THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 29, pp. 20577–20588, July 21, 2006

G-protein-coupled Receptor Kinase Specificity for β-Arrestin Recruitment to the β_2-Adrenergic Receptor Revealed by Fluorescence Resonance Energy Transfer*[S]

Received for publication, December 21, 2005, and in revised form, April 3, 2006 Published, JBC Papers in Press, May 10, 2006, DOI 10.1074/jbc.M513605200

Jonathan D. Violin[‡], Xiu-Rong Ren[‡], and Robert J. Lefkowitz[‡§¶1]

From the Departments of [‡]Medicine and [§]Biochemistry and [¶]Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710

The small family of G-protein-coupled receptor kinases (GRKs) regulate cell signaling by phosphorylating heptahelical receptors, thereby promoting receptor interaction with β-arrestins. This switches a receptor from G-protein activation to G-protein desensitization, receptor internalization, and β-arrestin-dependent signal activation. However, the specificity of GRKs for recruiting β-arrestins to specific receptors has not been elucidated. Here we use the β_2-adrenergic receptor (β_2AR), the archetypal nonvisual heptahelical receptor, as a model to test functional GRK specificity. We monitor endogenous GRK activity with a fluorescence resonance energy transfer assay in live cells by measuring kinetics of the interaction between the β_2AR and β-arrestins. We show that β_2AR phosphorylation is required for high affinity β-arrestin binding, and we use small interfering RNA silencing to show that HEK-293 and U2-OS cells use different subsets of their expressed GRKs to promote β-arrestin recruitment, with significant GRK redundancy evident in both cell types. Surprisingly, the GRK specificity for β-arrestin recruitment does not correlate with that for bulk receptor phosphorylation, indicating that β-arrestin recruitment is specific for a subset of receptor phosphorylations on specific sites. Moreover, multiple members of the GRK family are able to phosphorylate the β_2AR and induce β-arrestin recruitment, with their relative contributions largely determined by their relative expression levels. Because GRK isoforms vary in their regulation, this partially redundant system ensures β-arrestin recruitment while providing the opportunity for tissue-specific regulation of the rate of β-arrestin recruitment.

Arrestins and G-protein-coupled receptor kinases (GRKs)[2] are important regulators of heptahelical receptor function. The two nonvisual arrestins, β-arrestin1 and β-arrestin2, and the ubiquitous nonvisual GRKs, GRK2, GRK3, GRK5, and GRK6, together form an axis of receptor regulation critical to mammalian physiology (1–3).

The β-arrestin/GRK system was first described as a means of desensitizing receptors (4), but it is now known to mediate receptor internalization (5) and receptor-stimulated signals as well, including activation of Src, ERK1/2, Rho, and others (6). The primary switch in these regulatory pathways appears to be the recruitment of β-arrestins to receptors, a process facilitated by GRK-mediated receptor phosphorylation, which enhances binding affinity for β-arrestins (7). β-Arrestin binding to phosphorylated receptor sterically hinders G-protein coupling of the receptor (8) and also induces conformational changes in β-arrestin (9). These conformational changes regulate the ability of β-arrestin to couple to the endocytic machinery (10) and to stimulate β-arrestin-dependent signals. Indeed, it appears that β-arrestin may adopt functionally distinct conformations depending on specific features of receptor phosphorylation, as siRNA silencing of distinct GRKs has dramatically different effects on receptor internalization and β-arrestin-dependent signaling (11, 12).

GRKs appear to have low substrate specificity, perhaps to allow the small number of GRKs to regulate the diverse and numerous (more than 800) heptahelical receptors. To date there has been little success in describing specific roles for individual GRKs for the regulation of individual receptors. Although notable phenotypes arise from targeted deletion of individual GRKs in mice (13–16), it has been difficult to ascribe these effects to specific receptors. To the extent that GRKs may be attractive therapeutic targets (17), this confounds any mechanistic understanding of the roles of specific GRKs.

The β_2AR is the archetypal nonvisual heptahelical receptor (18) and has extensively characterized pharmacology and biochemistry (19). Indeed, the receptor has been a model for studying β-arrestins and GRKs (4, 20). However, efforts to elucidate the influence of specific GRKs on β_2AR function have relied primarily on overexpression. Given the low substrate specificity of GRKs, it is likely that overexpressed GRKs can exhibit compensatory phosphorylation, whereby one GRK can substitute for another GRK for a particular receptor substrate. Thus it has been difficult to ascribe roles for specific GRKs on the β_2AR or other receptors.

* This work was supported by National Institutes of Health Grants HL 16037 and HL 70631 (to R. J. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

[1] Investigator with the Howard Hughes Medical Institute. To whom correspondence should be addressed: Howard Hughes Medical Institute, Duke University Medical Center Box 3821, Durham, NC 27710. Tel.: 919-684-2974; Fax: 919-684-8875; E-mail: lefko001@receptor-biol.duke.edu.

[2] The abbreviations used are: GRK, G-protein-coupled receptor kinase; β_2AR, β_2-adrenergic receptor; siRNA, short interfering RNA; FRET, fluorescence resonance energy transfer; BRET, bioluminescence resonance energy transfer; mCFP, monomeric cyan fluorescent protein; mYFP, monomeric yellow fluorescent protein; HEK, human embryonic kidney; U2-OS, U2-osteosarcoma; PKA, cAMP-dependent protein kinase.

In this study, we use fluorescence resonance energy transfer (FRET) to report the interaction of β_2AR and β-arrestins fused to the cyan and yellow variants of the green fluorescent protein (mCFP and mYFP). We reasoned that this assay would elucidate the kinetics of β-arrestin recruitment, which should in turn reflect the activities of endogenous GRKs. In addition, a kinetic assay should be more sensitive to changes in GRK activity than measurements of receptor phosphorylation or β-arrestin recruitment at equilibrium. We set out to validate this approach and then to use the kinetics of β-arrestin recruitment to answer several outstanding questions about GRK regulation of the β_2AR. 1) What are the relative contributions of β_2AR phosphorylation and β_2AR agonist-induced conformational changes for generating affinity for β-arrestin? 2) Is there GRK specificity or redundancy for β-arrestin recruitment for the β_2AR? 3) Does GRK specificity for β_2AR phosphorylation correlate with GRK specificity for β-arrestin recruitment to the receptor? 4) Do different cell types, with differing GRK expression profiles, use distinct sets of GRKs to promote β-arrestin recruitment? The answers to these questions should illuminate the regulation of the many facets of β-arrestin functions.

EXPERIMENTAL PROCEDURES

Materials—Isoproterenol was obtained from Sigma. [^{125}I]Iodocyanopindolol was obtained from PerkinElmer Life Sciences. H-89 was obtained from EMD Biosciences. Anti-phospho-β_2AR (p355/p356) was from Santa Cruz Biotechnology. Anti-β-arrestin (A1CT) is described elsewhere (21). Anti-FLAG beads were from Sigma. All other reagents were from Sigma.

Plasmids—Rat β-arrestin1 and rat β-arrestin2 were amplified by PCR to encode HindIII and SalI restriction sites at the 5′ and 3′ ends, respectively, with the terminator codon replaced with a sequence encoding a diglycine linker. These products were cut, purified, and ligated into a pcDNA3.1-mYFP vector (22) to generate β-arrestin1-mYFP and β-arrestin2-mYFP. The β-arrestin-mYFP inserts were then transferred to a pcDNA3.1-zeo vector providing Zeocin resistance. The R169E mutation in β-arrestin1-mYFP was generated by the QuickChange protocol (Stratagene). Rat β_2AR was amplified by PCR to encode a HindIII restriction site, FLAG epitope, and signal peptide sequence at the 5′ end (23), and an XhoI restriction site at the 3′ end with the terminating codon replaced with a sequence encoding a diglycine linker. This product was cut, purified, and ligated into a pcDNA3.1-mCFP vector (22) to generate β_2AR-mCFP. All plasmids were amplified in bacteria, kit purified (Qiagen), and validated by capillary electrophoresis sequencing.

Small Interfering RNA (siRNA) Silencing of Gene Expression—Chemically synthesized double-stranded siRNA duplexes (with 3′ dTdT overhangs) were purchased from Dharmacon for the following targets, as described and validated elsewhere (11, 12): GRK2 (5′-AAGAAGUACGAGAAGCUGGAG-3′), GRK3 (5′-AAGCAAGCUGUAGAACACGUA-3′), GRK5 (5′-AAGCCGUGCAAAGAACUCUUU-3′), and GRK6 (5′-AACAGUAGGUUUGUAGUGAGC-3′). A nonsilencing RNA duplex (5′-AAUUCUCCGAACGUGUCACGU-3′) was used as a control for all siRNA experiments. A second GRK6 sequence (5′-AAGUGAACAGUAGGUUUGUAG-3′) was used to demonstrate that the siRNA effect was target-specific. HEK-293 cells were transfected with Gene Silencer (Gene Therapy Systems), and U2-OS cells were transfected with Lipofectamine 2000 (Invitrogen), according to manufacturers' instructions. Silencing was quantified by immunoblotting. Only experiments with verified silencing were used; average silencing for these experiments was as follows: for HEK-293 cells, GRK2 (91%), GRK3 (97%), GRK5 (85%), and GRK6 (96%); for U2-OS cells silencing was GRK2 (94%), GRK3 (99%), and GRK5 (97%); GRK6 was not detected in U2-OS cells.

