β-Arrestin-dependent, G Protein-independent ERK1/2 Activation by the β2 Adrenergic Receptor*

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Sudha K. Shenoy‡, 1, Matthew T. Drake‡, 1, Christopher D. Nelson§, Daniel A. Houtz*, Kunhong Xiao†, Srinivasan Madabushi§, Eric Reiter‡, 4, Richard T. Premont†, Olivier Lichtarge‡, 8, and Robert J. Lefkowitz†, 13

From the ‡Howard Hughes Medical Institute at Duke University Medical Center, Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710, the §Institut National De La Recherche Agronomique, 37380 Nouzilly, France, †Program in Structural and Computational Biology and Molecular Biophysics and the ‡Molecular and Human Genetics Department, Baylor College of Medicine, Houston, Texas 77030

Physiological effects of β adrenergic receptor (β2AR) stimulation have been classically shown to result from Gαi-dependent adenylyl cyclase activation. Here we demonstrate a novel signaling mechanism wherein β-arrestins mediate β2AR signaling to extracellular signal-regulated kinases 1/2 (ERK 1/2) independent of G protein activation. Activation of ERK1/2 by the β2AR expressed in HEK-293 cells was resolved into two components dependent, respectively, on Gαi/γ-protein kinase A (PKA) or β-arrestins. G protein-dependent activity was rapid, peaking within 2–5 min, was quite transient, was blocked by pertussis toxin (Gi inhibitor) and H-89 (PKA inhibitor), and was insensitive to depletion of endogenous β-arrestins by siRNA. β-Arrestin-dependent activation was slower in onset (peak 5–10 min), less robust, but more sustained and showed little decrement over 30 min. It was insensitive to pertussis toxin and H-89 and sensitive to depletion of either β-arrestin1 or -2 by small interfering RNA. In Gαi knock-out mouse embryonic fibroblasts, wild-type β2AR recruited β-arrestin2-green fluorescent protein and activated pertussis toxin-insensitive ERK1/2. Furthermore, a novel β2AR mutant (β2ART66F,Y132F,Y219F or β2ARTYY), rationally designed based on Evolutionary Trace analysis, was incapable of G protein activation but could recruit β-arrestins, underγ- β-arrestin-dependent internalization, and activate β-arrestin-dependent ERK. Interestingly, overexpression of GRK5 or -6 increased mutant receptor phosphorylation and β-arrestin recruitment, led to the formation of stable receptor-β-arrestin complexes on endosomes, and increased agonist-stimulated phospho-ERK1/2. In contrast, GRK2, membrane translocation of which requires Gβγ release upon G protein activation, was ineffective unless it was constitutively targeted to the plasma membrane by a prenylation signal (CAAX). These findings demonstrate that the β2AR can signal to ERK via a GRK5/6-β-arrestin-dependent pathway, which is independent of G protein coupling.

The β2-adrenergic receptor (β2AR) is a well studied member of the large and diverse group of seven transmembrane receptors (7TMRs), which have been shown classically to exert their intracellular effects through G protein activation (1–3). Agonist stimulation of the β2AR leads to Gαi-mediated activation of adenylyl cyclase, resulting in the production of cAMP and subsequent downstream signaling events. Moreover, additional studies both in cultured cell lines and in vitro have demonstrated that, in response to agonist, the β2AR can undergo PKA-dependent phosphorylation leading to activation of Gαi (a process referred to as G protein “switching”), thereby effectively changing the signaling specificity of the receptor (4).

Cessation of agonist-activated β2AR-Gαi-mediated signaling occurs via recruitment of modulatory proteins, β-arrestins, to the cytoplasmic surface of the receptor, a process that is enhanced by receptor phosphorylation by G protein-coupled receptor kinases (GRKs) (5). β-arrestin binding physically prevents receptor-Gαi interaction, leading to desensitization of receptor-mediated activation of Gαi. β-Arrestin binding further promotes the subsequent cytosol to membrane translocation of clathrin and adaptor protein AP-2, resulting in receptor endocytosis in clathrin-coated vesicles (6–8).

