Phosphorylation of the β_2-Adrenergic Receptor in Plasma Membranes by Intrinsic GRK5[†]

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ABSTRACT: Characterization of the GRKs participating in the phosphorylation of the β_2-adrenergic receptor (β_2AR) have in part been limited by the lack of a simple cell-free assay with membrane-bound β_2AR and GRKs. We describe here a cell-free assay for GRK phosphorylation of the β_2AR in a postnuclear 600*g* fraction and washed membranes by intrinsic GRK activity using the GRK phosphosite-specific antibody that recognizes pS(355,356). Treatment of these cell-free preparations with 1.0 μM isoproterenol (ISO) caused a rapid maximal 10−15-fold increase in GRK site phosphorylation of the β_2AR ($t_{1/2}$ = 1 min) with an EC_{50} for ISO stimulation of ∼80 nM. Extensively washed plasma membrane fractions retained the 10−15-fold ISO stimulation of GRK site phosphorylation and GRK5 levels while being depleted of GRK2 and GRK6. Stimulation of GRK site phosphorylation by a range of partial agonists correlated well with their intrinsic efficacy for stimulation of adenylyl cyclase. GRK phosphorylation of the β_2AR in the washed membrane fraction caused minimal desensitization of ISO stimulation of adenylyl cyclase activity. Association of GRK5 with the β_2AR in intact cells was demonstrated by a high level of basal $BRET^2$ using β_2AR-Rluc and GRK5-GFP^2 that was not diminished by agonist stimulation. $BRET^2$ between the β_2AR-Rluc and GFP^2-βarrestin 2 was increased by agonist, whereas $BRET^2$ between the β_2AR and GRK2-GFP^2 was not significant. On the basis of the level of GRK5-mediated phosphorylation we observe in isolated membrane fractions and co-localization of the β_2AR and GRK5, we conclude that GRK5 plays a distinctive role in the phosphorylation of the β_2AR.

Agonist stimulation of the β_2-adrenergic receptor (β_2AR[1]) in intact cells activates G_s/adenylyl cyclase and initiates the desensitization process involving PKA and GRK phosphorylation of the β_2AR followed by arrestin binding and internalization of the receptor. The PKA and GRK phosphorylation events and their relationship to desensitization have been analyzed in many studies involving both intact cell studies of phosphorylation and cell-free phosphorylation by both PKA (*1*−*3*) and GRKs (*4*−*7*). Previous work aimed at identifying the GRKs responsible for β_2AR phosphorylation has, for the most part, supported the hypothesis that GRK2 translocation to the plasma membrane, through its binding to the $\beta\gamma$ subunit, was the dominant activity in the desensitization of the β_2AR (*5*, *7*). However, it is clear as well that there is redundancy, and studies have demonstrated that purified GRKs1−6 are all capable of phosphorylating the β_2AR (*5*). A number of other approaches have been utilized to identify the GRKs involved in β_2AR regulation in cells such as dominant negative proteins, knockdowns and knockouts, overexpression, transgenic approaches, and inhibitors. Because of the problems with these various approaches it is not surprising that identification of the intact cell GRKs responsible for desensitization of the β_2AR in various cell types remains incompletely understood (*4*, *8*, *9*).

The original assay that led to the purification and characterization of GRK2 phosphorylation of the β_2AR involved the use of purified receptors reconstituted into liposomes followed by incubation with purified GRKs and reconstitutions with G proteins and arrestin to measure the effects of phosphorylation on activation (*10*, *11*). Determination of hormone-stimulated GRK activities using unperturbed receptors in plasma membrane preparations with *intrinsic* GRKs has not been described, although the β_2AR transiently expressed in HEK293 cell plasma membranes was shown to be phosphorylated by purified GRK2 and -3 (*12*). Also, use of urea stripping of membranes to deplete GRKs has been shown useful for several receptors for subsequent characterization of purified GRKs (*13*). Further, the study of the regulation of GRK2−6 activities by various protein kinases has for the most part also been accomplished utilizing an assay of heterologous GRK stimulation of rhodopsin phosphorylation (*4*, *7*); however, activities are much reduced in these heterologous systems relative to the rapid GRK phosphorylation of their receptors in intact cells.

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[1] Abbreviations: β_2AR, β_2-adrenergic receptor; GRK, G protein-coupled receptor kinase; PKA, cyclic AMP-dependent protein kinase; ISO, isoproterenol; HEK, human embryonic kidney; AT, ascorbate/thiourea; PAGE, polyacrylamide gel electrophoresis; BRET, bioluminescence resonance energy transfer; HE, Hepes/EDTA; PBS, phosphate-buffered saline; βarr, β-arrestin; Rluc, Renilla βarr luciferase.

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Recently, using a phosphosite-specific antibody for serines 355, 356 to monitor GRK site phosphorylation of the β_2-AR, we found that overexpression of GRK5 in either COS7 or HEK293 cells caused considerable constitutive GRK site phosphorylation in the absence of agonist stimulation (*14*, *15*) and that GRK5 was considerably more active than GRK2, consistent with the tight membrane association of GRK5 (*16*−*18*). Similar results were previously reported using ^{32}P labeling (*19*). Knockdown of GRKs2−6 using siRNA interference suggested that GRK5 and -6 play a role in GRK site phosphorylation of the β_2AR in HEK293 cells, although it was concluded that it was a minor role relative to GRK2 (*9*). β_2AR stimulation was also shown to elicit a GRK5- and -6-dependent stimulation of ERK activation that was independent of G protein involvement. Of interest, in the same study it was shown that GRK5 and -6 were actually stronger than GRK2 in phosphorylating a mutant, uncoupled β_2AR. Recent studies of other GPCRs have also raised the possibility that specific GRK activation and arrestin binding may nucleate downstream scaffolding for assembly of other proteins such as ERK that are unique and distinct relative to their role in desensitization, arrestin binding, and internalization (*6*, *20*).

The goals of the present work were to determine whether we could develop a method for assay of GRK site phosphorylation of the β_2AR cell free in plasma membranes with endogenous GRK activity and use this methodology to aid in the characterization of the GRK(s) involved in β_2AR phosphorylation. We report here the development of a cell-free assay for ISO stimulation of GRK phosphorylation of the β_2AR residues S355 and S356 and, based on a number of criteria, such as the rapidity and concentration dependency of activation of GRK site phosphorylation and the activity of a range of partial agonists, that the activity closely reflects that which occurs in intact cells. Unexpectedly we found that plasma membrane preparations, depleted of all GRKs except GRK5, displayed a robust ability to support agonist-dependent GRK site phosphorylation. Using BRET^2 methodology we also found evidence for the tight association of the β_2AR with GRK5, findings that are consistent with the membrane localization of GRK5. In summary, our study demonstrates that intrinsic GRK5 likely plays a distinctive role in the phosphorylation of the β_2AR, suggesting that the plasma membrane localization of GRK5 is an important mechanism for β_2AR regulation that perhaps subserves different downstream processes independent of G protein-dependent $\beta\gamma$ localization of GRK2 (*9*).

EXPERIMENTAL PROCEDURES

Materials. Cell culture reagents were purchased from Mediatech (Herndon, VA). Human Embryonic Kindney (HEK) cells were purchased from American Type Culture Collection (Manassas, VA). β_2AR agonists and antagonists, calyculin, calmodulin, PP2 inhibitor, and heparin were from Sigma-Aldrich (St. Louis, MO). The inhibitors KT5720, K252A, LY294002, and wortmannin were from Calbiochem (La Jolla, CA.) Peptide inhibitors were synthesized by Sigma Genosys (The Woodlands, TX.) Okadaic acid was obtained from Alexis Biochemicals (San Diego, CA). Peptide N-glycosidase F (PNGase F) was from New England Biolabs (Beverly, MA). Primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and HRP-conjugated secondary antibody and the Bio-Rad Protein Assay (cat no. 500−0006) were from BioRad. Recombinant human GRK2 with an S670A substitution that removes a potential MAP kinase phosphorylation site (*21*) and recombinant GRK6, a palmitoylation-deficient mutant in which three potential palmitoylation sites (located at Cys^{561}, Cys^{562}, and Cys^{565}) were converted to serine (*22*), were a kind gift from Dr. John Tesmer, The University of Michigan, Ann Arbor, MI.