Cell Culture—HEK-293 cells and U2-OS cells were maintained in modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution (Sigma). HEK-293 cells were transfected with FuGENE 6 (Roche Applied Science); U2-OS cells were transfected with Lipofectamine 2000 (Invitrogen). All transfections used 3.0 μg of plasmid in a 10-cm tissue culture plate. Cells expressing β_2AR-mCFP were selected with 400 ng/ml G418 (Sigma), and colonies of stable transfectants were isolated. A single line of stably transfected cells was chosen as representative based on membrane targeting and isoproterenol-induced internalization of the β_2AR-mCFP. Surface expression of β_2AR was measured by ^{125}I-cyanopindolol binding as described (24), and was determined to be 1.0 pmol of receptor/mg of protein for HEK-293 and 3.2 pmol/mg protein for U2-OS. Doubly stable cells were generated by transfecting β-arrestin2-mYFP-Zeo selecting single colonies with 150 μg/ml Zeocin (Invitrogen). β-Arrestin-mYFP overexpression was assessed by immunoblot and compared with endogenous β-arrestin (supplemental Fig. 1).

Imaging—Cells were washed once, placed in imaging buffer (125 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 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 200M microscope (Carl Zeiss MicroImaging, Inc.) with a Roper Micromax cooled charge-coupled device camera (Photometrics) controlled by SlideBook 4.0 (Intelligent Imaging Innovations). CFP and 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). YFP intensity was imaged through a 500/20 excitation filter, 515LP dichroic mirror, and 535/30 emission filter. All optical filters were obtained from Chroma Technologies. Excitation and emission filters were switched in filter wheels (Lambda 10–2; Sutter). Integration times were varied between 100 and 300 ms to optimize signal and minimize photobleaching. Spectral bleed through was determined by acquiring CFP, FRET, and YFP intensity images of samples expressing CFP only and YFP only and was linear with respect to fluorophore expression. This imaging system exhibits 43% CFP/FRET bleed through and 24% YFP/FRET bleed through. CFP/YFP bleed through was undetectable. FRET intensity corrected for bleed through (FRETc) was defined as $FRETc = FRET - 0.43 \times CFP - 0.24 \times YFP$. FRETc for all FRET images was calculated on a pixel-by-pixel basis for localization of FRET. In contrast, all graphs display

calculations based on intensity from whole cells or sets of cells.

Immunoblotting—Cells were lysed in SDS sample buffer and adjusted to equal protein concentration by protein assay of a parallel set of cells, as described (25). Equal amounts of protein were separated on 10% Tris-glycine polyacrylamide gels (Invitrogen) and transferred to polyvinylidine fluoride membranes for immunoblotting. GRKs were detected with isoform-specific antibodies from Santa Cruz Biotechnology for GRK2 (sc-562), GRK3 (sc-563), GRK5 (sc-565), and GRK6 (sc-566), according to the manufacturer's instructions. 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.

Receptor Phosphorylation—Three days after transfection, HEK-293 cells plated in 100-mm dishes were incubated at 37 °C for 60 min in phosphate-free minimum Eagle's medium containing [^{32}P]P_i (100 μCi/ml; 1 Ci = 37 GBq). The amount of $^{32}P_i$ in the medium was increased to 200 μCi/ml in the GRK6 siRNA-transfected cells to normalize for a 50% decrease in the uptake found in these cells. After agonist stimulation, the lysate protein concentrations were normalized according to the relative receptor expression. Immunoprecipitation was carried out to determine phosphorylation as described (11).

Coimmunoprecipitation—3 days after transfection with a FLAG-β_2AR plasmid and the different siRNAs, 100-mm plates were incubated in 4.0 ml of Dulbecco's phosphate-buffered saline with 10 mM HEPES for 1 h at 37 °C and then stimulated. Cells were subjected to cross-linking by dithiobis(succinimidyl-propionate) from Pierce (26). Immunoprecipitates were analyzed by immunoblotting with the A1CT polyclonal antibody (1:3,000 dilution), which detects β-arrestin1 and -2 (21).

Statistics—All statistics were performed with GraphPad Prism 3.02 software. For determining association kinetics, both one-phase and two-phase associations were compared to determine the best fit. For comparison of experimental treatments, one-way analysis of variance was used, with a Bonferroni multiple comparison test as appropriate.

RESULTS

In an attempt to elucidate β_2AR signal modulation by the arrestin/GRK axis, we chose to use the HEK-293 cell line as a model system. These cells express the four ubiquitously expressed nonvisual GRKs (2, 3, 5, and 6) and are readily transfected with either DNA plasmids or siRNA (11, 12). We chose to assay β-arrestin recruitment to the receptor by FRET to quantify both the kinetics and relative amount of β-arrestin recruitment. FRET has been used previously to assay β_2AR interaction with β-arrestin2 (27), as was the similar bioluminescence resonance energy transfer (BRET) assay (28). Whereas BRET is more simply adapted to plate reader systems than FRET, only FRET is compatible with single cell and imaging assays. We thus generated FRET constructs for an imaging assay, allowing us to evaluate both the subcellular location and single cell kinetics of β-arrestin recruitment. Briefly, the monomeric cyan and yellow variants of the green fluorescent protein (mCFP and mYFP) were fused to the human β_2AR and various β-arrestin constructs to generate β_2AR-mCFP and β-arrestin-

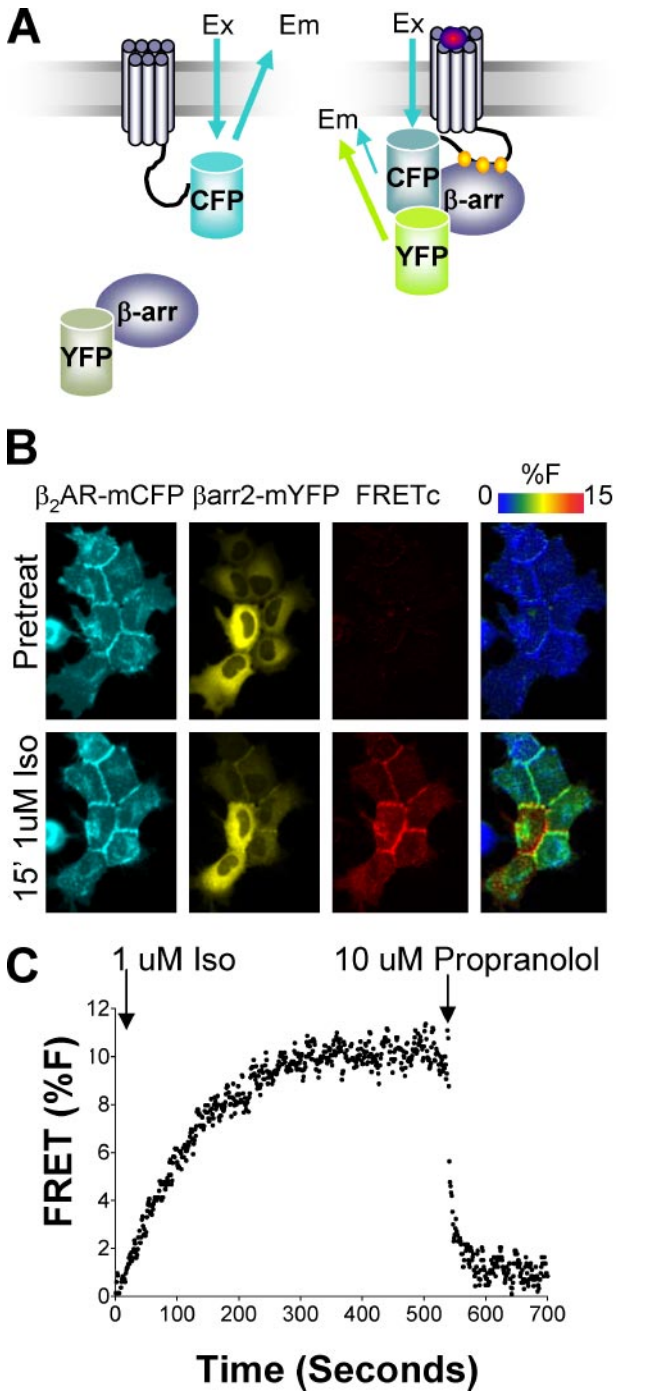


FIGURE 1. **Recruitment of β-arrestin to the β_2AR reported by FRET.** *A*, β_2AR-mCFP coexpressed with β-arrestin2-mYFP undergoes FRET upon agonist-stimulated phosphorylation of the receptor. *B*, FRETc, the FRET image corrected for spectral bleed through at each pixel, is detected 15 min after addition of 1 μM isoproterenol (*Iso*) and corresponds to translocation of β-arrestin2-mYFP to membrane. To best illustrate the localization of β-arrestin-bound β_2AR, %*F* is displayed as a pseudocolor spectrum, intensity-modulated to correspond to β_2AR-mCFP fluorescence to differentiate free β_2AR (no FRET, *blue*) and β-arrestin-β_2AR complex (high FRET, *red*). *C*, FRET is quantified as a percentage of whole-cell total CFP-excited fluorescence (%*F*) and shows monophasic kinetics for both isoproterenol-stimulated recruitment and ensuing dissociation by propranolol. Data are representative of three independent experiments.