Recent evidence has emerged, however, that, for a variety of receptors, β-arrestins can also function as molecular mediators of G protein-independent signaling by acting to scaffold a variety of signaling proteins. These include small-GTP-binding proteins, as well as members of the ERK-mitogen-activated protein kinase (MAPK) signal transduction pathway, among others (5). For example, angiotensin II receptor type 1a (AT1aR) signaling following activation by angiotensin II is known to promote AT1aR coupling to Gα11 (9). However, AT1aR activation by a peptide analogue of angiotensin II (termed SII), or angiotensin II activation of a mutant AT1aR (DRY → AAY) unable to couple to G proteins, results in activation of the MAPK cascade, which is Gαq-independent but β-arrestin-dependent (10–13). Further, these two independent signaling pathways show both distinct spatial (Gαq nuclear, β-arrestin cytosolic vesicles) and temporal (Gαq rapid/transient; β-arrestin slower and prolonged) characteristics with respect to ERK1/2 activation (14). Recent studies using siRNA have confirmed these results and have extended this general paradigm of distinct G protein/β-arrestin signaling to the V2 vasopressin receptor (V2R) (15).

7TMRs can be functionally divided into two broad categories based upon their interaction with β-arrestins following agonist activation. “Class B” receptors such as both the AT1aR and V2R form a very stable interaction complex with β-arrestins. “Class A” receptors, including the β2AR, on the other hand, are known to form only transient complexes regulated kinase; PKA, protein kinase A; siRNA, small interfering RNA; ET, Evolutionary Trace; GFP, green fluorescent protein; IBMX, 3-isobutyl-1-methylxanthine; HEK, human embryonic kidney; AT1aR, angiotensin II type 1a receptor; V2R, vasopressin type 2 receptor; DSP, dithiobissuccinimidyl propionate; MAPK, mitogen-activated protein kinase; WT, wild-type; PTX, pertussis toxin; pERK, phosphorylated ERK.
with β-arrestin (16). Whether β-arrestin-dependent ERK1/2 activation can occur via such Class A receptors is at present unknown. To investigate the potential for β-arrestin-dependent MAPK activation via a Class A receptor, we have used the β2AR to address these issues using gene silencing technology, Goα null cells, as well as a novel Evolutionary Trace-based mutant β2AR engineered to be uncoupled from G protein signaling.

**MATERIALS AND METHODS**

**Cell Lines, Biochemicals, and Plasmids—**HEK-293 and COS-7 cells were obtained from ATCC and maintained in designated culture media at 37 °C in a humidified 5% CO2 incubator. Goα null fibroblast cell line (17) was kindly provided by Dr. Jüppner (Massachusetts General Hospital) and maintained in Dulbecco’s modified Eagle’s medium/F-12 media supplemented with 5% fetal bovine serum, penicillin/streptomycin, and amphotericin B at 33 °C. Isoproterenol, propranolol, M2 anti-FLAG affinity agarose beads, G418, forskolin, mouse monoclonal anti-FLAG M2 antibody, and anti-mouse IgG conjugated to fluorescein isothiocyanate, were obtained from Sigma. Pertussis toxin was from List Biological Laboratories. H-89 was obtained from Calbiochem. DSP and GRK antibodies (18) were generated in the Lefkowitz laboratory. Detection of active ERK1/2 was with a rabbit polyclonal anti-phospho-p44/42 MAPK (Cell Signaling Technology Inc.). Horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences. [125I]iodocyanopindolol and [32P]P, were purchased from PerkinElmer Life Sciences. FLAG-β2AR/pcDNA3 (19), FLAG-β2AR[GRK-PKA-] (19), β-arrestin2-GFP (20), and GRK plasmids (13) have been described previously. The TYY mutant (β2AR[TYY]) was generated using a QuikChange multi-site-directed mutagenesis kit (Stratagene). All DNA constructs were verified by sequencing (Macrogen Inc., Seoul, South Korea).

**Generation of Cell Lines Stably Expressing Receptors—**Because HEK-293 cells have endogenously expressed β2ARs, higher expression levels of mutant receptors were necessary to correlate the effects attributable to the mutations. To maintain consistency of expression levels between different experiments, we created clonal HEK-293 cell lines with almost identical receptor expression levels for different experiments, we created clonal HEK-293 cell lines with almost identical receptor expression levels for different experiments, we created clonal HEK-293 cell lines with almost identical receptor expression levels for different experiments, we created clonal HEK-293 cell lines with almost identical receptor expression levels for different experiments, we created clonal HEK-293 cell lines with almost identical receptor expression levels for different experiments, we created clonal HEK-293 cell lines with almost identical receptor expression levels for different experiments.