Cell Culture. HEK293 stably expressing Flag-β_2AR (WT-β_2AR) at 2−4 pmol/mg membrane protein were grown in 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 200 μg/mL of G418 as previously described (*14*, *23*). COS7 and A431 cell lines were grown the same as described above for HEK293 cells (without G418), and transient transfection of these lines with the Flag-β_2AR was performed as previously described (*14*). When seeding cells for experiments, dishes were coated with poly-L-lysine to aid attachment.

Intact Cell Phosphorylation. Intact cell GRK site phosphorylation was performed as previously described (*23*). Briefly, 100 mm dishes of confluent WT-β_2AR cells were treated with β_2AR agonists in 0.1 mM ascorbate/1 mM thiourea pH 7 (AT) or with AT alone. The cells were then washed three times in cold HE buffer (20 mM Hepes/1 mM EDTA pH 7.7), and the contents of each dish were scraped into 1.5 mL of solubilization buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 0.9% dodecyl-β-D-maltoside, 20 mM Na_4-PPi, 10 mM NaF, 20 μM Na_3VO_4, 10 μg/mL benzamidine, 10 μg/mL trypsin inhibitor, 10 μg/mL leupeptin, and 100 nM okadaic acid.) The samples were rocked for 30 min at 4 °C and centrifuged for 15 min at 21 000*g*. The supernatants (160 μL) were treated for 2 h at 37 °C with 3 μL (150 U) of PNGase F to deglycosylate the β_2AR. The samples were heated to 65 °C in SDS sample buffer for 15 min, resolved on 12% SDS-PAGE, transferred to nitrocellulose, and immunoblotted first with phosphosite-specific antibody against GRK site serines 355, 356, and then stripped and reprobed with antibody to the C-tail as previously described (*14*, *23*). Westerns were visualized on film using ECL-Plus from GE Healthcare and quantitated using Syngene software. GRK site antibody data were normalized to total receptor levels (anti-C-tail) and then to the maximal level. When multiple gels were run for one experiment the data were normalized to samples giving the highest values that were replicated on each gel as internal controls.

Cell-Free GRK Site Phosphorylation. Preparation of the 600g Supernatant and 21K Pellet Fractions. Four 100 mm dishes of confluent WT-β_2AR cells were rinsed twice in PBS, and the contents of each dish were scraped into 1.5 mL of cell-free buffer A (50 mM Tris pH 7, 0.1 mM EDTA pH 7, 40 μg/mL BSA, 50 nM calyculin, 10 mM DTT, 50 mM KCl, 5 mM $MgCl_2$, 10 μg/mL leupeptin, 1 mM benzamidine, and 1 mM PMSF). The cells were pooled (6.0 mL), homogenized with seven strokes of a type B Dounce homogenizer, and centrifuged at 600*g* for 5 min at 4 °C to remove all intact cells and nuclei. A 1.5 mL aliquot of the 600*g* supernatant was used directly for cell-free phosphorylation, and 1.5 mL was used for preparing each of the 21K pellets as follows. The post 600*g* supernatant was centrifuged for 10 min at 21 000*g*, and the pellet was resuspended and washed either once (21K pellet 1) or twice (21K pellet 2) with buffer A

(all steps at 4 °C). The pellets were then suspended in 1.5 mL of buffer A and used for cell-free phosphorylation. In addition to preparation of the 21K fraction we also examined GRK site phosphorylation in plasma membrane fractions isolated on sucrose gradients as previously described (*23*). Typical total proteins levels ($n = 5$) in 1.5 mL of the 600*g* and 21K pellets 1 and 2 were 2.8 ± 0.25, 0.33 ± 0.005, and 0.275 ± 0.021 mg, respectively. β_2AR levels in 1.5 mL of these fractions were 1079 ± 148, 599 ± 103, and 483 ± 108 fmol, respectively. β_2AR levels were measured with ^{125}-ICYP as previously described (*24*).

Cell-Free GRK Site Phosphorylation Assay. A 1.5 mL amount of either the freshly prepared 600*g* supernatant or the 21K pellet fractions was used for the cell-free assay. Just prior to assay, 100 μL of the ATP regenerating system was added to the 1.5 mL fractions, giving final concentrations of 500 μM ATP, 8 mM creatine phosphate, and 16 U/mL creatine phosphokinase and a total volume of 1.6 mL. Aliquots of 100 μL were placed in tubes and incubated with or without agonists or other additions at 30 °C for the desired time. The assay was terminated by addition of 100 μL of 2× solubilization buffer. Samples were rocked for 30 min, treated with PNGase F, and 5X SDS sample buffer (50 μL) as described above. An aliquot of 20 μL (of the 250 μL sample) was used for SDS-PAGE representing 0.5% of the starting cell lysate. GRK site phosphorylation was measured as described above for intact cell phosphorylation. Relative GRK levels were also determined in select samples by Westerns. To quantitate the levels of GRKs, standard curves of purified GRK2 and -6 and GST-GRK5 (0.8−67 ng) were run in conjunction with samples from the post 600*g* and 21K pellets 1 and 2. GST-GRK5 construct was generated by ligating full-length GRK5 into pGEX4T-1-GST at the 3′ end with restriction sites Bam H1 and EcoR1. The plasmid was grown and GST-GRK5 purified on glutathione-sepharose as described in the Amersham Biosciences protocol.

Measurement of Cell-Free Desensitization of Adenylyl Cyclase Activity. 600g Supernatant. WT-β_2AR cells in 100 mm dishes were washed twice in PBS, scraped in buffer A, and homogenized as described above. Samples were centrifuged for 5 min at 600*g*, and the regenerating system was added to the supernatants. The samples were then treated for 5 min ± 1 μM ISO and cooled. The post 600*g* fraction was washed twice in buffer A by consecutive centrifugation steps at 21 000*g* and then suspended in HE buffer and assayed for adenylyl cyclase activity. Alternatively the post 600*g* fraction was placed over sucrose step gradients (23% and 43% sucrose) and the plasma membrane fraction assayed for adenylyl cyclase activity as previously described (*24*).

21K Pellet. Cells were washed, homogenized in buffer A, and centrifuged at 600*g* as described above. The supernatants were centrifuged for 10 min at 21 000*g*, and the pellets were suspended in 6 mL of buffer A. The regenerating system was added, and the samples were treated for 5 min ± 1 μM ISO, then centrifuged at 21 000*g*, and washed twice with buffer A. The samples were then suspended in HE buffer and assayed for adenylyl cyclase.

BRET^2 Assays. Human βarr2 N-terminally tagged with GFP^2 (GFP^2-βarr2) and the Renilla Luciferase (RLuc) cDNA were purchased from Perkin-Elmer, Wellesley, MA. Human GRK2 and GRK5 cDNA were cloned from a pancreatic cDNA library (Clontech, Palo Alto, CA) and inserted into pcDNA3.1+ (Invitrogen, San Diego, CA). GRK2-GFP^2 and GRK5-GFP^2 were generated by subcloning GRK2 and GRK5 lacking the stop codon in a 5′ position of GFP^2. To make the β_2AR-RLuc construct β_2AR lacking the stop codon was inserted in a 5′-position of RLuc. All cDNA clones were verified by sequencing. The membrane-tagged GFP^2 was constructed by adding the cDNA sequence encoding the membrane localizing N-terminal 14 amino acid residues (MGCVQCKDKEATKL) from p59Fyn in the 5′-position of GFP^2.

HEK293 cells were transiently transfected by calcium phosphate precipitation according to previously reported methods (*25*). For BRET^2 saturation experiments, a range of transfections were made with a constant level of β_2AR-RLuc cDNA and increasing amounts of GRK5-GFP^2, GRK2-GFP^2, or GFP^2-βarr2 cDNA. Cells were harvested 48 h after transfection.