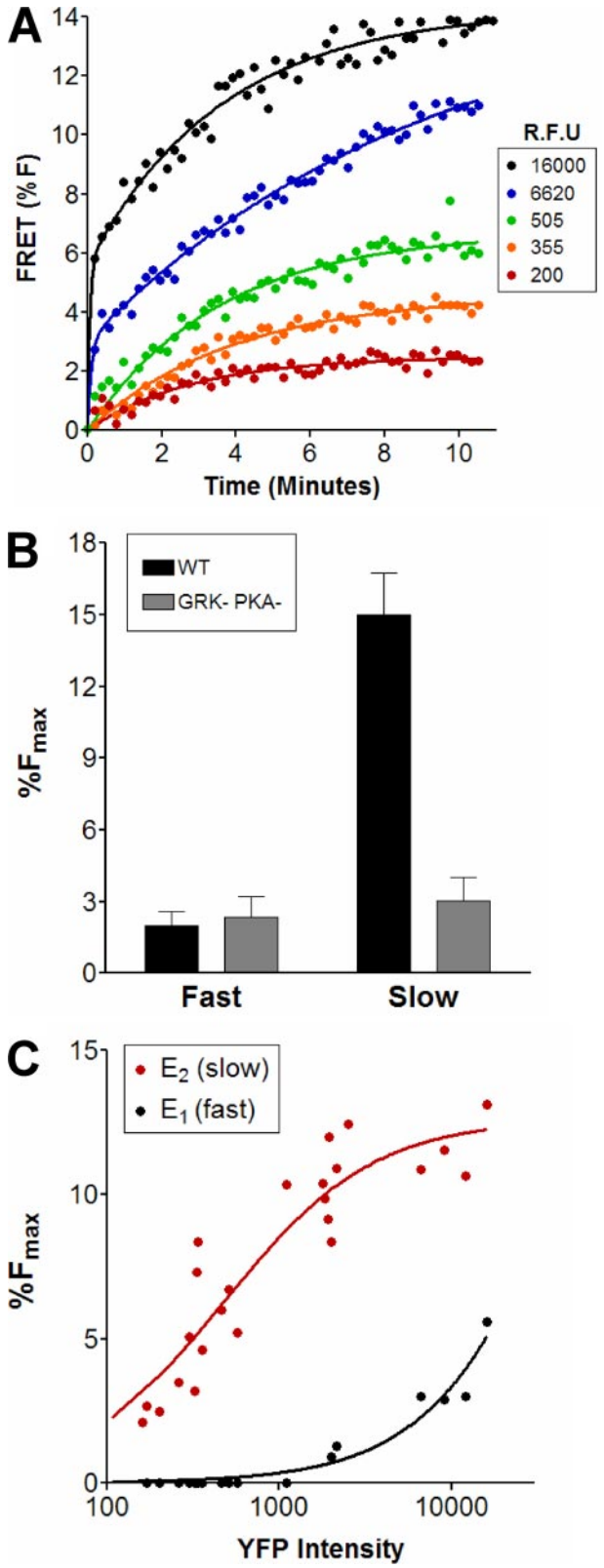


FIGURE 2. **β_2AR-mCFP has two binding affinities for β-arrestin2-mYFP.** *A,* at low expression of β-arrestin2-mYFP (*R.F.U.*, relative fluorescent units of YFP) isoproterenol-stimulated association is monophasic with a half-time of 90 s; at higher expression levels, association is biphasic, with half-times of 2 and 90 s. Data are from 5 individual cells representative of 25 from six separate experiments. *B,* phosphorylation-deficient β_2AR-mCFP exhibits a rapid association similar to wild-type (*WT*) β_2AR-mCFP but is markedly impaired in the slow association, consistent with a phosphorylation-independent rapid association and phosphorylation-dependent slow association. Data are average ± S.E. of three separate experiments. *C,* slow association is much higher affinity than the fast association, as described by the fit of a saturable binding curve for each phase. Data are from single cells from six independent experiments.

mYFP for coexpression. These constructs interact after addition of the agonist isoproterenol, and a portion of excited state CFP is nonradiatively transferred to YFP, resulting in yellow rather than cyan emission (Fig. 1*A*). The proportion of energy thus transferred (FRETc, corrected for spectral overlap (29)) is quantifiable as a percentage of total fluorescence emitted %*F*, analogous to fractional receptor occupancy as shown in Equation 1,

$$\text{FRET}\ (\%F) = 100 \times \frac{\text{FRETc}}{\text{CFP} + \text{FRETc}} \qquad \text{(Eq. 1)}$$

%*F* differs from absolute FRET efficiency (%*E*) only in the absence of a term correcting for hardware-specific differences in the quantum yield of CFP and FRET emission (29). In addition, FRET can be imaged after correcting for spectral overlap, allowing localization of the β_2AR-β-arrestin interaction (Fig. 1*B*). At higher resolution, this method of FRET imaging discriminates between β_2AR-mCFP bound to β-arrestin-mYFP at the plasma membrane and internalized vesicular β_2AR-mCFP, which has lost the association with β-arrestin-mYFP (supplemental Fig. 2). When FRET is measured for a field of cells, the kinetics of this interaction are monoexponential and rapidly reversed with addition of the antagonist propranolol (Fig. 1*C*).

We tested the kinetics of recruitment in cells stably transfected with β_2AR-mCFP and transiently transfected with β-arrestin2-mYFP. These cells express constant amounts of β_2AR-mCFP (1 pmol/mg protein; data not shown) and varying amounts of β-arrestin2-mYFP. We used transiently transfected cells, for which single cell overexpression is highly variable, to assess the effects of β-arrestin expression on the β-arrestin-β_2AR interaction. We found that in cells with low to moderate expression of β-arrestin-mYFP, as measured by single cell YFP fluorescence, isoproterenol-induced recruitment is monoexponential (Fig. 2*A*). Furthermore, higher β-arrestin2-mYFP expression yielded increased recruitment, as measured by maximum fractional FRET. In contrast, in cells with high expression of β-arrestin2-mYFP, recruitment is biphasic, exhibiting a rapid association (less than 5 s half-time) as well as the slow association seen with low expression. At all levels of β-arrestin2-mYFP expression, the slow rate was the same ($t_{1/2}$ = 2.5 ± 0.2 min, n = 15), with no correlation between β-arrestin2-mYFP expression and $t_{1/2}$ (R^2 = 0.18). This is consistent with a bimolecular interaction dependent on a single rate-limiting activity. Presumably, this limit is set by the rate of receptor phosphorylation by GRKs. To test this, we transiently expressed β-arrestin2-mYFP with either wild-type β_2AR-mCFP or with a nonphosphorylatable mutant β_2AR-mCFP (GRK−/PKA−) (30). As expected, the nonphosphorylatable receptor exhibits very low agonist-induced FRET (Fig. 2*B*). For this experiment, β-arrestin was highly expressed, and wild-type and GRK−/PKA− receptor recruited similar amounts of rapidly associating β-arrestin. The defect in GRK−/PKA− recep-

tor is thus only in the slow phase of β-arrestin recruitment. This is consistent with conformation-dependent rapid association and phosphorylation-dependent slow association of β-arrestin2 with the β_2AR. Indeed, as shown below, GRK activity influences only the slow phase of β-arrestin recruitment.

We next established the relative affinities (k_F) of these two states (agonist-induced conformation and the phosphorylated state) for β-arrestin2-mYFP by fitting the amplitude of slow and fast FRET changes with varying amounts of β-arrestin2-mYFP expression to a saturable binding model (Fig. 2*C*) shown in Equation 2,

$$\text{FRET}\ (\%F) = \frac{\%F_{max} \cdot \text{YFP}}{\text{YFP} + K_{\text{FRET}}} \qquad \text{(Eq. 2)}$$

It was not possible to accurately measure the relative affinity of the rapid, phosphorylation-independent component because the extremely high expression levels of β-arrestin2-mYFP necessary to saturate this component lead to YFP aggregation and altered receptor trafficking (data not shown). However, it is apparent from the data that the affinity of β-arrestin2-mYFP for the agonist-occupied phosphorylated receptor is at least 2 orders of magnitude higher than for the agonist occupied receptor prior to phosphorylation.

These results suggest that β-arrestin2 recruitment kinetics can be used to assess both agonist-induced receptor conformation and agonist-induced receptor phosphorylation. β-Arrestin1 is reported to be less effective than β-arrestin2 for β_2AR internalization (31), so we compared the kinetics and relative binding affinity of β-arrestin1-mYFP and β-arrestin2-mYFP. In addition, we tested the "phosphorylation-independent" mutant β-arrestin1-mYFP R169E (32). This mutant exhibits a disrupted "polar core," which consists of closely aligned amino acid side chains of opposite charge. The R169E mutation mimics the disruption of this region that is thought to occur upon β-arrestin binding phosphorylated receptor, leading to a conformational change with higher affinity for receptor and several β-arrestin-scaffolded proteins (7). We found that β-arrestin1-mYFP is recruited with a slow rate similar to β-arrestin2-mYFP but with a lower affinity (Fig. 3). Phosphorylation-independent β-arrestin1-mYFP (R169E) displays more pronounced biphasic association, with a much larger rapid association than β-arrestin1-mYFP or β-arrestin2-mYFP at equivalent expression levels. However, there is also a large slow component of β-arrestin1-mYFP (R169E) recruitment, most likely signifying a phosphorylation-dependent affinity. Interestingly, the relative affinity of this mutant is similar to that of β-arrestin2-mYFP. Together, these results suggest that β-arrestin2 binds the β_2AR with higher affinity than β-arrestin1, but with similar kinetics, limited by the rate of receptor phosphorylation. Phosphorylation-independent β-arrestin1-mYFP (R169E) appears in this assay to be partially phosphorylation-independent, with an enhanced affinity contributed by the agonist-induced, unphosphorylated receptor.

