitation filter (20 nm bandpass centered at 436 nm), a 455DCLP (dichroic longpass mirror), and separate emission filters (480/30 for CFP and 535/30 for FRET). Yellow fluorescent protein (YFP) inten-
the plasma membrane. This translocation increases the effective concentration of DAGR at the membrane, resulting in increased intermolecular FRET. This mechanism is distinguished from conformational change-induced intramolecular FRET changes (8) by its dependence on DAGR concentration. Whereas intramolecular, conformation-dependent FRET changes are concentration independent, intermolecular FRET is proportional to the amount of DAGR present. Increasing DAGR concentration, measured by the FRET-independent YFP intensity of single transiently transfected cells, causes increasing FRET changes between untreated cells and cells treated with a saturating dose of the DAG analogue phorbol 12-myristate 13-acetate (supplemental Fig. S1A), confirming the mechanism of action of DAGR.

We then used DAGR to monitor the dynamics of Angiotensin II (AngII) stimulation of the AT1A R. To avoid concerns of AT1A R dysregulation caused by expression above physiological levels, we generated clonal HEK-293 cell lines stably transfected with tetracycline-inducible AT1A R to control receptor expression. In HEK-TetON-AT1A R cells expressing moderate amounts of AT1A R (150 fmol/mg protein) and transfected with DAGR, time lapse DAGR experiments reveal both the kinetics and subcellular location of DAG signals (Fig. 1B). Prior to agonist addition, DAGR is cytosolic (shown with the FRET-independent YFP image) and FRET is low (blue pseudocolor). Thirty seconds after AngII addition, DAGR translocates from the cytosol to the plasma membrane, where FRET increases (red pseudocolor). Quantification of the FRET ratio time lapse (FRET intensity/monomeric CFP intensity, normalized to base line) in single cells showed that AngII led to a rapid increase in DAG (peaking after ~15 s), followed by a slower return to base line (after ~5 min) (Fig. 1C). This DAG transient was completely blocked by the angiotensin receptor blocker valsartan.

Because DAGR functions by binding DAG and could potentially buffer DAG concentrations, we addressed concerns that DAGR could perturb normal AT1A R signaling. We evaluated signal output downstream of DAG by measuring angiotensin-stimulated protein kinase C activity with the fluorescent biosensor CKAR (8). 100 nM AngII stimulated transient protein kinase C (PKC) activity, which was entirely dependent on phospholipase C, DAG, and PKC as assessed by a panel of inhibitors...
(supplemental Fig. S1B). When CKAR is co-expressed with a red fluorescent analogue of DAGR (mRFP1-C1-mRFP1) that translocates after AngII stimulation to a similar extent as DAGR, PKC activity was indistinguishable from CKAR expressed with free mRFP1 (Fig. 1D). This indicates that the amount of DAG bound by DAGR did not significantly affect DAG-dependent signaling and thus validates DAGR as a non-destructive assay of AT1AR signaling.