BRET^2 measurements were made using a Mithras LB 940 plate reader (Berthold Technologies, Bad Wildbad, Germany). Following harvesting, 180 μL of resuspended HEK293 cells was distributed in 96-well microplates (white Optiplate; Perkin-Elmer, Wellesley, MA) resulting in a density of ∼200 000 cells/well, and all subsequent steps were performed at 22 °C. Agonist or buffer was added manually, and substrate DeepBlueC (Perkin-Elmer, Wellesley, MA) was injected to a final concentration of 5 μM 2 s before reading in all BRET^2 measurements. The reading time after agonist addition was 5 min based on pilot studies of the time course of development of the BRET^2 signal. The signals detected at 400 and 515 nm were measured sequentially, and the 515/400 ratios were calculated. The background signal from Rluc was determined by coexpressing the β_2AR-Rluc construct with empty vector, and the BRET^2 ratio generated from this transfection was subtracted from all other BRET^2 ratios. Expression levels of Rluc- and GFP^2-tagged constructs for each BRET^2 experiment were monitored by luminescence and fluorescence measurements, respectively. Luminescence was measured on the Mithras LB 940 plate reader 2 s after DeepBlueC addition. For fluorescence measurements, cells from the same transfections were plated in a black clear-bottom microplate (ViewPlate; PackardBioScience, Inc., Montreal, Canada) at a density of $\sim 5 \times 10^4$ cells per well. After 1 h incubation in darkness, the total fluorescence was measured using the NovoStar microplate reader (BMG LabTech, Offenburg, Germany) with an excitation line at 485 nm and an emission filter at 520 nm. Background values obtained with untransfected cells were subtracted in both measurements, and the means of quadruplicate wells/sample were then calculated.

RESULTS

Characterization of GRK Site Phosphorylation Cell Free. To critically evaluate whether we could observe GRK site phosphorylation cell free, we first examined the post-nuclear 600*g* fraction of the WT-β_2AR cells since previous results suggested that GRK2 was the primary GRK mediating β_2AR phosphorylation and that it is localized to the cytosol. Our previous work in intact cells demonstrated that epinephrine stimulation of GRK site phosphorylation was rapid ($t_{1/2} \approx 30-40$ s) and that the initial rate of phosphorylation was occupancy dependent, displaying an EC_{50} of approximately

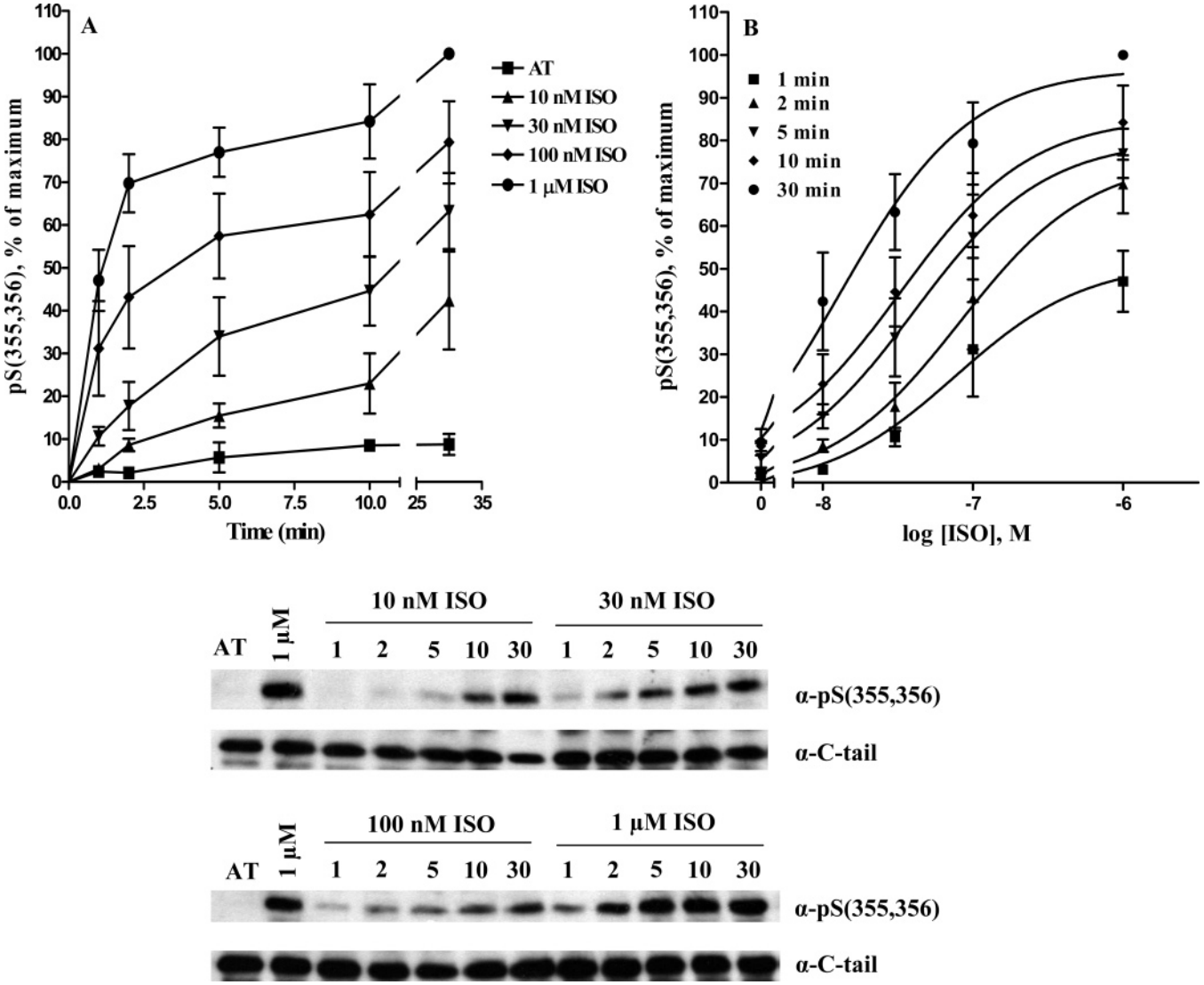


FIGURE 1: Effects of time and ISO concentration on GRK site phosphorylation. The post 600*g* supernatant was prepared from four 100 mm dishes of WT-β_2AR as described in Experimental Procedures. The supernatants were treated at 30 °C with various concentrations of ISO for the times indicated, and the GRK site phosphorylation was assessed by Western blotting. The GRK site data were normalized first to the total β_2AR (C-tail antibody) and then to the 1.0 μM ISO point at 30 min. Values are the means ± SEM of five separate experiments, with one representative Western blot. The data were plotted as a function of the time (A) and concentration of ISO (B).

200 nM (*13*). We also showed that while lower concentrations resulted in proportionately slower initial rates of phosphorylation, the eventual level of phosphorylation after 20 min with 50 nM epinephrine (≈10% occupancy) was nearly equivalent to that with 10 μM epinephrine (97% occupancy). The data in Figure 1A demonstrate that we observe a rapid and profound concentration-dependent ISO stimulation of GRK site phosphorylation in the post 600*g* supernatant. 100 nM ISO produces a level of GRK site phosphorylation that is about 80% that of 1.0 μM ISO. The data plotted as a function of ISO concentration (Figure 1B) demonstrate that the EC_{50} for the 1 and 2 min data (initial rates) is ~80 nM and for the 10 and 30 times of stimulation ~ 33 and 13 nM, respectively. These data collectively show good agreement with those observed in intact cells for GRK site phosphorylation (*14*).