We next set out to address the roles of individual GRKs in regulating β-arrestin recruitment. To minimize variability in our β-arrestin recruitment assay, we generated cell lines stably expressing either β-arrestin1-mYFP, β-arrestin2-mYFP, or β-arrestin1-mYFP (R169E) in addition to β_2AR-mCFP. Based on the findings above, these cell lines allow us to test the GRK requirements for both slow, phosphorylation-dependent, and rapid, phosphorylation-independent, β-arrestin recruitment. These cell lines each express 8–30-fold β-arrestin-mYFP over endogenous β-arrestin as shown by immunoblot (supplemental Fig. 1); however, because the phosphorylation-dependent phase of the β-arrestin-β_2AR interaction is independent of β-arrestin expression (Fig. 2*A*), results from these cell lines can be extrapolated to GRK function for endogenous β-arrestins.

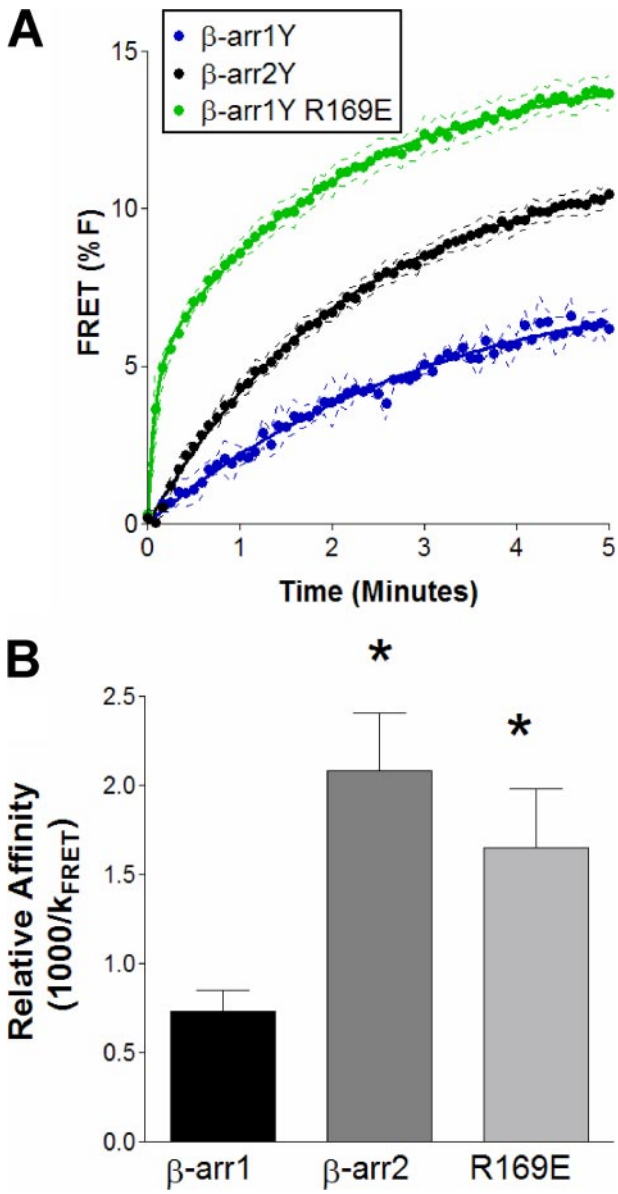


FIGURE 3. **A phosphorylation-independent β-arrestin (*β-arr*) mutant displays enhanced low affinity binding.** *A,* β-arrestin1/2 recruitment is monophasic at moderate expression levels, but the recruitment of the phosphorylation-independent mutant β-arrestin1-mYFP R169E expressed at similar levels is biphasic, with an enhanced rapid recruitment. *B,* comparing the maximum FRET of β-arrestin isoforms and phosphorylation-independent mutants across a range of expression levels reveals that β-arrestin2 binds the β_2AR with significantly higher affinity than β-arrestin1. The β-arrestin1 R169E has significantly increased affinity than wild-type β-arrestin1. *, $p < 0.05$. All data are average ± S.E. of three independent experiments.

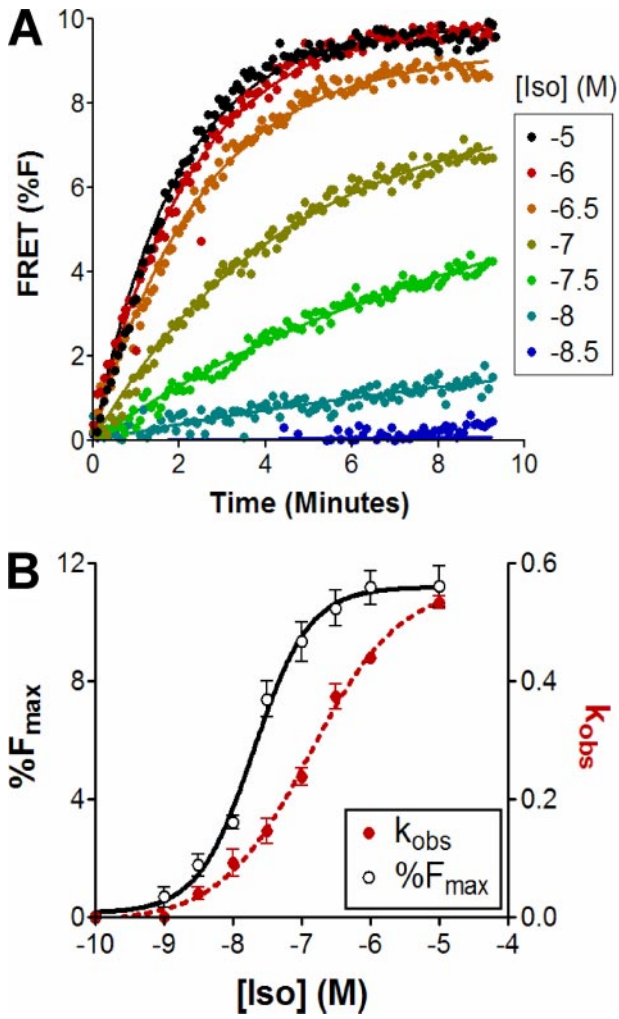


FIGURE 4. **The rate and magnitude of β-arrestin recruitment depend on agonist concentration.** *A,* cells stably expressing both β_2AR-mCFP and β-arrestin2-mYFP were stimulated with varying concentrations of isoproterenol (*Iso*). Data are from one of three separate experiments. *B,* the rate (k_{obs}) of β-arrestin-mYFP recruitment is less sensitive to isoproterenol than the final amount of recruitment (%F_{max}). Data are average ± S.E. of three independent experiments.

We first tested the dose-response relationship of the kinetics and amplitude of β-arrestin2-mYFP recruitment to the β_2AR-mCFP. Increasing concentrations of isoproterenol caused increasing rate and amount of recruitment (Fig. 4*A*). The rate and amplitude of recruitment after 1 μM isoproterenol was similar for the double-stable transfectants and transiently transfected cells, indicating that the double-stable transfectants are representative of the parent cell lines (data not shown). Quantification of the dose-response relationship reveals that the amount of β-arrestin recruited (%F_{max}) fits a saturable one-site binding model (Fig. 4*B*) and is indistinguishable from predicted receptor occupancy (33). In contrast, the rate of β-arrestin recruitment is less sensitive to isoproterenol and exhibits a "shallow slope" with a Hill coefficient of 0.67. This most likely reflects the kinetics of the receptor-ligand interaction and receptor conformational change. We decided to continue our studies using high agonist concentration (1 μM) to maximize both amount and rate of β-arrestin recruitment, using both of these parameters to test GRK activities.

We assessed GRK activity by the following two methods: GRK overexpression and GRK silencing by siRNA. We found that overexpression of GRK2 increased the rate of β-arrestin2-mYFP recruitment without altering recruitment amplitude (Fig. 5*A*). Similar results were found with each of the ubiquitous GRKs (GRK2, GRK3, GRK5, and GRK6) (data not shown). This suggests that any of these GRKs are capable, with overexpression, of phosphorylating the β_2AR to induce β-arrestin recruitment. However, this does not address which GRKs are relevant at endogenous expression levels. To address this question, we silenced endogenous GRKs with siRNA as described earlier (11, 12). We first tested the effects of GRK6 silencing on stably expressed β-arrestin1-mYFP and β-arrestin1-mYFP (R169E); both YFP constructs were expressed to similar levels, and β_2AR-mCFP expression and distribution were comparable (data not shown). Cells transfected with a scrambled control siRNA displayed monophasic and biphasic recruitment for β-arrestin1 and β-arrestin1 (R169E), respectively, just as in untransfected cells (Fig. 5*B*). Transfection of siRNA to silence GRK6 resulted in a reduced rate of slow-phase recruitment, without altering the rapid recruitment of β-arrestin1-mYFP (R169E). Similar results, with a less pronounced effect, were noted with GRK2 silencing (data not shown). This is consistent with a GRK-independent rapid recruitment and GRK-mediated phosphorylation-dependent slow recruitment. Importantly, the final amplitude of recruitment is unaffected by GRK siRNA, suggesting that the primary defect noted with GRK silencing is kinetic. Protein kinase A, which also phosphorylates and desensitizes the β_2AR, does not appear to affects β-arrestin recruitment, as 20 μM H-89 does not alter either the rate or amplitude of isoproterenol-induced FRET (data not shown). Additionally, 20 μM H-89 does not detectably alter the appearance of isoproterenol-stimulated vesicles containing β_2AR-mCFP (data not shown). This is consistent with the inability of PKA to mediate phosphorylation relevant for β-arrestin recruitment to the β_2AR.