The transient nature of the AngII-stimulated DAG signal reflects rapid rates of AT1AR desensitization as well as DAG clearance by diacylglycerol kinases. Receptor desensitization is mediated by either GRK receptor phosphorylation and β-arrestin recruitment (1) or by second messenger-regulated kinase receptor phosphorylation (identified for the AT1AR as PKC) (17–19). To test the effect of receptor phosphorylation on DAG, we generated a mutant angiotensin II receptor with all 13 serines and threonines in the carboxyl-terminal tail (subsequent to the seventh transmembrane helix) mutated to alanine, named “13A”. These residues are required for agonist-induced AT1AR phosphorylation, β-arrestin recruitment, and receptor internalization (20–24). We compared the signal kinetics of WT and 13A AT1AR and found the two display strikingly different patterns of DAG generation (Fig. 2A). Compared with the transient DAG signal from WT AT1AR, 13A AT1AR exhibits a larger and prolonged DAG signal. This difference is consistent with a rapid (<5 min) phosphorylation-mediated desensitization of the AT1AR. This result is not unique to HEK-293 cells, as preliminary comparison of WT and 13A in TetOn-U2-osteosarcoma cells gave the same result (data not shown). Similar results were found in HEK-293 cells with CYPHR, a FRET sensor for phosphatidylinositol bisphosphate/inositol triphosphate, that like DAGR reflects phospholipase C activity (8) (supplemental Fig. S1C). Consistent with these results, AT1AR internalization, a phosphorylation-dependent process (22), is rapid and nearly complete for cells expressing wild-type (WT) receptor, whereas mutant 13A AT1AR completely fails to internalize (Fig. 2B). We also evaluated calcium signals as a more distal measure of receptor function. In contrast to DAG, calcium dynamics do not correlate well with receptor desensitization; WT and 13A receptors gave similar calcium responses (Fig. 2C), consistent with post-receptor mechanisms of calcium regulation (reviewed in Refs. 25, 26). We thus conclude that DAGR can measure Gq-coupled receptor regulation with high temporal fidelity, which allows us to explore the regulation of receptor desensitization.

Using tetracycline induction of receptor expression, we then determined the effect of receptor density on DAG kinetics and receptor desensitization. As already noted in Fig. 1C, we found that at low receptor expression (<100 fmol/mg protein, equivalent to 90,000 receptors/cell) AngII-stimulated DAG is transient, returning to baseline after 2–3 min (Fig. 3A). This represents a surprisingly rapid and profound desensitization of the AT1AR. In contrast, at increasingly higher, but still moderate, receptor expression (up to 375 fmol/mg protein), the rapid phase of desensitization is progressively lost, leading to sustained DAG. An expanded time scale (Fig. 3B) shows that the initial rate of DAG accumulation correlates with receptor expression and illustrates how rapidly desensitization occurs (within 30 s) at low receptor density.

We next tested the effect of altering 13A AT1AR expression on DAG. As expected, this mutant fails to desensitize even at very low density (30 fmol/mg protein), and the primary effect of increasing receptor expression is increased rate of DAG generation and higher maximum DAG signal (Fig. 3C). An expanded time scale (Fig. 3D) shows the effect of receptor expression on rate of accumulation and the contrast with the kinetics of

![FIGURE 2. DAGR reports AT1AR desensitization. A, AngII-induced diacylglycerol from the wild-type AT1AR (WT) rapidly returns to base line, whereas the signal from a nonphosphorylatable, non-desensitizing AT1AR (13A) remains elevated. Receptor surface expression was comparable for WT and 13A (150 fmol/mg protein). B, 100 nM AngII induces rapid internalization of wild-type AT1AR (WT) but fails to internalize a nonphosphorylatable AT1AR mutant (13A). C, Calcium dynamics display a more complicated relationship to desensitization, with only an elevated plateau of intracellular calcium to distinguish WT from 13A. All data are means with standard errors from three to five experiments.](image-url)
WT-AT1AR-stimulated DAG transients. The initial rate of DAG accumulation (10–20 s after agonist addition) is highly correlated with receptor expression \( r^2 \approx 0.98 \), supplemental Fig. S1D) and is similar for both WT and 13A AT1AR. This indicates that at these low and moderate receptor concentrations, receptor expression is the limiting factor in a DAG response. Furthermore, the similarity of initial DAG accumulation for WT and 13A AT1AR indicates that the 13A mutant elicits normal G protein coupling to phospholipase C.

Because AT1AR desensitization is dramatically impaired even at moderate expression levels, we investigated the mechanisms of desensitization for this receptor. Because \( \beta \)-arrestins desensitize signaling by binding receptors and sterically interfering G protein coupling, the desensitization capacity of \( \beta \)-arrestins could become stoichiometrically limited by AT1AR expression levels above the concentration of endogenous \( \beta \)-arrestins. To test this hypothesis, we used a stably transfected HEK-293 cell line with dramatic overexpression of the AT1AR (1200 fmol/mg protein). Cells transfected with a vector plasmid display sustained DAG after AngII stimulation, typical of lost desensitization (Fig. 4A). However, we found that overexpression of either \( \beta \)-arrestin1 or 2 could fully restore normal desensitization kinetics to this cell line. This led us to conclude that 1) \( \beta \)-arrestins are the limiting component to AT1AR desensitization at high receptor expression, and 2) both \( \beta \)-arrestin1 and \( \beta \)-arrestin2 are capable of desensitizing the AT1AR.