**siRNA Transfection—**Chemically synthesized, double-stranded siRNAs, with 19-nucleotide duplex RNA and 2-nucleotide 3′-dTdT overhangs were purchased from Xeragon (Germantown, MD) in deprotected and desalted form. The siRNA sequences targeting human β-arrestin1 and β-arrestin2 were 5′-AAGGCUCUCCGCGCAGAAU-3′ and 5′-AAGGACCGAAGUGUUUGUG-3′ corresponding to positions 439–459 and 148–168 relative to the start codon, respectively. A non-silencing RNA duplex (5′-AAUUCUGCAACGUGUCGCU-3′), as the manufacturer indicated, was used as a control. For siRNA experiments, early passage HEK-293 cells that were 40–50% confluent on 100-mm dishes were transfected with 20 μg of siRNA, using the Genesilencer transfection reagent. Forty-eight hours later cells were split into either 6- or 12-well dishes for pERK assays and 12-well (Biocoat) dishes for radioligand binding.

**Phospho-ERK Assay—**HEK-293 cells on 6- or 12-well plates were starved for at least 4 h in serum-free medium prior to stimulation. After stimulation, cells were solubilized by directly adding 2× SDS-sample buffer, followed by sonication with a microtip for 15 s or by boiling at 100 °C for 5 min. For each transfection, an equal portion of the cells was set aside for protein determination (Bradford). Equal micrograms of cellular extracts were separated on 4–20% (for ERK1/2 detection) or 10% (for β-arrestins 1 and 2 detection) Tris-glycine polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes for immunoblotting. Phosphorylated ERK1/2, total ERK1/2, and β-arrestin antibodies were detected by immunoblotting with rabbit polyclonal anti-phospho-p44/42 MAPK (Cell Signaling, 1:2,000), anti-MAPK 1/2 (Upstate Technology Inc.). Detection of active ERK1/2 was with a rabbit polyclonal anti-phospho-p44/42 MAPK (Cell Signaling Technology Inc.). Horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences. [125I](–)iodocyanopindolol binding on monolayers of cells—Receptor expression was measured by [125I](–)iodocyanopindolol radioligand binding on monolayers of cells on poly-d-lysine-coated 12-well dishes (Biocoat) in Dulbecco’s modified Eagle’s medium buffered with 10 mM HEPES (pH 7.5) and 5 mM MgCl2. Binding was performed in triplicate with 400 μM 125I(–)iodocyanopindolol in the presence or absence of the hydrophobic antagonist propranolol (10 μM, to define nonspecific binding). After incubation at 37 °C for 30 min, the cells were placed on ice and washed several times with phosphate-buffered saline buffer containing calcium and magnesium. Cells were solubilized in 0.1 N NaOH and 0.1% SDS and counted for 125I.

**Internalization—**FLAG epitope-tagged receptors expressed in COS-7 cells in twelve-well dishes were treated with or without agonist for 30 min in serum-free medium at 37 °C. Cell-surface receptors were labeled with M2 FLAG monomolecular antibody and fluorescein isothiocyanate-conjugated goat antibody to mouse IgG as secondary antibody. Receptor internalization was quantified as the loss of cell-surface receptors, as measured by flow cytometry (Flow Cytometry Facility, Duke University).