We next tested each of the ubiquitous nonvisual GRKs; siRNA silencing of GRK2 and GRK6 significantly slows β-arrestin2-mYFP recruitment (Fig. 5*C*), but GRK6 has the most profound effect. We verified that this is not an off-target effect of the particular GRK6 siRNA sequence by showing comparable results with a second, independent siRNA sequence for GRK6 (data not shown). The efficiency of GRK silencing was shown to be greater than 90% by immunoblot (Fig. 5*C*, *bottom*). Importantly, GRK silencing had no significant effect on the maximum FRET signal, consistent with an unchanged amount of β-arrestin2 recruited at equilibrium (Fig. 5*D*). However, we cannot exclude the possibility that the nature of the receptor-β-arrestin interaction is altered by GRK silencing, altering FRET efficiency and masking differences in the amount of β-arrestin recruited. Notably, GRK silencing had the same effects on rate and amplitude of β-arrestin1-mYFP recruitment as observed for β-arrestin2-YFP (data not shown).

It is most likely that the slowed rate of FRET increase corresponds to a slowed association of β-arrestin with β_2AR. However, because FRET depends on fluorophore orientation as well as proximity, it is also possible that some of the FRET increase we detect is caused by conformational changes of the β-arres-

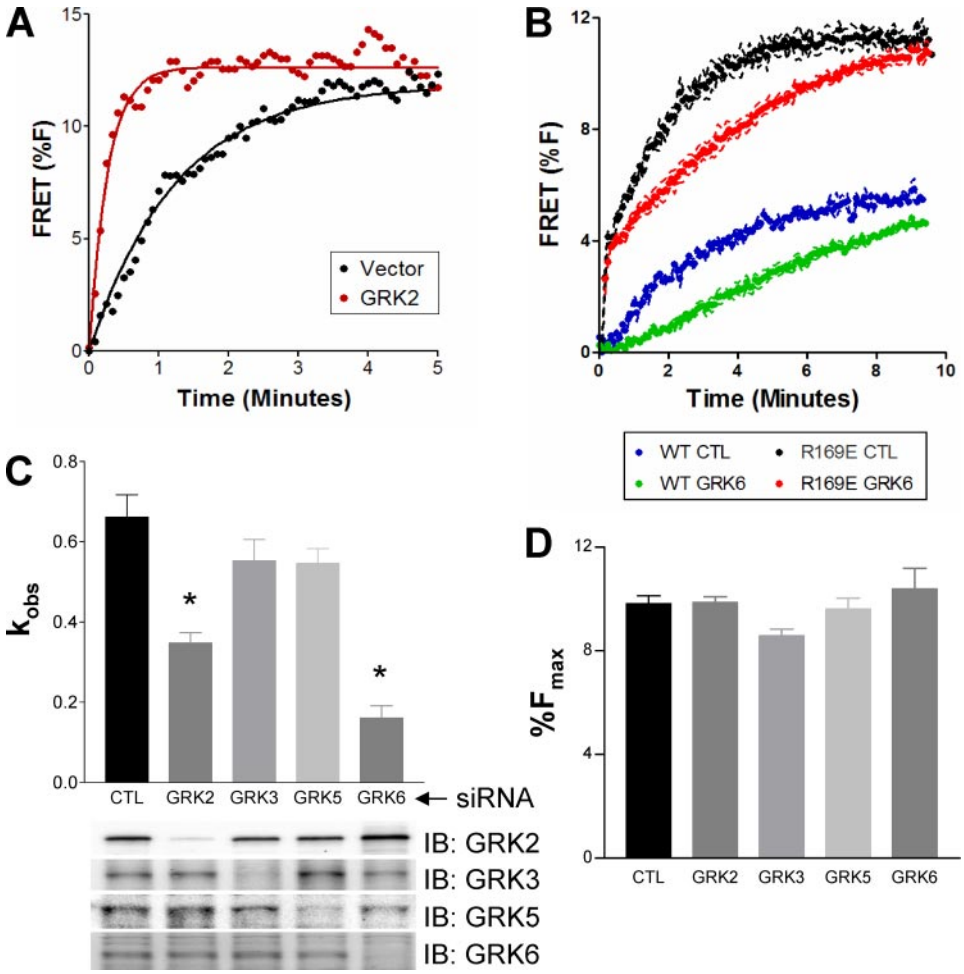


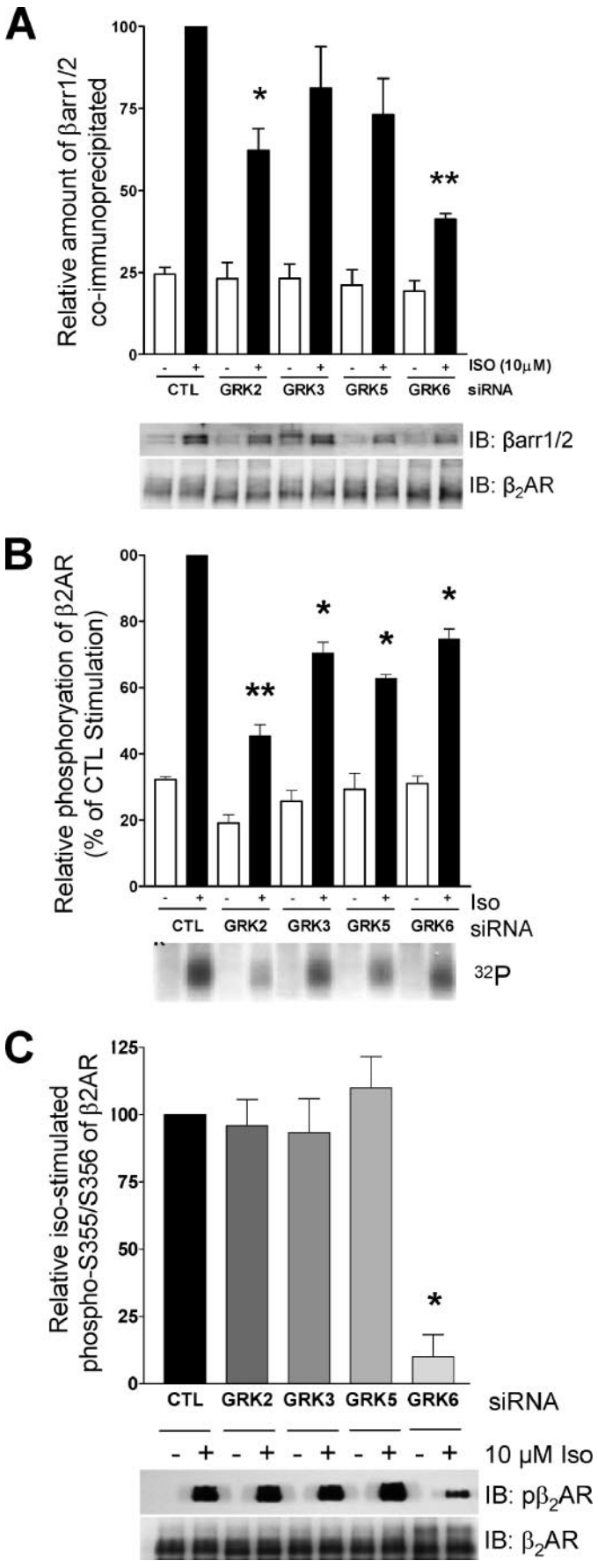
FIGURE 5. **High affinity β-arrestin2-mYFP binding is kinetically limited by GRKs.** *A,* overexpression of GRK2 increases the rate of β-arrestin2-mYFP recruitment compared with vector alone. Data are representative of three separate experiments. *B,* siRNA silencing of GRK6 reduces the rate of recruitment of β-arrestin1-mYFP (*WT siGRK6*) compared with a scrambled siRNA (*WT CTL*) and reduces only the slow rate of phosphorylation-independent β-arrestin1-mYFP (*R169E siGRK6* and *R169E CTL*) without affecting the rapid association. Data are average ± S.E. of three separate experiments. *C,* siRNA silencing of GRK2, GRK3, GRK5, and GRK6 reveals that GRK2 and GRK6 have significant effects on rate (k_{obs}) of β-arrestin2-mYFP recruitment (*, $p < 0.001$). The effect of GRK6 siRNA is most profound and was noted with two siRNA sequences (GRK6-1 and GRK6-2). A representative immunoblot (*IB*) for each GRK shows the effectiveness of siRNA silencing. Data are average ± S.E. from three independent experiments performed in triplicate. *D,* the final amount of β-arrestin recruited ($\%F_{max}$) is not altered by GRK silencing, suggesting an enzymatically limited recruitment of β-arrestin. Data are average ± S.E. of the same experiments as in *C*.

tin-β_2AR complex after the initial interaction. We discriminated between these two possibilities by measuring β-arrestin2-mYFP redistribution from cytosol to membrane by tracking directly excited YFP fluorescence in user-defined regions of cytosol and membrane. After isoproterenol stimulation, membrane YFP intensity increases and cytosolic YFP intensity decreases. The ratio of these two signals serves as a direct measure of β-arrestin translocation and is independent of FRET. As expected, GRK siRNA slows translocation compared with a control siRNA, consistent with a GRK-controlled rate of β-arrestin association with β_2AR (supplemental Fig. 3). Furthermore, this finding is inconsistent with FRET changes induced by a GRK-controlled conformational change of the β-arrestin-β_2AR complex.