GRK Site Phosphorylation in Washed Particulate Preparations. To better define the agonist-stimulated cell-free phosphorylation, we isolated crude membrane fractions (21K pellets) from the post 600*g* fraction by three successive centrifugation steps (21 000*g*) as described in Experimental Procedures. We then compared the time courses of 100 nM ISO stimulation of GRK site phosphorylation of the two washed membrane fractions (21K pellets 1 and 2) with the starting post 600*g* fraction (Figure 2). We predicted that our activity would be greatly reduced if GRK2 (or GRK3) was the major activity since it would be removed by preparation of the washed particulate fractions. Surprisingly we found the rate of activation of the two membrane pellet fractions to be identical, and their activities were only slightly reduced relative to the post 600*g* fraction (Figure 2). Also, the concentration and time dependence for ISO activation was nearly identical to that in the post 600*g* preparation (data not shown). To further define the buffer used for cell-free phosphorylation in the washed 21K membrane fractions, the necessity of various components of the GRK phosphorylation buffer A were examined, and only ATP and $MgCl_2$ were required. The EC_{50} for ATP was 30 μM.

Since it was expected that there would be minimal GRK activity in the washed particulate fractions, we assessed the levels of GRK2, -5, and -6 in the post 600*g* and two particulate fractions. We found that the levels of GRK2 and

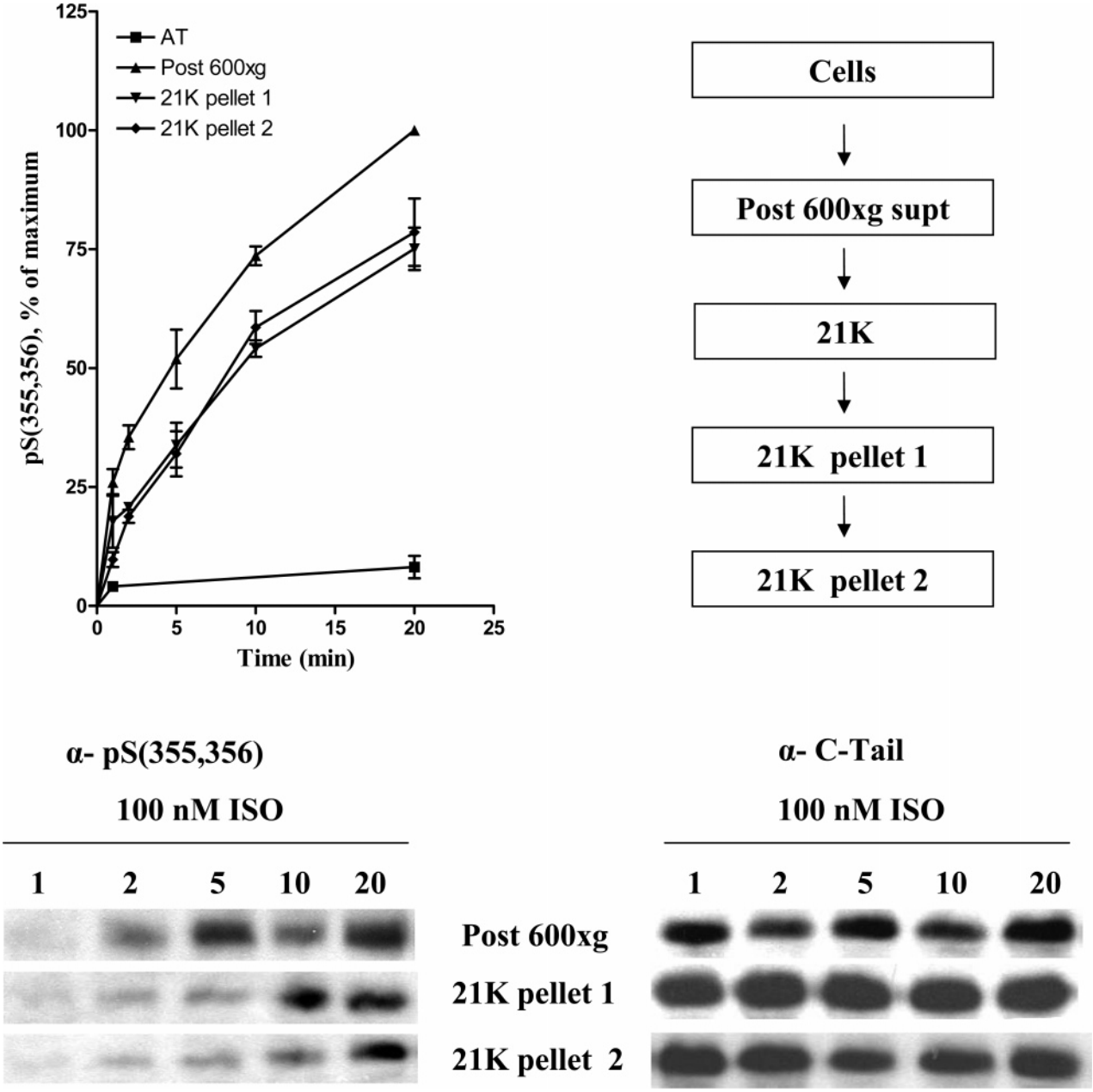
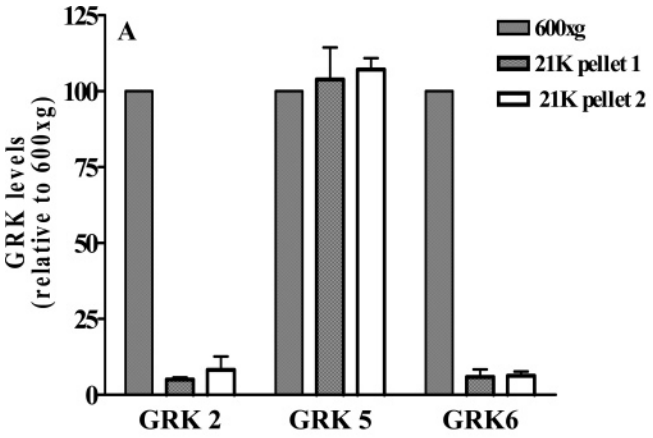


FIGURE 2: Comparison of post 600*g* supernatants and 21K pellets with 1 or 2 washes. Post 600*g* supernatant was prepared from four 100 mm dishes of WT-β_2AR cells and split into two aliquots. One was kept on ice, while the other was further split into two aliquots. Both were centrifuged for 10 min at 21 000*g* (21K). One pellet was washed once in cell-free buffer A with a 21K spin (21K pellet 1), and the other was washed twice (21K pellet 2). Both were suspended in cell-free buffer A, then treated with 100 nM ISO for the times indicated, and processed as described for Western blotting. The data were normalized to the total receptor levels (C-tail antibody) and then to the 20 min, 1.0 μM ISO point. Values shown are the means ± SEM from three separate experiments.

-6, while high in the post 600*g* fraction, were essentially eliminated by the first 21K step (Figure 3A), and the supernatant fraction contained the GRK2 and -6. In contrast, the levels of GRK5 in the two particulate preparations were maintained. To quantitate the levels of GRK2, -5, and -6 standard curves were generated and run with samples from the 600*g* and 21K pellets 1 and 2 (Figure 3B). GRK2 and -6 were reduced from 34.4 ± 5 and 26.6 ± 9 ng/20 μL to essentially undetectable levels in the 21K pellet 2, while GRK5 levels were maintained (from 34.7 ± 7 to 27.3 ± 7.2 ng/20 μL). As can be seen in Figure 3B the levels of the GRKs varied considerably in the various fractions (with the exception of GRK2 and -6 in the 21K pellets that were indistinguishable from background). Levels of GRK5 in the 600*g* fraction and 21K pellet 2 varied from 11 to 63 ng/20 μL and 6 to 51 ng/μL. When normalized to the mean total protein content (as given in Experimental Procedures) the levels of GRK5 varied from ~0.03% to 0.2% of the total protein in the 600*g* fraction and from 0.2% to 1.4% in the 21K pellet. Since the β_2AR in the pellet fractions [measured as previously described by ^{125}ICYP binding (*24*)] was between 2 and 4 fmol/20 μL in these samples, it can be calculated that the GRK5 level exceeded that of the receptor on the order of 100-fold. Further, these fractions were also probed by Westerns for β-arrestin 1 and 2 levels and GRK3, and while the post 600*g* had significant levels, these proteins were eliminated in the 21K pellet fractions (data not shown). Thus, we conclude that the GRK site phosphorylation in the washed particulate fractions was attributable exclusively to GRK5.