Because other work has shown that overexpressed, angiotensin-stimulated AT1AR co-immunoprecipitates both endogenous \( \beta \)-arrestins (12), we have further concluded that the two \( \beta \)-arrestins are redundant for AT1AR desensitization. To confirm the importance of receptor phosphorylation for \( \beta \)-arrestin-mediated AT1AR desensitization, we repeated these experiments with 13A AT1AR. As expected, exogenous \( \beta \)-arrestin expression did not restore desensitization to the non-phosphorylatable 13A AT1AR (Fig. 4B). This result solidifies the role of phosphorylation as a necessary intermediate between receptors and \( \beta \)-arrestins in the desensitization process.

In contrast to \( \beta \)-arrestins, overexpression of GRK2, the kinase responsible for most AT1AR phosphorylation (12), suppressed detectable DAG accumulation altogether (Fig. 4A). However, similar results for GRK2 were obtained using the 13A mutant receptor (Fig. 4B) and with a kinase-inactive K220M GRK2 mutant (27) for the WT AT1AR (data not shown). These results suggest this effect is not related to GRK2 kinase activity and most likely corresponds to G protein sequestration ascribed to an RGS (regulator of G protein signaling)-like domain of GRK2 (28, 29). Interestingly, overexpression of GRK3, the closest homologue of GRK2, did not suppress the DAG signal (data not shown). We confirmed G\( \alpha_q \) as the locus of the profound signal inhibition caused by GRK2 overexpression: G\( \alpha_q \) overexpression, presumably saturating the RGS activity of GRK, partially rescued the signal suppression (supplemental Fig. S2). Despite this, the finding that 13A AT1AR completely fails to desensitize in the presence of endogenous levels of GRK2 (Fig. 2A) suggests that...
GRK2 kinase activity, and not the RGS function, is the primary regulator of acute AT1AR desensitization. Given these results and the concern that results derived from overexpressed GRKs and β-arrestins may not reflect physiologically relevant functions, we evaluated the effects of siRNA-mediated silencing of the endogenous β-arrestin/GRK system. Although individual silencing of neither β-arrestin1 nor β-arrestin2 significantly altered WT AT1AR desensitization (data not shown), simultaneous silencing of both β-arrestins significantly abrogated desensitization in comparison to a non-functional control siRNA (Fig. 5A). Average β-arrestin silencing was 80%; we found it impossible to completely eliminate β-arrestin expression (Fig. 5B) but believe that our results can be qualitatively extrapolated to indicate that a large portion of AT1AR desensitization in our HEK-293 cells is β-arrestin dependent. Importantly, AT1AR expression was unchanged by β-arrestin silencing. In contrast, GRK2 silencing up-regulated surface AT1AR expression 2-fold. To compensate for this, we treated control siRNA-transfected cells with a dose of doxycycline (0.05 μg/ml) empirically determined to result in AT1AR expression equivalent to that found after GRK2 silencing. In experiments with equivalent AT1AR expression, desensitization is dramatically compromised by the loss of GRK2 expression (Fig. 5C). Relative to β-arrestin silencing, GRK2 silencing was very effective (Fig. 5D), averaging 90–95%. In contrast to the dramatic effect of GRK2 silencing on desensitization, similarly effective silencing of GRK3, GRK5, or GRK6 had no significant effect on AT1AR desensitization (supplemental Fig. S3). Thus, we conclude that GRK2 is the primary regulator of AT1AR desensitization in our HEK-293 cells and that GRK2 functions predominantly through phosphorylation of the carboxyl-terminal tail of AT1AR, inducing β-arrestin translocation and inhibition of receptor-G protein coupling.