We confirmed the effect of GRK silencing on β-arrestin recruitment using coimmunoprecipitation of endogenous β-arrestins. Immunoprecipitation of a FLAG-tagged β_2AR showed increased β-arrestin association following 5 min of 10 μM isoproterenol (Fig. 6*A*). This effect was inhibited by silencing either GRK2 or, even more effectively, GRK6. These data confirm our FRET assay results (Fig. 5*C*) and suggest that the β_2AR exhibits some specificity for GRK-mediated β-arrestin recruitment. We then assessed β_2AR phosphorylation by ^{32}P incorporation to test if the GRKs that mediate bulk phosphorylation are the same as those required for endogenous β-arrestin recruitment. To simplify the interpretation of this experiment, we used a mutant β_2AR that lacks PKA phosphorylation sites but has unaltered phosphorylation by GRKs (30). Total phosphorylation after 5 min is most effectively reduced by GRK2 silencing (Fig. 6*B*). This indicates that not all β_2AR phosphorylation is equivalent for recruiting β-arrestin and that there is functional specificity for β_2AR regulation by different GRKs. To further examine this possibility, we tested β_2AR phosphorylation by using an antibody that specifically recognizes phosphorylation of serines 355 and 356 on the β_2AR (34). In contrast to bulk β_2AR phosphorylation, this site appears to be exclusively phosphorylated by GRK6 (Fig. 6*C*), confirming that GRKs target different sites on the β_2AR.

Because GRK silencing does not appear to alter the amount of β-arrestin recruited, but only the rate at which recruitment occurs, it is apparent that either 1) the residual GRK expressed after silencing is sufficient, with impaired kinetics, to drive β-arrestin recruitment, or 2) the untargeted GRK isoforms are responsible for recruitment of β-arrestin2 after individual GRK silencing by a mechanism of compensatory phosphorylation. Because multiple GRKs (most prominently GRK2 and GRK6) contribute to β-arrestin recruitment in the HEK-293 cells used here, we favor the latter hypothesis. Indeed, simultaneous silencing of GRK2 and GRK6 reduced the rate of β-arrestin2-mYFP recruitment, as measured by FRET, more than any single GRK siRNA alone (Fig. 7*A*). Thus GRK2 and GRK6 can compensate for each other for β-arrestin recruitment. The most likely mechanism of this GRK2 and GRK6 interplay is either shared substrate residues on the β_2AR or different substrate sites with equal affinity for β-arrestin2.

If β_2AR phosphorylation sites responsible for β-arrestin recruitment are shared by GRKs, then β-arrestin recruitment



in a cell type with a different GRK expression pattern should exhibit different sensitivity to GRK siRNA, corresponding to the relative expression of each GRK isoform. To test this, we generated a U2-OS osteosarcoma cell line stably expressing β_2AR-mCFP and β-arrestin2-mYFP. We compared GRK expression between these cells and the β_2AR-mCFP/β-arrestin2-mYFP HEK-293 cell line by immunoblot (Fig. 7*B*). In comparison to HEK-293 cells, U2-OS express relatively more GRK3 and GRK5 but little to no GRK6. GRK silencing in the U2-OS line revealed that GRK2 and especially GRK3 are most efficacious at promoting β-arrestin2 recruitment, in correlation with the expression pattern of GRKs in the U2-OS line (Fig. 7*C*). As in HEK-293 cells, we found an additive effect of simultaneous silencing the two GRKs that individually contribute most to β-arrestin recruitment, in this case GRK2 and GRK3 (Fig. 7*D*). Thus we conclude that β_2AR regulation by GRK-mediated β-arrestin recruitment strongly depends on a cell's complement of GRKs.

DISCUSSION

FRET has been used extensively as a nondestructive way of measuring protein-protein interactions, protein conformational changes, and physiochemical properties in living cells (35). These approaches have been put to use in many fluorescent biosensors to report intracellular signals. Here we use FRET between mCFP-tagged β_2AR and mYFP-tagged β-arrestins as a measure of GRK activity. Other groups have shown FRET or BRET, a related biophysical phenomenon, between receptors and β-arrestins as a method of reporting β-arrestin recruitment (28). We chose to develop a FRET assay because, unlike BRET, FRET can be used as an imaging technique, allowing single cell and subcellular measurements of receptor-β-arrestin interaction. One previous report shows FRET between β_2AR and β-arrestins, which required overexpressed GRK2 (27); our assay differs in that we measure the receptor-β-arrestin interaction driven by endogenous GRKs. This allowed us to make several novel observations pertaining to GRK regulation of β-arrestin function for the β_2AR as follows: 1) the rate of β-arrestin recruitment is a specific reporter of endogenous or exogenous GRK activity, and 2) the β_2AR exhibits cell type-dependent GRK specificity for its regulation by β-arrestin. We also show, for the first time, the rapid agonist-induced conformational change of the β_2AR in live cells, as detected by the low affinity interaction of β_2AR-mCFP and β-arrestin-mYFP.

FIGURE 6. **β_2AR immunoprecipitation reveals distinct GRK specificity for β-arrestin (β-*arr*) association, bulk phosphorylation, and phosphorylation of a single site in the absence of overexpressed β-arrestins.** *A,* immunoprecipitation of transiently transfected FLAG-β_2AR shows agonist-induced β-arrestin association with the β_2AR after 5 min of stimulation with 10 μM isoproterenol (*Iso*). Silencing of GRK2 or GRK6 significantly impairs this association (*, $p < 0.01$; **, $p < 0.001$). A representative experiment is shown, including immunoblots (*IB*) for immunoprecipitated β_2AR and associated β-arrestin1/2. *B,* PKA-independent phosphorylation of the β_2AR was assessed by ^{32}P incorporation into a β_2AR mutated at the PKA phosphorylation sites. Silencing of any GRK reveals a loss of agonist-stimulated receptor phosphorylation (*, $p < 0.001$), but GRK2 silencing is significantly more potent than any other GRK (**, $p < 0.01$). A representative autoradiograph is shown. *C,* immunoblotting with an antibody specific for phosphorylation at serine residues 355 and 356 reveals that GRK6 silencing dramatically inhibits phosphorylation at this site (*, $p < 0.001$). A representative experiment is shown, including immunoblots (*IB*) for phosphorylated and total immunoprecipitated receptor. All data are average ± S.E. of three independent experiments.