B **Quantitation of GRK levels**[a]

	GRK 2	GRK 5	GRK 6
Preparation	Mean SEM (ng)	Mean SEM (ng)	Mean SEM (ng)
600xg	34.4 ± 4.6	34.7 ± 7.2	26.6 ± 9.0
21K pellet 1	1.8 ± 2.0	38.3 ± 8.7	-0.1 ± 0.8
21K pellet 2	0.1 ± 1.6	27.3 ± 7.2	-1.2 ± 0.6

[a] GRK values are in ng/20 µl

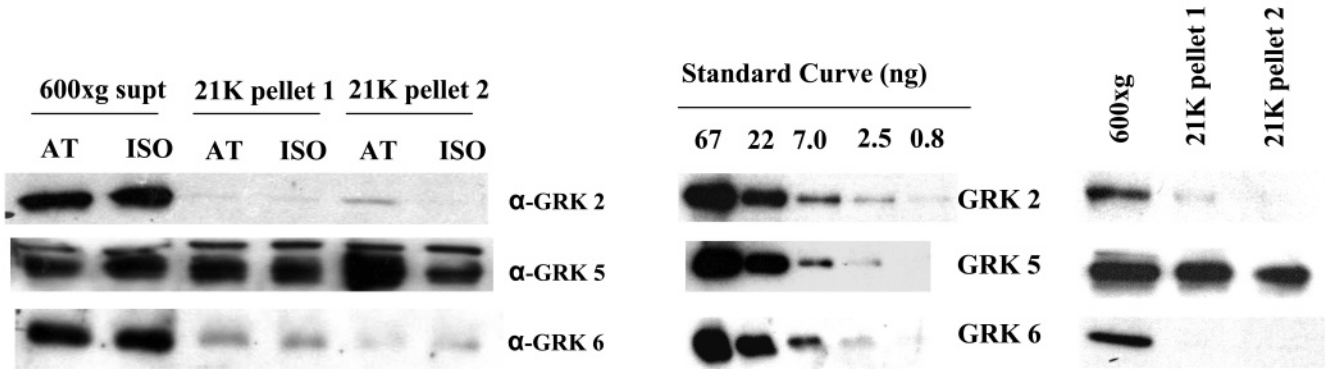


FIGURE 3: Levels of GRKs 2, 5, and 6 in the post 600*g* supernatant and the 21K pellets. Samples (20 μL) of the post 600*g* and 21K pellets prepared as described in the legend to Figure 2 were run on SDS-PAGE, transferred, and probed with antibodies to GRK2, GRK5, and GRK6. (A) Values shown are the means ± SEM from three experiments, with a representative blot shown below the graph. (B) To quantitate the levels of GRKs, standard curves were generated using purified GRK2 and -6 and GST-tagged GRK5 and run with the samples. GRK levels in the samples were calculated from the standard curves, and the results are shown in the table (means ± SEM, $n = 6$). The values in the table (ng/20 μL) correspond to 0.5% of the 1.5 mL extract from one 100 mm dish as explained in Experimental Procedures.

We also investigated whether membranes prepared on continuous sucrose gradients would show a similar level of GRK site phosphorylation, and we found that the plasma membrane fraction showed a strong ISO stimulation of GRK site phosphorylation equivalent to the 21K pellets (data not shown). Further, to demonstrate that the GRK5 phosphorylation was not restricted to the Flag-tagged WT-β_2AR, we found that an HEK293 line previously generated that stably expresses an HA-tagged β_2AR (*26*) showed similar GRK5 phosphorylation (1.0 μM ISO, 5 min) in the doubly washed 21K pellet preparation. We also examined the level of GRK site phosphorylation in 21K pellet preparations of both A431 and COS7 cells after transient transfection with the WT β_2-AR. We found a level of GRK site phosphorylation that was approximately 60% of that in the HEK293 cell lines, demonstrating that the intrinsic GRK5 phosphorylation was not restricted to HEK293 cells (data not shown).

Evidence That a Substantial Fraction of Intact Cell GRK Site Phosphorylation Is Retained in Cell-Free Preparations. Since we routinely measured a 10−15-fold stimulation of GRK phosphorylation in the washed 21K membranes the question arose as to how the levels of agonist-stimulated GRK site phosphorylation compared in the post 600*g*, the doubly washed 21K particulate fraction, and intact cells. To obtain a comparison of the relative levels the following experiments were performed. Intact cells were stimulated with 1.0 μM ISO for 5 min, a time that gives near maximal GRK site phosphorylation (*14*, *23*). In the same experiment with cells grown under identical conditions, the 600*g* and doubly washed 21K pellet fractions were also stimulated with ISO for 5 min. To achieve equivalent levels of reactivity with saturating levels of the anti-GRK site and C-tail antibodies for comparison of the relative levels of GRK site phosphorylation in the three preparations, similar amounts of receptor from each of the three preparations were run on one gel. The gels were cut in half, and one-half was developed with the anti-GRK site antibody and the other with the C-tail antibody under identical exposure times with primary and secondary antibodies. Figure 4 shows a comparison of the intact cell GRK-mediated phosphorylation with the post 600*g* and 21K. The ratios of GRK site antibody reactivity to the C-tail antibody were not significantly different between the cell-free preparations and the intact cells, demonstrating that the levels attained cell free were nearly equivalent to the intact cells. Overall the data indicate that approximately 80% of the intact cell phosphorylation is retained in the 600*g* and 21K washed fractions, with the caveat that there was considerable variation between experiments rendering any quantitative comparisons tenuous at best.

Another way of approaching the same question as to whether cell-free GRK site phosphorylation reached saturation relative to intact cells was to determine if further phosphorylation occurred with addition of purified GRK2 and GRK6 (palmitoylation minus). To resolve this, the 21K fraction was incubated with or without agonist and additionally with either GRK2 or GRK6. The data show that GRK6, but not GRK2, gave some significant additional phosphorylation (Figure 5).

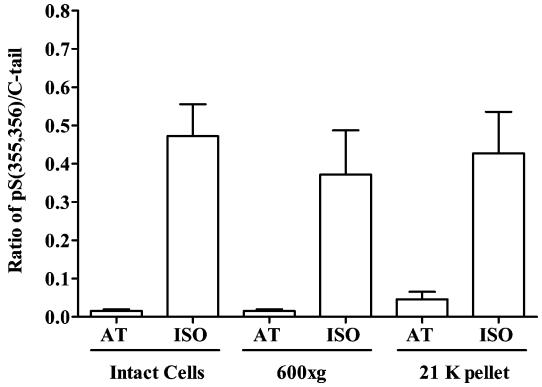


FIGURE 4: Comparison of ISO-induced phosphorylation in intact cells, post 600*g* supernatant, and 21K pellets. For the intact cell part of the experiment, WT-β_2AR cells in 100 mm dishes were treated with ±1 μM ISO for 5 min, then washed, scraped in 1.5 mL of solubilization buffer, and processed as described. For the cell-free part of the experiment, the post 600*g* supernatant was prepared from four 100 mm dishes. One-half was kept on ice, and the other half was used to prepare a twice washed 21K preparation (21K pellet 2). The post 600*g* and 21K pellet 2 were treated for 5 min ± 1 μM ISO as described in Experimental Procedures. Westerns were performed on the intact cell and cell-free preparations on one gel under identical conditions to assess GRK site phosphorylation and total β_2AR levels. Values shown are the ratios of GRK site phosphorylation to the C-tail antibody and are the means ± SEM of five experiments. There were no significant differences between preparations (ANOVA).