**Metabolic Labeling—**HEK-293 cells stably expressing the β2AR or mutant receptors were incubated at 37 °C for 60 min in phosphate-free minimal essential medium containing [32P]P (100 μCi/ml). After isoproterenol treatment for 5 min at 37 °C, FLAG receptors were immunoprecipitated and samples separated by SDS-PAGE. Gels were dried and exposed to a PhosphoImage screen, and the [32P] incorporation was quantified.
Confocal Microscopy—HEK-293 cells stably expressing the WT, TYY, or GRK-PKA-receptors on 10-cm dishes were transiently transfected with β2AR or β2ARTYY that are FLAG epitope-tagged utilizing Lipofectamine 2000 reagent. Twenty-four hours post-transfection, cells were plated on collagen-coated 35-mm glass bottom plates. On the following day, cells were starved for at least 2 h in serum-free medium before stimulation. α-MSH and isoproterenol were added to the dishes with slow and constant agitation. After 20-min incubation at room temperature, the DSP reaction was quenched by adding Tris-HCl, pH 7.5 (final concentration of 25 mM). Cells were solubilized, and receptors were immunoprecipitated with DSP Cross-linking—HEK-293 cells stably expressing either β2AR or β2ARTYY that are FLAG epitope-tagged were used for these experiments. Cells on 100-mm dishes were incubated in 4.0 ml of Dulbecco’s phosphate-buffered saline plus 10 mM HEPES for 1 h at 37 °C and subsequently stimulated with 10 μM isoproterenol for 5 min. A membrane-permeable, hydrolyzable covalent cross-linker dithiobissuccinimidyl propionate (DSP, from Pierce) was added to the dishes with slow and constant agitation. After 20-min incubation at room temperature, the DSP reaction was quenched by adding Tris-HCl, pH 7.5 (final concentration of 25 mM). Cells were solubilized, and receptors were immunoprecipitated with DSP Cross-linking—HEK-293 cells stably expressing either β2AR or β2ARTYY that are FLAG epitope-tagged were used for these experiments. Cells on 100-mm dishes were incubated in 4.0 ml of Dulbecco’s phosphate-buffered saline plus 10 mM HEPES for 1 h at 37 °C and subsequently stimulated with 10 μM isoproterenol for 5 min. A membrane-permeable, hydrolyzable covalent cross-linker dithiobissuccinimidyl propionate (DSP, from Pierce) was added to the dishes with slow and constant agitation. After 20-min incubation at room temperature, the DSP reaction was quenched by adding Tris-HCl, pH 7.5 (final concentration of 25 mM). Cells were solubilized, and receptors were immunoprecipitated with DSP Cross-linking—HEK-293 cells stably expressing either β2AR or β2ARTYY that are FLAG epitope-tagged were used for these experiments. Cells on 100-mm dishes were incubated in 4.0 ml of Dulbecco’s phosphate-buffered saline plus 10 mM HEPES for 1 h at 37 °C and subsequently stimulated with 10 μM isoproterenol for 5 min. A membrane-permeable, hydrolyzable covalent cross-linker dithiobissuccinimidyl propionate (DSP, from Pierce) was added to the dishes with slow and constant agitation. After 20-min incubation at room temperature, the DSP reaction was quenched by adding Tris-HCl, pH 7.5 (final concentration of 25 mM). Cells were solubilized, and receptors were immunoprecipitated with
FIGURE 3. Effects of β-arrestin siRNA on β2AR stimulated pERK. HEK-293 cells stably expressing the β2AR were transfected with the indicated siRNAs. Serum-starved cells were treated with 100 nM isoproterenol for the indicated times and cell lysates were analyzed for pERK and ERK (A and C) and β-arrestin (B). pERK bands were quantified and normalized to ERK levels and plotted in the graph shown in A. Signal at each point is expressed as percentage of the maximal pERK signal with CTL siRNA (5 min). The quantification is mean ± S.E. from six separate experiments (A); *, p < 0.05; ***, p < 0.001 compared with the control treatment. Data in D represent quantification of pERK detected at various time points after 10 μM isoproterenol treatment of cells stably expressing β2AR. These cells were transfected with the indicated siRNA and preincubated with 20 μM H-89 for 15 min before stimulation. The graphs represent mean ± S.E. from three independent experiments.

FLAG beads. Coimmunoprecipitated β-arrestins were detected by immunoblotting with a rabbit polyclonal anti-β-arrestin1/2 antibody (A1CT).

Evolutionary Trace—The relative importance of transmembrane sequence residues was computed from an Evolutionary Trace (21) of 129 visual opsins, 69 bioamine, 58 olfactory, and 82 chemokine receptors. Comparison to the literature revealed that the residues ranked in the top 20th percentile, a structural cluster of seven likely to participate in G protein-coupling in all rhodopsin-like receptors: Thr-68, Tyr-132, Ala-134, Tyr-219, Leu-275, Tyr-326, and Pro-330, using human-β2AR numbering (22). These were the starting point for the rational design of the TYY mutant described below. Four of these were eliminated, because mutational data either: linked them to increased G protein activation (Leu-275) (23–26) or to decreased sequestration (Tyr-326) (27, 28) or was insufficient (Asn-330 and Ala-134). By contrast, the remaining three residues had mutations shown to decrease G protein signaling in multiple receptors and with no data suggesting a decrease in internalization.