**DISCUSSION**

This study analyzed real-time AT1AR signal kinetics as a model for heptahelical receptor desensitization, using the fluorescent biosensor DAGR. DAGR is a non-disruptive reporter and does not alter receptor function as measured by the effect of a DAGR analogue on downstream DAG-dependent PKC activity (Fig. 1D). This contrasts with phosphatidylinositol hydrolysis, the standard second messenger assay for Gq-coupled receptors, which requires lithium to block analyte degradation (3, 30), altering downstream and feedback signaling. Other assays for Gq-coupled signals include high pressure liquid chromatography, used to measure DAG directly (31), and a DAG assay using recombinant DAG kinase for the generation of 32P-labeled phosphatidic acid from crude membranes (32). However, none of these assays are amenable to real-time analysis.

In contrast, sufficient dynamic sensitivity is readily obtained with calcium binding fluorophores such as FURA-2-AM that are commonly used to detect receptor activation in living cells. However, as shown in Fig. 2C, these indicators do not effectively discern differences in receptor desensitization, Addition-ally, receptor desensitization has been inferred from β-arrestin recruitment assays (10), but these require overexpressed receptor and β-arrestin and thus alter receptor function.

Future studies using DAGR to measure receptor signal kinetics might take advantage of Gα15 subunits or chimeric Gα subunits that are commonly overexpressed to couple any 7TMR to phospholipase C and DAG/Ca2+ generation (33). This may

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**FIGURE 4.** Desensitization of high expression AT1AR, assessed by DAGR kinetics, is restored by β-arrestin overexpression. A, overexpression of either β-arrestin1 or β-arrestin2 restores rapid desensitization of highly expressed AT1AR (1200 fmol/mg protein) compared with transfection of pcDNA3.1 vector alone. Overexpression of GRK2 completely blocks the DAG signal. B, in contrast, overexpression of neither β-arrestin1 nor β-arrestin2 restores desensitization to the nonphosphorylatable AT1AR 13A. Overexpression of GRK2 blocks the DAG signal as in panel A. All data are means with standard errors for three to five experiments. Overexpression was confirmed by immunoblot, with β-actin immunoblot shown as control for protein loading.
allow a more comprehensive evaluation of the kinetics and GRK specificity for 7TMR family regulation.

Despite the utility of DAGR as a non-disruptive sensor, the DAGR signal is a complex readout, dependent on the dynamic balance of DAG generation and clearance. We have isolated the acute phase of AT1AR desensitization mediated by GRK2 phosphorylation of the receptor tail and β-arrestin recruitment, but it remains possible that dynamic regulation of DAG clearance by diacylglycerol kinase activity may play a role in shaping DAG signals. This should prove to be a fruitful area of further study.

Perhaps the most provocative findings revealed by this study relate to the relationship between the amount of receptor expression and desensitization. Typically, studies of the molecular regulation of receptors like the AT1AR have been performed in transfected cellular systems with receptor densities of pmol/mg protein. Here we have shown that these levels saturate the ability of cells to desensitize second messenger signaling. This reflects stoichiometric β-arrestin function via a mechanism of steric blockade; in contrast, GRKs function enzymatically and can compensate for receptor overexpression.

In contrast to the very high receptor densities frequently used in molecular studies, tissues and cell lines typically express ~100 fmol/mg protein (equal to ~90,000 receptors/cell in this study) of endogenous AT1AR (34–39). As shown by DAGR transients in HEK-TetOn-AT1AR cells (Fig. 3A), these expression levels are well within the range of the capacity of cells to desensitize receptor signaling. Consequently, we believe that the tetracycline-inducible cell system affords a biologically relevant level of receptor expression.