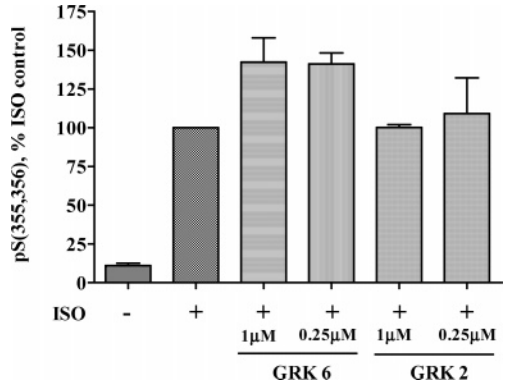


FIGURE 5: Effect of GRK2 or -6 on ISO-induced phosphorylation in washed 21K membranes. WT-β_2AR cells were homogenized in buffer A and the doubly washed 21K pellet 2 prepared. Pellets were suspended in buffer A and treated with 100 nM ISO for 5 min either with or without addition of purified GRK6 or GRK2. GRK site phosphorylation was assessed by Westerns. Data shown are the means ± SEM of three experiments.

Evidence for Association of β_2AR with GRK5. Our cell-free GRK site phosphorylation and the Westerns showing retention of GRK5 suggested that there may be a tight complex of the receptor and GRK5. To investigate this possibility HEK293 cells were transiently transfected with β_2AR-Rluc and either GFP^2-βarr2, GRK5-GFP^2, GRK2-GFP^2, or membrane-tagged GFP^2. The membrane tag used was a 14 amino acid sequence derived from p59Fyn that was placed on the N-terminus of GFP^2. This sequence contains a myristoylation and palmitoylation site, and it has been demonstrated to be sufficient for membrane-localizing proteins (*27*). Pilot studies of the time course of BRET^2 with the GRK5-GFP^2/β_2AR-Rluc interaction showed a high level of BRET^2 that was stable over a time period of 2 s to 20 min with or without 1.0 μM ISO, whereas BRET^2 with GFP^2-βarr2/β_2AR-Rluc interaction peaked at 3−5 min (data not shown), consistent with the time course of arrestin binding and internalization of the receptor previously reported (*14*, *28*, *29*). On the basis of this result, the cells were stimulated in the presence or absence of 1.0 μM ISO for 5 min, and the BRET^2 level was determined at various ratios of GFP^2-constructs/β_2AR-Rluc as described in Experimental Procedures. The results shown in Figure 6A (no agonist) demonstrate a high level of BRET^2 of the β_2AR-Rluc with GRK5-GFP^2 in the basal unstimulated state with a half-maximal saturation at a ratio of 1.3 (arbitrary units). Further, the BRET^2 was not altered by addition of agonist (Figure 6B). In contrast, cotransfection of β_2AR-Rluc with GRK2-GFP^2 gave a minimal basal signal in either the presence or the absence of ISO. With β-arrestin2 there was also a minimal basal signal in the absence of ISO; however, in the presence of ISO the BRET^2 signal increased with a half-maximal saturation at a ratio of 0.6. As expected the membrane-localized GFP^2 showed a progressive increase in BRET^2 that did not saturate with an increasing acceptor/donor ratio. The GRK5-GFP^2 data are consistent with the cell-free GRK site phosphorylation data, indicating that GRK5 plays a role in GRK phosphorylation of the receptor attributable to its co-localization to the plasma membrane. Finally, to confirm that the GRK5-GFP^2 construct was functional, we transiently overexpressed the construct and compared its constitutive phosphorylation of the β_2AR with that of untagged GRK5 as previously reported (*14*, *15*). The GRK phosphorylation of the β_2AR observed with the transient transfection of the GRK5-GFP^2 was equivalent to that observed with untagged GRK5 (data not shown), demonstrating that the tag did not alter GRK5-mediated phosphorylation. Furthermore, it has been shown that GRK5-GFP can phosphorylate rhodopsin in vitro (*17*).

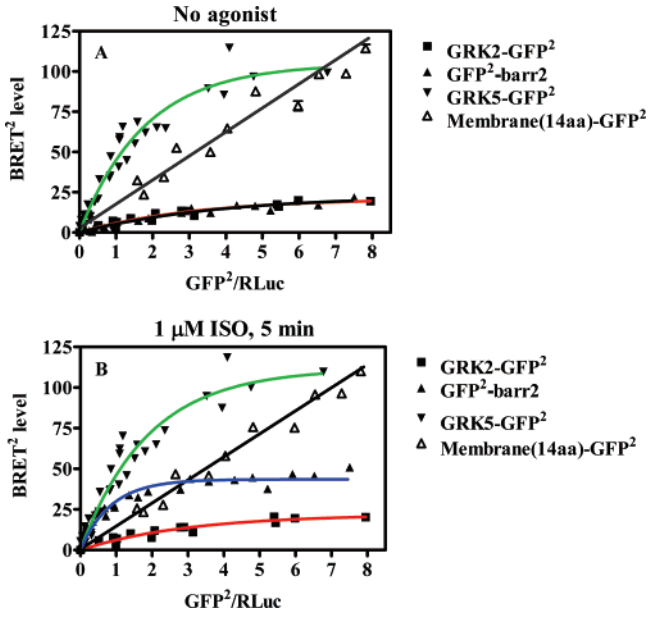


FIGURE 6: BRET^2 of the β_2AR-Rluc with either GRK5-GFP^2, GRK2-GFP^2, GFP^2-βarr, or membrane-tagged GFP^2. BRET^2 experiments were performed with varying ratios of GFP-tagged proteins to the β_2AR-Rluc following 5 min incubation either without (A) or with 1.0 μM ISO (B) as described in Experimental Procedures. Data shown are means from three independent experiments.

Lack of Desensitization after Cell-Free Agonist-Stimulated GRK Phosphorylation. Work from our group (*23*) showing

Table 1: Lack of Desensitization after Cell-free GRK Site Phosphorylation[a]

	EC_{50} (nM)			V_{max} (pmol/min/mg)		
	treatment			treatment		
fraction	AT	ISO	ratio of EC_{50}	AT	ISO	ratio of V_{max}
600*g*	2.01 ± 0.28	2.85 ± 0.20	1.42 ± 0.10	62.0 ± 0.15	50.5 ± 2.8	0.81 ± 0.04
600*g* + gradient	1.33 ± 0.11	1.52 ± 0.12	1.16 ± 0.18	183 ± 38	153 ± 30	0.83 ± 0.01
21K pellet	2.22 ± 0.06	3.27 ± 0.67	1.32 ± 0.17	74.9 ± 18	49.1 ± 3.2	0.69 ± 0.12

[a] The post 600*g* supernatant and 21K pellet fractions were treated with either the carrier AT or 1.0 μM ISO for 5 min. After stimulation the preparations were washed as described in Experimental Procedures, and adenylyl cyclase was assayed to determine the EC_{50} and V_{max} for ISO stimulation. The data are the mean ± range ($n = 2$).

resensitization with no diminishment of GRK site phosphorylation, as well as that of others in reconstituted systems with purified components (*10*, *30*), suggested that GRK site phosphorylation in the absence of arrestin caused little desensitization. Our cell-free assays provided another opportunity to test this proposal since the 21K preparation was free of arrestin and the arrestin in the 600*g* fraction was greatly diluted. In studies of intact cell desensitization we routinely observe 6−10-fold increases in the EC_{50} for ISO activation of adenylyl cyclase in response to a 5 min pretreatment with 1.0 μM ISO (*24*, *29*, *31*, *32*). To determine if the cell-free GRK site phosphorylation caused desensitization, the post 600*g* fraction and twice-washed 21K fractions were incubated with 1.0 μM ISO for 5 min and then used to determine the EC_{50} for ISO stimulation of adenylyl cyclase. The data in Table 1 show that there was only a slight increase in the EC_{50}s for either the post 600*g* or the 21K particulate fractions (∼40% or 30% increase, respectively). The 600*g* fraction purified over sucrose gradients showed better activity but still exhibited no significant desensitization. The decreases in V_{max} were consistently observed in the range of 20−30%, but these are not receptor-level events as previously reported (*24*, *29*). We conclude that the lack of both arrestin binding and internalization in the cell-free preparations is the likely explanation for the minimal desensitization, consistent with previous work (*10*, *23*, *30*). While there is arrestin present in the post 600*g* fraction, it is likely that dilution of arrestin in this supernatant relative to intact cells prevents sufficient levels of binding.