RESULTS

Isoproterenol-dependent, H-89/PTX-insensitive pERK Stimulated by the β2AR in HEK-293 Cells—Two different β2AR-mediated pathways of G<sub>i</sub>-dependent ERK1/2 activation have been described in HEK-293 cells (29–31). Both pathways involve the activity of PKA, in one case to activate downstream effectors such as the small G protein Rap and the other to phosphorylate the β2AR itself, thereby switching its coupling to G<i>k</i> proteins. To evaluate the extent of PKA dependence of ERK1/2 activation in HEK-293 cells, we examined the time course of isoproterenol-induced pERK in the presence and absence of H-89, a well defined PKA inhibitor. β2AR is endogenously expressed in HEK-293 cells (~40 fmol/mg of cellular protein) and mediates a modest and transient activation of ERK1/2 with peak signal at 5 min after the addition of 10 μM isoproterenol (Fig. 1, A and B). The level of pERK declines to 22% (of maximal response) at 15 min and returns almost to basal levels after 30 min. Stable overexpression of a FLAG-tagged β2AR (2 pmol) augments the overall ERK1/2 activation by 4-fold at early time points and up to 8-fold beyond 10 min of agonist treatment (Fig. 1, C and D, and data not shown). However, the overall pattern of ERK1/2 activation is identical for both endogenous and exogenous receptor expression. Under both conditions, H-89 abolishes most of the early activity but, quite surprisingly, pERK at later time points is unaffected, indicating the presence of H-89-insensitive, isoproterenol-dependent signals beyond 5 min (Fig. 1, A–D). H-89 treatment precludes β2AR-G<i<k</i> coupling due to the absence of G<i>k</i>/G<i>i</i> switching (4). However, to independently assess the role of G<i>k</i> in the activation of ERK at late time points, we also determined the effects of pertussis toxin on the time course of isoproterenol-stimulated pERK. Pertussis toxin preincubation of HEK-293 cells expressing the β2ARs had similar effects on isoproterenol-dependent ERK activation as H-89 treatment, especially at the late time points of isoproterenol stimulation. The signals beyond 5 min were resistant to pertussis toxin (Fig. 2, A–D) suggesting that the late activity occurs in the absence of G<sub>i</sub> or G<sub>k</sub> coupling.

Effects of β-Arrestin siRNA on β2AR-stimulated pERK—We hypothesized that the ERK activity at later time points that is insensitive to
H-89 and PTX is mostly independent of G protein activity and might be regulated by β-arrestins. To test the potential role of β-arrestins, we analyzed the time course of isoproterenol-induced pERK after depleting cellular levels of β-arrestin1 or -2 by transfecting siRNA specifically directed against each isoform. In the presence of a non-targeting control siRNA, isoproterenol stimulated ERK phosphorylation in cells stably expressing the β2AR is identical to that observed without any siRNA transfection (compare Figs. 1 C and 3A). However, both β-arrestin1 and -2 siRNA individually reduce the 5-min signal by ~50% and essentially eliminate signals beyond 10 min (Fig. 3, A and C). Minimal decrements were observed at 1 and 2 min suggesting that most of the early signals are due to G protein activation. In these experiments, we could achieve at least 90% reduction of the two isoforms by RNA interference. A representative Western blot of the levels of β-arrestin1 and -2 in control and siRNA-treated cells is shown in Fig. 3B. We also observed similar β-arrestin1/2-dependent ERK activation by the β2ARs upon transient expression (300–800 fmol/mg of protein) in HEK-293 cells (data not shown). Furthermore, β-arrestin siRNA transfection led to complete elimination of the H-89-insensitive pERK (Fig. 3D) suggesting that most of the late activity that is PKA-independent is in fact β-arrestin-dependent. Our attempts to determine PTX effects in siRNA-transfected cells were unsuccessful for technical reasons. Unfortunately, the siRNA-transfected cells could not withstand the prolonged starvation conditions (16 h) used for PTX pretreatment.

An Evolutionary Trace-based Mutant β2AR Uncoupled from G protein.—We have previously shown that a mutant Angiotensin II 1a receptor (AT1AR 

DRY → AAY), which is completely uncoupled from Gq proteins is nonetheless able to activate ERK1/2 in response to angiotensin stimulation, in a β-arrestin2-dependent manner (12). To rationally design an analogous β2AR mutant, we employed Evolutionary Trace analyses (see “Materials and Methods”) and altered three residues, Thr-68, Tyr-132, and Tyr-219 by site-directed mutagenesis. These residues are indicated in an amino acid sequence alignment of the β2AR and rhodopsin (Fig. 4A). The relative positions of the corresponding residues are shown in a structural model of rhodopsin (Fig. 4B). These were mutated together to new side chains that were charge neutral, non-conservative, and not found at cognate positions.