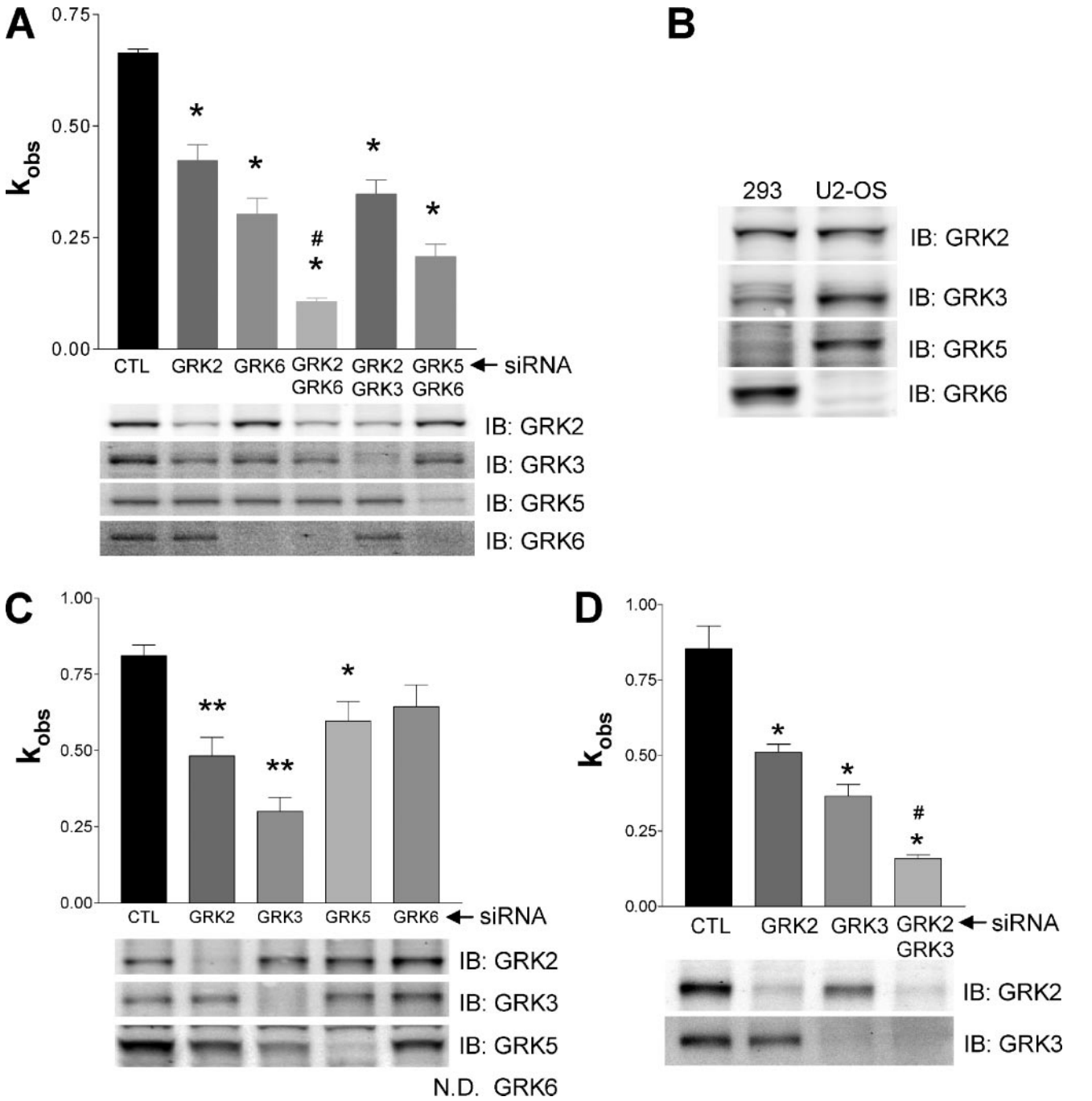


FIGURE 7. **U2-OS cells utilize a different set of GRKs for β-arrestin recruitment than HEK-293 cells as measured by FRET.** *A,* simultaneous silencing of GRK2 and GRK6 slows recruitment more than silencing either GRK alone (#, $p < 0.01$), consistent with a mechanism of compensatory phosphorylation. All treatments were significantly different from control (*, $p < 0.001$). An immunoblot (*IB*) is shown for each GRK to verify silencing. *B,* immunoblotting reveals that U2-OS cells express relatively more GRK3 than HEK-293 cells and less GRK6. For each GRK, an immunoblot is shown of equal amounts of total protein from each cell line. *C,* siRNA silencing of GRKs reveals that GRK2 and GRK3 are most important for β-arrestin2-mYFP recruitment (each treatment compared with control: *, $p < 0.05$; **, $p < 0.001$). An immunoblot is shown for each detectable GRK to verify silencing (*N.D.,* not detected). *D,* silencing GRK2, GRK3, or GRK2 and GRK3 together significantly slows the rate of β-arrestin recruitment (*, $p < 0.001$). Simultaneous silencing of GRK2 and GRK3 is more effective at reducing the rate of β-arrestin2-mYFP recruitment than either GRK alone (#, $p < 0.05$), consistent with compensatory phosphorylation between these GRKs. An immunoblot is shown for GRK2 and GRK3 to verify silencing. All data are average ± S.E. of three independent experiments performed in triplicate. All immunoblots are representative of three independent experiments.

FRET efficiency (the proportion of donor excitation emitted as FRET) depends upon the proximity (<10 nm) and orientation of the two interacting fluorophores (in this study, mCFP and mYFP). Changes in either proximity or orientation can alter FRET efficiency. Therefore, it is important to note that FRET efficiency does not necessarily directly reflect the amount of interaction. For instance, it is conceivable that β-arrestin-mYFP can bind the β_2AR-mCFP in different conformations, leading to a different CFP-YFP orientation and thus different FRET efficiencies. However, the kinetics and relative affinities of such interactions are independent of maximum FRET efficiency, so these param-

eters are more rigorous measures of β-arrestin-mYFP recruitment to β_2AR-mCFP.

The β_2AR-β-arrestin interaction, as reported by FRET, was agonist-dependent, rapidly reversible, and localized to the plasma membrane (Fig. 1), as expected from previous work showing redistribution of green fluorescent protein-tagged β-arrestin (36). This association displayed both phosphorylation-dependent and -independent affinities. We assessed these affinities by measuring single cell FRET over time in cells with constant levels of β_2AR-mCFP but varying amounts of β-arrestin-mYFP. These studies revealed a fast, low affinity phosphorylation-independent association and a slower GRK phosphorylation-dependent high affinity interaction (Fig. 2, *A* and *C*). The phosphorylation-independent affinity is insensitive to phosphoacceptor site mutation (Fig. 2*B*) and GRK silencing (Fig. 5*B*), consistent with a low affinity of β-arrestin for the agonist-induced "active" receptor conformation. In contrast, the much higher affinity slow component is both sensitive to phosphoacceptor site mutation (Fig. 2*B*) and GRK overexpression or siRNA (Fig. 5, *A* and *B*). The rate of this slow association is independent of β-arrestin-mYFP expression (Fig. 2*A*), suggesting that the GRK sensitivity of this assay can be extrapolated to endogenous β-arrestin. Importantly, we can only detect the phosphorylation-independent association with very high β-arrestin expression, so it is unclear if this low affinity state has any physiological relevance. Nonetheless, this rapid, low affinity binding is a direct readout of receptor conformational change, and comports with other assays of receptor conformation, both with purified proteins (37) and in live cells (38, 39).

Interestingly, the major effect of altered GRK expression is kinetic. The rate of the slow, high affinity β-arrestin interaction is enhanced by GRK overexpression (Fig. 5*A*) and reduced by GRK silencing (Fig. 5, *B* and *C*). In contrast, neither overexpression nor silencing of GRKs appreciably alters the final maximum amount of FRET. As noted, FRET efficiency depends on both proximity and orientation of the interacting fluorophores. This indicates that the amount of β-arrestin recruited at equilibrium is the same in all conditions, but we cannot exclude the possibility that differences in β-arrestin recruitment are masked by parallel differences in β-arrestin or receptor conformation that inversely alter FRET to result in an unchanged FRET signal. Regardless, because GRKs are the rate-limiting step in β-arrestin recruitment, the rate of FRET increase is a functional reporter of GRK activity; any regulation or intervention that alters relevant GRK activity would be expected to alter the rate at which β-arrestin associates with receptor. Because β-arrestin binding desensitizes and internalizes the receptor, and results in a set of β-arrestin-dependent signals (24), such rate changes may have distinct signaling effects. As we refine our understanding of heptahelical receptor signaling, it will be important to address both the amount and rate of β-arrestin recruitment as important parameters of receptor regulation.

It is clear from this work that, as shown previously *in vitro* (40), β_2AR affinity for β-arrestin is regulated by two factors, the agonist-induced receptor conformation and receptor phosphorylation. The agonist-induced conformational change is very rapid (37) but induces significant amounts of β-arrestin recruitment only when β-arrestin expression is very high (Fig. 2*A*). GRK-mediated receptor phosphorylation leads to higher affinity binding not at a rate set by receptor occupancy directly but rather by GRK activity. Presumably, the relative contributions of receptor conformation and phosphorylation vary for different receptors; for example, the LTB4 receptor appears to recruit β-arrestin completely independently of phosphorylation (41).

Interestingly, it seems that the phosphorylation-independent β-arrestin1 R169E mutant displays enhanced affinity for the unphosphorylated, agonist-induced receptor conformation. However, this mutant is still sensitive to receptor phosphorylation and thus is, in fact, only partially phosphorylation-independent. Although this mutant is a useful probe for agonist-induced receptor conformation (mimicking very high expression of wild-type β-arrestin), it is unclear if under physiological conditions any endogenous β-arrestin associates with unphosphorylated β_2AR. Certainly, the fact that nonphosphorylatable β_2AR is deficient in desensitization and internalization suggests that these β-arrestin functions require receptor phosphorylation (30).

One implication of our results is that β-arrestin expression levels affect the amount of receptor-β-arrestin complex formed. This may have profound implications for the desensitization, surface expression, and β-arrestin signaling of receptors in cells with different β-arrestin expression levels. For assay systems that rely on exogenous β-arrestin, such as FRET and BRET, it is important to consider the effect of β-arrestin overexpression on the assay readout. To manage this concern, we generated cell lines that stably express both β_2AR-mCFP and β-arrestin-mYFP. These cells allowed us to monitor very reproducible β-arrestin recruitment even when measuring relatively few cells. We noted that both the rate and amount of β-arrestin recruitment depend on agonist concentration (Fig. 3). Interestingly, the relative amount of β-arrestin recruited correlates well with receptor occupancy, although the rate of β-arrestin recruitment is less sensitive. This likely reflects the kinetics of receptor-ligand interaction and receptor conformational change, and may allow the receptor to discriminate agonist concentrations across a wider range than would be predicted by receptor occupancy at equilibrium.

Because receptor phosphorylation is rate-limiting for β-arrestin recruitment and function, we measured the effect of individual GRKs by siRNA-mediated silencing. Results from two cell types lead us to the following striking conclusions: 1) β-arrestin recruitment to the β_2AR is not specific to a single GRK, and 2) different cell types can use different GRKs to accomplish β-arrestin recruitment to the same receptor. We deduce this from the fact that GRK silencing does not appear to reduce the amount of β-arrestin-receptor complex. Instead, several GRKs contribute to β-arrestin recruitment in a complementary manner, as GRK silencing reduces the rate of β-arrestin recruitment. In the absence of any particular GRK, other GRKs still result in complete β-arrestin recruitment but with slowed kinetics. This is consistent with the following two possible mechanisms: compensatory GRK activity and compensatory β-arrestin binding. Compensatory kinase activity would occur

if GRKs shared the same phosphoacceptor sites on the receptor; the silencing of one GRK would result in the same phosphorylated residues but with delayed kinetics as the remaining GRKs take longer to complete the process. In this schema, total GRK activity is the relevant parameter for β-arrestin recruitment, and silencing of one GRK is akin to partial inhibition of total GRK activity. Compensatory β-arrestin binding, on the other hand, could result if GRKs phosphorylate different residues, but β-arrestin cannot distinguish between them. Such a mechanism would be consistent with β-arrestin having no set sequence specificity for binding but instead requiring some net charge introduced by phosphorylation (7). This would also be consistent with a kinetic role for individual GRKs; in the absence of a given GRK, it may take longer for the required number of phosphorylations to accumulate on the amino acids targeted by the remaining GRKs. Either of these mechanisms is consistent with our findings. The most promising method of distinguishing these hypotheses is a proteomic characterization of β_2AR phosphorylation sites for each GRK. Indeed, preliminary work suggests this is a feasible approach (42). It may be possible, by combining phosphorylation site identification with GRK silencing, to determine what specificity exists for GRK phosphorylation of the β_2-adrenergic receptor and other receptors. This work, however, suggests that such putative specificity plays a role only in the kinetics of β-arrestin recruitment.