Effects of Partial Agonists on GRK Site Phosphorylation. In our prior studies we compared agonist stimulation of GRK site phosphorylation in intact cells with the intrinsic efficacy for activation of adenylyl cyclase (*14*, *32*) and found an excellent correlation. Another critical test of the relationship of cell-free GRK site phosphorylation to intact cells was the effect of stimulation with a range of agonists from the strongest (ISO) to the weakest (ephedrine). In the experiment shown in Figure 1 (Supporting Information), the post 600*g* supernatant and washed 21K pellet were stimulated with either carrier AT or saturating concentrations of the various agonists for 2 min, and GRK site phosphorylation was then determined. Since we found that stimulation of phosphorylation was similar for both the 600*g* and the washed 21K preparations, the results shown in Figure 1 (Supporting Information) are for both preparations. Our results demonstrate that the GRK phosphorylation was proportional to agonist strength (intrinsic efficacy) for activation of adenylyl cyclase, thus providing further evidence that the GRKs involved responded nearly identically over a range of agonist strength as with our previous studies of intact cells (*14*). The antagonists propranolol and ICI-118551 showed no significant GRK site phosphorylation (data not shown).

Inhibition of Agonist-Stimulated GRK5 Phosphorylation in the 21K Pellet. Previous work demonstrated that GRK5 phosphorylation of rhodopsin in vitro was inhibited by calmodulin (*33*, *34*). To determine if calmodulin inhibited the GRK5-mediated phosphorylation of β_2AR, the doubly washed 21K pellet 2 in buffer A supplemented with 1 mM $CaCl_2$ was stimulated with 100 nM ISO for 5 min in the presence or absence of various calmodulin concentrations and the extent of phosphorylation determined. We found that 4 μM calmodulin gave approximately an 80% inhibition, and the IC_{50} was ∼1.0 μM (data not shown), providing further evidence that the GRK site phosphorylation in the 21K pellet showed characteristics of GRK5. However, a prior study with ROS rhodopsin and purified GRKs demonstrated an IC_{50} of ∼50 nM for calmodulin inhibition of GRK5, considerably more potent than that we observe (*33*). This possibly reflects the greater affinity of GRK5 for the β_2AR relative to rhodopsin that in turn could inhibit calmodulin binding to the GRK5. Also, the strong membrane binding of intrinsic GRK5 in the 21K pellet through the N-terminus (*17*) could alter the surface it presents to calmodulin, which could be quite different from that with rhodopsin as substrate.

We also determined the effect of a range of inhibitors on the cell-free GRK phosphorylation in the 21K fraction following stimulation with 100 nM ISO. We found significant inhibition with 10 μM K252A (52 ± 11%), which is a PKG/PKA inhibitor, and 1.0 μM heparin #1 (52 ± 11%). We also found that 10 and 30 μM H89 blocked phosphorylation (73 ± 12% and 89 ± 4%); however, this effect occurred by H89 competition with ISO binding as previously shown (*32*, *35*). To confirm this we stimulated the 21K fraction with 100 μM ISO and found no significant inhibition with 30 μM H89. There was also no significant inhibition found in this assay with either heparin #2 (1 μM), the Src inhibitor P2 (10 μM), the PI-3-kinase inhibitors LY294002 (25 μM) and wortmannin (10 μM), or the PKA inhibitor KT5720 (5 μM). We also examined the GRK site peptide (CKAYGNGYSSNGN-NH2) either with or without phosphorylation of the two serines and the β_2AR-IL1 peptide that was shown to inhibit GRK2 and -5 phosphorylation of rhodopsin (*36*). Neither peptide showed any inhibition.

DISCUSSION

Our results demonstrate the feasibility of cell-free GRK site phosphorylation of the β_2AR and revealed, unexpectedly, the remarkable capability of intrinsic GRK5 to phosphorylate the β_2AR in membrane fractions from HEK293 cells. Agonist

activation of GRK site phosphorylation in membranes with intrinsic GRK has not been reported, although phosphorylation of the β_2AR in plasma membrane preparations by exogenous purified GRK2 and -3 (*12*) and α_2-adrenergic receptor phosphorylation using urea-washed membrane preparations and purified GRKs have been demonstrated (*37*). Cell-free studies of GRK activities have depended primarily on use of either reconstitution of purified receptor with purified GRKs or heterologous substrates such as rhodopsin or synthetic peptides (*7*). While much progress has been made in defining the phospholipid composition that promotes GRK phosphorylation of receptors reconstituted in liposomes (*38*, *39*), it is unlikely that it fully replicates the complex native membrane environment.

In this study several lines of evidence support our conclusion that a significant fraction of GRK phosphorylation of the β_2AR is mediated by GRK5. First, we show that GRK5 phosphorylation of the β_2AR at serines 355,356 is stimulated by agonist with remarkably similar characteristics to intact cells with regard to the kinetics of activation, concentration dependency of ISO stimulation, relative activity of partial agonist activation, and calmodulin inhibition. Second, we show that the GRK phosphorylation of the β_2AR in the doubly washed 21K pellet is caused by GRK5 since Western blotting and quantitative analysis of GRK levels revealed the near complete absence of GRKs 2, 3, and 6. Third, we find that the level of GRK5 phosphorylation in the 21K pellet is substantial relative to intact cells.

A fourth line of evidence in support of a role for GRK5 is based on our BRET^2 studies that were undertaken to probe the possibility that there is either a close association of the β_2AR and GRK5 in the plasma membrane or a relatively stable complex of the two proteins. Our findings suggest that there is a relatively stable complex of the β_2AR with GRK5 since we found a strong basal BRET^2 signal that showed saturation as we increased the GRK5-GFP/β_2AR-RLuc ratio. The BRET^2 signal was not diminished with agonist stimulation, suggesting that the phosphorylation and internalization that occur over this time span did not alter the association. This may be attributable to two factors. First, we reported that ISO-stimulated internalization is much diminished with transient transfection of the β_2AR and GFP-β-arr2 (*29*). Second, we previously reported that transient overexpression of GRK5 in HEK293 and COS7 cells causes a constitutive GRK site phosphorylation of the β_2AR (using the anti-pS-(355,356) antibody) to a level about 30−40% of agonist-stimulated levels (*14*, *15*). Both of these events would be expected to diminish the effect of agonist on the BRET^2 signal. That the β_2AR is responsive to agonist was demonstrated by agonist stimulation of receptor association with βarr2, which agrees well with the rate of β_2AR internalization in these cells (*14*, *23*, *24*, *32*, *40*). The GFP^2/RLuc ratio resulting in a half-maximal BRET^2 signal (the $BRET_{50}$ value) was modestly lower for GFP^2-βarr2 compared to GRK5-GFP^2 coexpressed with β_2AR-RLuc, suggesting that GRK5 interacts with β_2AR with a slightly lower affinity than agonist-recruited βarr2. We have not been able to show co-immunoprecipitation of the β_2AR and GRK5 either with or without agonist stimulation (data not shown), indicating that either even though the two are co-localized to the membrane their association constant is not sufficient to allow co-immunoprecipitation or more likely the detergent used in the solubilization protocol uncoupled their association. Thus, at present we cannot rule out the possibility that the receptor and GRK5 interact stably either directly or perhaps indirectly through a larger complex. There is precedent for a stable association of GRK5 with a GPCR. The V1R vasopressin receptor in which the C-terminus was truncated was demonstrated to form a stable complex with GRK5 by co-immunoprecipitation (*41*).

We were unable to detect a BRET^2 signal with the β_2AR and GRK2, suggesting that in these cells there is not a quantitatively stable or agonist-induced association of GRK2 and β_2AR. Although BRET^2 is a relatively sensitive assay for measuring the dynamics of protein−protein interactions in living cells, we cannot exclude the possibility that GRK2 translocates to a subpopulation of β_2ARs in a highly transient manner. Recruitment of GRK2-GFP^2 to the glucagon-like peptide-1 receptor measured 5 min after agonist addition has recently been demonstrated using BRET^2 (*42*) as has transient agonist-induced recruitment of GRK2 to the oxytocin receptor (*43*). However, it appears plausible that the close association of the β_2AR with GRK5 as we have shown would favor GRK5-mediated phosphorylation of the receptor relative to GRK2.