**FIGURE 4.** A novel ET-based β2AR mutant that is uncoupled from G protein. A, sequence comparison of bovine rhodopsin and human β2AR in the indicated transmembrane regions displaying the sites of mutagenesis leading to β2AR TTYY. B, a structural model of bovine rhodopsin (53) indicating the corresponding sites of mutations. C, cAMP generated upon stimulation of HEK-293 cells that are transfected with either pCDNA3 (endogenous βARs), β2AR or β2AR TTYY plasmids. Cells were incubated with IBMX and then treated with increasing doses of isoproterenol. Isoproterenol-dependent cAMP values were normalized to forskolin-induced levels. Data represent mean values ± S.E. from 6 – 8 independent experiments. D, HEK-293 cells transfected with pCDNA3, FLAG-β2AR receptor, or FLAG-β2ARTYY were stimulated or not with 10 μM isoproterenol. FLAG receptors were immunoprecipitated and probed with an antibody specific to phospho-seryl 345 and 346 on the C-tail of the β2AR (upper panel). This is a consensus site for PKA phosphorylation. The same blot was reprobed with a β2AR antibody (H-20, Santa Cruz Biotechnology) as shown in the lower panel. These blots are representative of four identical experiments.
in any of the receptors traced for this study. This T68F-Y132G-Y219A mutant was predicted to drastically alter the component of G protein coupling located at the boundary between the transmembrane domain and the intracellular loops while leaving intact the cytoplasmic interaction of these loops with GRK and $\beta$-arrestin.

To differentiate characteristics of the mutant $\beta$2AR TYY from those of the endogenously expressed $\beta$2AR in HEK-293 cells, experimental determinations were made only when the mutant receptor was overexpressed at >20- to 50-fold. Under such conditions, no difference was observed between untransfected cells (endogenous receptors), and $\beta$2AR TYY-transfected cells (Fig. 4C) with respect to either the levels of cAMP accumulation or the half-maximal stimulating concentrations of isoproterenol. On the other hand, when the $\beta$2AR was expressed in these cells at levels comparable to the $\beta$2AR TYY (i.e., >20–50 times in excess of endogenous receptors) cAMP response was much more robust with a greatly increased $V_{\text{max}}$ and reduced $EC_{50}$ for isoproterenol as compared with the endogenous receptors. The dose-response curves shown in Fig. 4C establish that $\beta$2AR TYY does not stimulate cAMP accumulation beyond that elicited by the endogenous $\beta$2AR, confirming its lack of coupling to $G_{s}$.

Previous studies have demonstrated that cAMP increase and subsequent PKA activation lead to PKA-mediated phosphorylation of the $\beta$2AR on serine residues within the consensus motif RRSS (Ser-261 and Ser-262 in the third loop and Ser-345 and Ser-346 in the carboxyl tail) (30, 32). Because $\beta$2AR TYY does not stimulate a cAMP response, it would be predicted not to undergo the feedback PKA phosphorylation that occurs in the $\beta$2AR. To test this, we employed a commercially available PKA site-specific antibody that recognizes phosphoserines (Ser-345 and Ser-346) and analyzed receptor immunoprecipitates for phosphorylation in a Western blot (Fig. 4D). A basal level of phosphorylation is detected for the $\beta$2AR in the absence of isoproterenol stimulation (lane 3 in the upper panel, Fig. 4D), and a 5-min agonist treatment leads to a marked increase in phosphorylation. In contrast, both basal- and agonist-induced phosphorylations are absent in the $\beta$2AR TYY samples (lanes 5 and 6 in the upper panel, Fig. 4D). Both the $\beta$2AR and $\beta$2AR TYY immunoprecipitates contained equal amount of receptor protein as detected by a $\beta$2AR-specific antibody (lower panel, Fig. 4D). These data confirm that $\beta$2AR TYY does not provoke feedback phosphorylation by PKA.