Recent results suggest that GRKs can have specific effects on β-arrestin signaling distinct from their effects on β-arrestin recruitment (11, 12). If these discrepancies are also found for the β_2AR, it would suggest that not all β-arrestin recruitment is equivalent. For example, the rate and order of receptor phosphorylations might not affect the amount of β-arrestin recruited but could place the β-arrestin in either distinct conformations or orientations on the cytoplasmic receptor surface, leading to different regulatory effects on the receptor signal output. Such differences in β-arrestin conformation/orientation were not evident by FRET in our system. However, our β_2AR-mCFP and β-arrestin-mYFP are constructed with flexible linkers attaching the fluorescent protein; this is expected to mask many subtle conformational differences in the receptor-arrestin interaction and instead biases our assay for simple proximity. Protein or small molecule fluorophores attached in a more rigid manner, or to different sites on the receptor or β-arrestin, should be more sensitive to conformational changes and may help elucidate such effects.

In conclusion, we have established a FRET system for detecting β-arrestin recruitment to the β_2AR. This system elucidates both properties of β-arrestin binding affinity for the receptor and the series of events (agonist-induced conformational change and phosphorylation) required for that binding affinity. We have discovered that β-arrestin recruitment kinetics serve as an assay for GRK activity, which can be used to monitor the interplay of multiple GRKs regulating β_2AR function. This system should be useful both to study the properties of different ligands for the β_2AR and to study GRK regulation as a mechanism of signaling feedback and cross-talk. Our findings indicate that the role of individual GRKs is to determine the rate of β-arrestin binding to the β_2AR. Because individual GRKs have disparate regulatory mechanisms (1), this affords the cell a subtle way to control the lifetime of the ligand-occupied receptor and its effects, prior to β-arrestin binding and the initiation of G-protein desensitization, receptor internalization, and β-arrestin signaling.

Acknowledgments—We thank Donna Addison and Elizabeth Hall for excellent secretarial assistance.

REFERENCES

1. Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) *Annu. Rev. Biochem.* **67,** 653–692
2. Shenoy, S. K., and Lefkowitz, R. J. (2003) *Biochem. J.* **375,** 503–515
3. Ferguson, S. S. (2001) *Pharmacol. Rev.* **53,** 1–24
4. Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., and Lefkowitz, R. J. (1990) *Science* **248,** 1547–1550
5. Ferguson, S. S., Downey, W. E., III, Colapietro, A. M., Barak, L. S., Menard, L., and Caron, M. G. (1996) *Science* **271,** 363–366
6. Lefkowitz, R. J., and Shenoy, S. K. (2005) *Science* **308,** 512–517
7. Gurevich, V. V., and Gurevich, E. V. (2004) *Trends Pharmacol. Sci.* **25,** 105–111
8. Lohse, M. J., Andexinger, S., Pitcher, J., Trukawinski, S., Codina, J., Faure, J. P., Caron, M. G., and Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267,** 8558–8564
9. Han, M., Gurevich, V. V., Vishnivetskiy, S. A., Sigler, P. B., and Schubert, C. (2001) *Structure (Camb.)* **9,** 869–880
10. Kim, Y. M., and Benovic, J. L. (2002) *J. Biol. Chem.* **277,** 30760–30768
11. Ren, X. R., Reiter, E., Ahn, S., Kim, J., Chen, W., and Lefkowitz, R. J. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 1448–1453
12. Kim, J., Ahn, S., Ren, X. R., Whalen, E. J., Reiter, E., Wei, H., and Lefkowitz, R. J. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 1442–1447
13. Jaber, M., Koch, W. J., Rockman, H., Smith, B., Bond, R. A., Sulik, K. K., Ross, J., Jr., Lefkowitz, R. J., Caron, M. G., and Giros, B. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 12974–12979
14. Peppel, K., Boekhoff, I., McDonald, P., Breer, H., Caron, M. G., and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272,** 25425–25428
15. Gainetdinov, R. R., Bohn, L. M., Walker, J. K., Laporte, S. A., Macrae, A. D., Caron, M. G., Lefkowitz, R. J., and Premont, R. T. (1999) *Neuron* **24,** 1029–1036
16. Fong, A. M., Premont, R. T., Richardson, R. M., Yu, Y. R., Lefkowitz, R. J., and Patel, D. D. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 7478–7483
17. Iaccarino, G., and Koch, W. J. (1999) *Expert Opin. Investig. Drugs* **8,** 545–554
18. Collins, S., Lohse, M. J., O'Dowd, B., Caron, M. G., and Lefkowitz, R. J. (1991) *Vitam. Horm.* **46,** 1–39
19. Johnson, M. (1998) *Am. J. Respir. Crit. Care Med.* **158,** S146–S153
20. Benovic, J. L., Strasser, R. H., Caron, M. G., and Lefkowitz, R. J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 2797–2801
21. Attramadal, H., Arriza, J. L., Aoki, C., Dawson, T. M., Codina, J., Kwatra, M. M., Snyder, S. H., Caron, M. G., and Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267,** 17882–17890
22. Violin, J. D., Zhang, J., Tsien, R. Y., and Newton, A. C. (2003) *J. Cell Biol.* **161,** 899–909
23. Guan, X. M., Kobilka, T. S., and Kobilka, B. K. (1992) *J. Biol. Chem.* **267,** 21995–21998
24. Shenoy, S. K., Drake, M. T., Nelson, C. D., Houtz, D. A., Xiao, K., Madabushi, S., Reiter, E., Premont, R. T., Lichtarge, O., and Lefkowitz, R. J. (2006) *J. Biol. Chem.* **281,** 1261–1273
25. Ahn, S., Shenoy, S. K., Wei, H., and Lefkowitz, R. J. (2004) *J. Biol. Chem.* **279,** 35518–35525
26. DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D., and Bunnett, N. W. (2000) *J. Cell Biol.* **148,** 1267–1281
27. Krasel, C., Bunemann, M., Lorenz, K., and Lohse, M. J. (2005) *J. Biol. Chem.* **280,** 9528–9535

28. Azzi, M., Charest, P. G., Angers, S., Rousseau, G., Kohout, T., Bouvier, M., and Pineyro, G. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 11406–11411
29. Gordon, G. W., Berry, G., Liang, X. H., Levine, B., and Herman, B. (1998) *Biophys. J.* **74,** 2702–2713
30. Seibold, A., Williams, B., Huang, Z. F., Friedman, J., Moore, R. H., Knoll, B. J., and Clark, R. B. (2000) *Mol. Pharmacol.* **58,** 1162–1173
31. Kohout, T. A., Lin, F. S., Perry, S. J., Conner, D. A., and Lefkowitz, R. J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 1601–1606
32. Kovoor, A., Celver, J., Abdryashitov, R. I., Chavkin, C., and Gurevich, V. V. (1999) *J. Biol. Chem.* **274,** 6831–6834
33. Benovic, J. L., Shorr, R. G., Caron, M. G., and Lefkowitz, R. J. (1984) *Biochemistry* **23,** 4510–4518
34. Tran, T. M., Friedman, J., Qunaibi, E., Baameur, F., Moore, R. H., and Clark, R. B. (2004) *Mol. Pharmacol.* **65,** 196–206
35. Zhang, J., Campbell, R. E., Ting, A. Y., and Tsien, R. Y. (2002) *Nat. Rev. Mol. Cell Biol.* **3,** 906–918
36. Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S., and Caron, M. G. (2001) *J. Biol. Chem.* **276,** 19452–19460
37. Swaminath, G., Xiang, Y., Lee, T. W., Steenhuis, J., Parnot, C., and Kobilka, B. K. (2004) *J. Biol. Chem.* **279,** 686–691
38. Liapakis, G., Chan, W. C., Papadokostaki, M., and Javitch, J. A. (2004) *Mol. Pharmacol.* **65,** 1181–1190
39. Hoffmann, C., Gaietta, G., Bunemann, M., Adams, S. R., Oberdorff-Maass, S., Behr, B., Vilardaga, J. P., Tsien, R. Y., Ellisman, M. H., and Lohse, M. J. (2005) *Nat. Methods* **2,** 171–176
40. Gurevich, V. V., Dion, S. B., Onorato, J. J., Ptasienski, J., Kim, C. M., Sterne-Marr, R., Hosey, M. M., and Benovic, J. L. (1995) *J. Biol. Chem.* **270,** 720–731
41. Jala, V. R., Shao, W. H., and Haribabu, B. (2005) *J. Biol. Chem.* **280,** 4880–4887
42. Trester-Zedlitz, M., Burlingame, A., Kobilka, B., and von Zastrow, M. (2005) *Biochemistry* **44,** 6133–6143