On the basis of our findings and others of the membrane locale of GRK5 it was crucial to have a control for the obvious problem that any two proteins localized to the membranes will show BRET^2 from random association as the acceptor/donor ratio is increased (*44*, *45*). This was accomplished by demonstrating that GFP^2 tagged with the membrane localizing peptide from p59 Fyn (*27*) showed a linear increase in BRET^2 that failed to saturate, consistent with predictions.

In the aggregate our findings using these novel approaches for the study of GRK phosphorylation of the β_2AR highlight a significant role for GRK5 in HEK293 cell lines and also suggest that GRK5 plays a role that is unique relative to the other GRKs and dependent on its membrane localization. Our results do not distinguish the relative contributions of the various GRKs in the intact cell for promoting β_2AR phosphorylation. In that regard the similar levels of GRKs 2, 5, and 6 we found in the post-600*g* fraction show that all are present at sufficient concentrations to mediate the GRK phosphorylation. Further, simple interpretation of our cell-free results may be clouded by the possibility that there is likely a mixture of inside-out and right-side-out vesicles, which may inhibit GRK2 phosphorylation of the receptors. Also GRK2, being a cytoplasmic protein, may be sufficiently diluted in the 600*g* preparation to impair its activity, although it was notable that addition of GRK2 to the 21K preparation had no effect. In contrast GRK6 did give some additional stimulation of phosphorylation of the receptor in the 21K pellet. Previous cell-free studies of highly purified GRK6 clearly show that it phosphorylates purified β_2AR reconstituted into liposomes and that palmitoylation considerably amplifies the stimulation apparently by increasing its membrane association (*46*, *47*). Using select siRNA knockdown of GRKs it was shown that GRK6 knockdown, but not that of GRK2 or -5, had a dramatic effect on β_2AR GRK site phosphorylation using the anti-pS(355,356) antibody but not nearly as dramatic an effect on β_2AR phosphorylation by ^{32}P-labeling, indicating both that there is specificity for GRK6 phosphorylation for the pS(355,356) site relative to other

GRKs and that GRK6 plays the major role in S355,356 phosphorylation in intact cells (*48*). Also, there is evidence GRK6 plays a role in β-adrenergic receptor desensitization in rat uterine smooth muscle (*49*).

While our results do not address the relative roles of the GRKs in the intact cell they do demonstrate that the anti-pS(355,356) works well for quantitating GRK5-mediated phosphorylation in the 21K pellet, and our findings show excellent correspondence with our intact HEK293 cell studies (*14*). Recently two separate studies using the anti-pS(355,-356) antibody to detect β_2AR phosphorylation demonstrated that a mutated β_2AR (β_2AR^{tyy}) that uncouples the receptor from G_s was phosphorylated in response to transient transfection of GRK5 and -6 (*9*) and that the mutant β_2AR (Y326A) was phosphorylated following GRK2 transfection (*50*). Further, as previously discussed above, we have shown in HEK293 and COS7 cells that with maximal transient overexpression, GRK2 and GRK5 resulted in constitutive phosphorylation of these residues as detected with the anti-pS(355,356) GRK site antibody (*14*, *15*). Of interest, with limited overexpression we found that GRK5 constitutive phosphorylation of these residues in HEK293 cells expressing the Flag-tagged WT β_2AR greatly exceeds that of GRK2 (unpublished data), which could be either attributed to the membrane localization of GRK5 or a consequence of the differential phosphorylation of residues on the β_2AR by GRK2 versus GRK5 (*48*). A recent mass spectrometric study of β_2AR phosphorylation in HEK293 cells showed that a peptide, amino acids 349−372, that includes serines 355, 356 was the only peptide covering the C-tail that was phosphorylated in HEK293 cells overexpressing the β_2AR (*51*). ISO stimulation resulted in addition of two phosphates in this peptide, consistent with our phosphosite-specific antibody results, although this group was not able to identify the sites by sequencing. Additionally it was found that there was a constitutive phosphorylation of another site on the 349−372 peptide. Our earlier findings with site-directed mutagenesis also indicated a role for a third site, namely, S364, in GRK-mediated desensitization. These results provide a possible explanation for the discrepancy we (*24*) and the study referred to above (*48*) have reported in comparisons of ^{32}P-labeling versus use of the anti-pS(355,356) GRK site antibody, since the extended preincubation with ^{32}P required by this method would label this site. Thus, in the aggregate while we feel that monitoring overall GRK site phosphorylation with the anti-pS(355,356) antibody is a reasonable approach, considerable effort will be required, perhaps combining siRNA approaches with mass spectroscopy to resolve the questions remaining concerning the relative contributions and specificity of the GRKs in intact cells for phosphorylation of the β_2AR. This approach in turn should be coupled with studies with purified GRKs and receptor, although a previous effort using this approach (*52*) to identify GRK sites revealed significant differences with intact cell (*24*, *31*) and the mass spectroscopy studies (*51*).

Our demonstration of the feasibility of monitoring cell-free GRK5 phosphorylation in crude plasma membrane fractions opens up the possibility of screening inhibitors of GRKs without the necessity of getting the inhibitors past the membrane barrier. This should be particularly useful for screening peptides, which are notoriously difficult to screen without permeabilization procedures that may disrupt the signaling apparatus because of its sensitivity to detergents. Our preliminary screen of commonly used inhibitors demonstrates that at least one inhibitor frequently used for blocking PKA and other protein kinase activities in cells, K252A, appears to inhibit GRK5 activity. Heparin, which previous studies have indicated is a potent inhibitor of GRK2 activity, was relatively weak in our GRK5 assay and dependent on a short variety of available heparin preparations. Recent X-ray studies of GRK2 and GRK6 indicate that the peptide binding site near the catalytic center of GRK2 has a greater distribution of positive charges (*22*), possibly providing a rationale for heparin selectivity for GRK2. In intact cells there is the problem of specificity; e.g., heparin was shown to block GRK activity, but its site of action is not clear since it also binds arrestin, and PKA phosphorylation was a confounding factor when assessing receptor phosphorylation by ^{32}P-labeling in intact cells. Not unexpectedly we found that a peptide of the region incorporating the GRK site serines 355 and 356 was not an effective inhibitor since the high affinity binding of the receptor to GRKs likely involves intracellular domains other than the C-tail as has been shown for rhodopsin activation of GRK1 (*53*). We also found that a synthetic peptide identical in structure to the N-terminus of the first intracellular loop of the β_2AR that was reported to give a potent inhibition of GRK2 (and to a lesser extent GRK5) phosphorylation of rhodopsin (*36*) was also ineffective in our cell-free assay. The reasons for the lack of effect of the peptide in our assay could reflect that GRK5 binding to and phosphorylation of rhodopsin in the heterologous assay is far weaker than endogenous GRK5 phosphorylation of the membrane-localized β_2AR.

In summary, our findings indicate that GRK5 may play a unique role in phosphorylation of the β_2AR in HEK293 cells, attributable to its tight binding to the plasma membrane and stable association with the receptor that provides the means for the rapid agonist-induced phosphorylation of the receptor without the necessity of $\beta\gamma$ release and GRK2 translocation. Further, localization of the receptor and GRK5 to the plasma membrane and their apparent tight association may be important factors to consider in the differential downstream signaling in addition to possible differences in the sites that may be phosphorylated by this GRK (*6*, *20*). Our demonstration of the feasibility and ease of observing a marked cell-free GRK phosphorylation of the β_2AR in its membrane locale also opens many possibilities for future studies, such as identifying both the receptor and the GRK domains that interact upon agonist activation, for cell-free studies of GRK5 regulation such as that we demonstrated with calmodulin inhibition, and for screening of potential inhibitors of the β_2AR/GRK5 binding and activation.

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SUPPORTING INFORMATION AVAILABLE

Comparison of ISO- and partial agonist-induced phosphorylation in the post 600*g* supernatant and 21K fraction. This

material is available free of charge via the Internet at http://pubs.acs.org.

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