FIGURE 5. $\beta$-arrestin recruitment to the $\beta$2AR TYY. A, HEK-293 cells stably expressing $\beta$2AR, $\beta$2AR TYY, or $\beta$2AR GRK,PKA were transiently transfected with $\beta$-arrestin2-GFP. Displayed panels represent cells fixed after 15 min of 1 $\mu$m isoproterenol treatment. $\beta$-Arrestin was evenly distributed in the cytosol before stimulation (not shown). Identical results were obtained in four experiments. B, cells stably expressing the $\beta$2AR or $\beta$2AR TYY were stimulated with 10 $\mu$m isoproterenol for 5 min and FLAG receptors immunoprecipitated after chemical cross-linking with DSP. The IP was probed with a $\beta$-arrestin antibody (upper panel) and a FLAG M2 monoclonal antibody (lower panel). The bar graph represents quantification of $\beta$-arrestin in the IP from three independent experiments. p = 0.002 according to paired t test. C, COS-7 cells were transiently transfected with FLAG-$\beta$2AR or FLAG-$\beta$2AR TYY with or without $\beta$-arrestin2. After serum starvation, cells were treated with 10 $\mu$m isoproterenol for 30 min at 37 °C. Cell-surface receptors before and after agonist treatment were determined by Flow cytometry. Data represent the mean ± S.E. of 3–5 independent experiments done in triplicate. #, p = 0.008, WT versus WT + $\beta$-arrestin2, ***, p < 0.0001; TYY, TYY + $\beta$-arrestin2 according to unpaired t test.
β-Arrestins and Adrenergic Signaling

β-Arrestin Binds and Functions as an Endocytic Adaptor for β2AR\textsuperscript{TYY}

To determine if β-arrestin can interact with β2AR\textsuperscript{TYY}, we utilized confocal microscopy to visualize the translocation of β-arrestin2-GFP to agonist-activated receptors. HEK-293 cells stably expressing ~2 pmol of either β2AR or β2AR\textsuperscript{TYY} receptors were transiently transfected with β-arrestin2-GFP. Prior to isoproterenol stimulation, β-arrestin is distributed uniformly in the cytosol (not shown). In cells expressing β2AR\textsuperscript{TYY} less robust plasma membrane translocation was observed upon isoproterenol stimulation in comparison to cells harboring the β2AR (Fig. 5A, first two panels). In contrast, β-arrestin2 recruitment to β2AR\textsuperscript{TYY} was more pronounced when compared with cells stably expressing a phosphorylation-defective β2AR mutant lacking all phosphorylation sites, which has virtually no β-arrestin binding properties (Fig. 5A, last panel).

We also determined the association of endogenous β-arrestins with the stably expressed receptors (β2AR or β2AR\textsuperscript{TYY}) by immunoprecipitation assays performed in the presence of chemical cross-linkers (Fig. 5B). Detection of β-arrestins utilized an antibody that recognizes both β-arrestin isoforms. In these assays, no β-arrestin binding was observed prior to agonist treatment. Upon isoproterenol stimulation, robust β-arrestin2 and weak β-arrestin1 recruitment was seen for both β2AR and β2AR\textsuperscript{TYY}. However, much less β-arrestin was bound to β2AR\textsuperscript{TYY} amounting to ~23% of the levels communoprecipitated with β2ARs (Fig. 5B).

It has been reported that β2AR internalizes poorly in COS-7 cells, which express very low levels of endogenous β-arrestin. Furthermore, exogenous expression of β-arrestin2 has been shown to enhance the isoproterenol-induced internalization of β2AR in COS-7 cells (33). As seen in Fig. 5C, FLAG epitope-tagged β2AR\textsuperscript{TYY} internalized to the same extent (~10%) as the FLAG-β2AR in COS-7 cells as measured by the disappearance of cell-surface receptors after a 30-min isoproterenol treatment. Additionally, expression of β-arrestin2 increased the internalization of the WT as well as the mutant by ~3-fold (Fig. 5C). These data suggest that the mutant receptor utilizes β-arrestin-dependent endocytic mechanisms similar to the WT receptor.