Regardless of receptor density, overexpression of GRK2 completely inhibited any DAG accumulation in response to AngII. This is unrelated to AT1AR desensitization or GRK2 kinase function because an identical result was obtained with GRK2 K220R, a kinase-inactive mutant, and also by transfecting the wild-type GRK2 with the 13a AT1AR. Instead, in the context of GRK2 overexpression, DAG signals are blocked through Goq sequestration by the RGS homology domain of GRK2 (28, 29, 40). Because AT1AR desensitization requires both intact AT1AR phosphoacceptor sites and GRK2 expression and the RGS homology function is independent of these phosphoacceptor sites, we conclude that the primary mechanism of AT1AR desensitization is GRK2 phosphorylation of the AT1AR carboxyl-terminal tail. However, because receptor, β-arrestin, and GRK isoform concentrations vary across cell type, we expect that other tissues and cell lines can use different GRKs to mediate AT1AR desensitization, as recently shown for the β2-adrenergic receptor (41). Additionally, in cells with sufficiently high concentrations of GRK2, the RGS homology domain function may be an important component of AT1AR desensitization.

The role of GRK2 in AT1AR phosphorylation and desensitization is consistent with data showing that GRK2, of all the ubiquitously expressed GRKs, is the single largest contributor to β-arrestin recruitment to the AT1AR in our HEK-293 cells. 5

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However, this contrasts with findings in these same cells that GRK6 is the largest contributor to β-arrestin recruitment to the β_{2}AR receptor (41). This demonstrates a receptor specificity of GRK function. It is likely that this specificity varies among cell and tissue types, illustrated by the fact that β_{2}AR regulation by GRK6 is absent in U2-osteosarcoma cells, which instead use GRK2 and 3 to regulate β_{2}AR function (41). This complexity can be regulated further by "biased agonists" such as the AngII analogue 3Sar, 4lle, 4lle-Ang-II, which recruits β-arrestins in the absence of G protein stimulation and is regulated by GRK6 instead of GRK2. This may provide opportunities for novel, more subtle pharmacological manipulation of receptor systems beyond the classic spectrum of agonists and antagonists of G protein-mediated signaling. Indeed, as shown here, the effect of altering GRK activity on receptor signaling can be quite profound.

Of course, there are other means of altering receptor signaling. The receptor, GRK, and β-arrestin system comprise a mechanism for homologous desensitization that contrasts with heterologous desensitization mediated by receptor phosphorylation by second messenger kinases. For Go_{q}α-coupled receptors like the AT1_{A}R, PKC has been shown to mediate receptor phosphorylation and heterologous desensitization as measured by increased signaling after suppression of PKC activity (17–19). However, we did not evaluate heterologous, PKC-mediated desensitization with the DAGR reporter because of the complexities of the PKC feedback network. In addition to phosphorylating receptor, PKC has been found to phosphorylate and inhibit phospholipase C-β (42). Hence, PKC inhibition simultaneously increases positive signaling (by blocking phospholipase C-β phosphorylation) and decreases negative feedback (by blocking heterologous AT1_{A}R desensitization). Indeed, treatment with Ro-31-8425 delays the return of AngII-stimulated DAG to base line, but we have not determined the relative contributions of PKC to phospholipase C regulation and AT1_{A}R heterologous desensitization. The methods described here should provide fruitful exploration of these issues. However, this work suggests that independent of other signaling feedback mechanisms, GRK2 is required for AT1_{A}R desensitization in HEK-293 cells.

More generally, this work provides the technical and conceptual framework to evaluate receptor desensitization in a new light. Although the classical definition of desensitization involves measuring responses to repeated stimulation, we believe that kinetic measurements of receptor signaling in the presence of constant agonist provide a more accurate and physiologically relevant description of receptor regulation. Our data highlight the critical importance of receptor expression, GRK phosphorylation, and β-arrestin binding in determining the rate of receptor desensitization. The extrapolation of these methods to other cell lines, tissues, and receptors will provide a more comprehensive understanding of heptahelical receptor regulation and may provide more precise methods of evaluating drug candidates that influence the amplitude and kinetics of receptor signals.

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