ERK Activation by β2AR and β2AR\textsuperscript{TYY}

We next evaluated whether β2AR\textsuperscript{TYY}, which does not stimulate cAMP accumulation, could nonetheless stimulate cellular ERK1/2. We treated untransfected HEK-293 cells (endogenous WT receptors) and either WT or TYY stable expressers (~2 pmol of receptors/mg) with a range of isoproterenol concentrations for 5 min and analyzed whole cell lysates for pERK and ERK1/2 content (Fig. 6, A and B). Even at 10 pm isoproterenol, ERK activation was detected in the β2AR stable cells. Peak activation was reached at ~10–20 nM isoproterenol. On the other hand, 1 nM isoproterenol could only weakly activate ERK via the endogenous and β2AR\textsuperscript{TYY} receptors. Peak activity for the endogenous receptors was reached at 100 nM isoproterenol. However, at this agonist concentration, β2AR\textsuperscript{TYY}-mediated stimulation of pERK was much greater than that mediated by the endogenous receptors. At maximal agonist concentration, β2AR\textsuperscript{TYY} (10 μM)-mediated ERK1/2 activation was 3- to 4-fold more than what was induced by the endogenous receptors and was essentially equivalent to that observed by the β2AR. Thus, β2AR\textsuperscript{TYY} can initiate robust signals via the effector pERK in the absence of any second messenger generation.

To further validate our findings with the β2AR\textsuperscript{TYY} that ERK activation can proceed in the absence of Gs coupling, we tested Gs null mouse embryonic fibroblasts for isoproterenol-stimulated pERK. Isoproterenol treatment of these cells did not yield any detectable cAMP accumulation (data not shown) (17). A time course of pERK stimulated in these cells by 10 μM isoproterenol is shown in Fig. 7A (left panel). The ERK activity has a slow onset and peaks ~10 min and returns to basal levels by 30 min. This pERK is insensitive to pertussis toxin treatment. Accordingly, the ERK activation by endogenous βARs in these cells, which lack Grα, does not proceed via Gs. As a comparison, a time course of ERK activation in the presence and absence of pertussis toxin was determined in wild-type MEF cells (Fig. 7A, right panel). The pattern of

![Figure 6. Isoproterenol-stimulated pERK in HEK-293 cells expressing WT, TYY, or endogenous β2ARs.](image-url)

HEK-293 cells or clonal cells stably expressing β2AR or β2AR\textsuperscript{TYY} were treated with the indicated concentrations of isoproterenol for 5 min at 37 °C. Whole cell lysates were prepared and analyzed for pERK and ERK content by Western blot. Panel A represents the quantification of pERK bands by densitometry. pERK amount was normalized to the total ERK protein. All the points are represented as a percentage of maximal activity observed. Data represent mean ± S.E. of three independent experiments. Curve fitting was performed with the GraphPad PRISM software. Representative blots of pERK and ERK are shown in panel B.
ERK activation is reminiscent of what is seen in HEK-293 cells, such that peak activity occurs between 2 and 5 min. Furthermore just as observed in HEK-293 cells, the pERK time course includes an early phase sensitive to pertussis toxin and a late phase that is not (compare with Fig. 2A). These results suggest that pertussis toxin-sensitive Gi-mediated ERK activation can occur only after $G_{i1}/H_{9251}$ coupling.

In the $G_4$ null cells a normal "Class A" pattern of β-arrestin2-GFP recruitment was observed with isoproterenol stimulation (Fig. 7B). Iso-
restin depletion on the β2AR-mediated pERK in Gs null cells. The lower temperature required for culturing these cells (33 °C) (17) probably prevented efficient siRNA uptake. On the other hand, we could demonstrate that the Gs-independent pERK generated by the β2AR TYY was in fact β-arrestin-dependent (see below).

pERK Stimulated by β2AR TYY Is β-Arrestin-dependent—To determine if the ERK1/2 activation elicited by β2AR TYY is transduced by β-arrestin proteins, we next examined the effect of β-arrestin depletion by RNA interference on the β2AR TYY-mediated ERK response (Fig. 8, A and C). HEK-293 cells stably expressing β2AR TYY (2 pmol/mg) were transfected with control, β-arrestin1, or β-arrestin2 siRNA. Under control conditions, after isoproterenol stimulation, peak activity occurred at 5 min and decreased to 20% of maximal levels at 30 min. β-Arrestin1 as well as β-arrestin2 siRNA dramatically decreased the 5-min signal to 15–18% and completely abolished the late activity. The reductions in pERK levels in the presence of β-arrestin siRNA correlate with similar amounts of reduction in β-arrestin levels (Fig. 8B). These data indicate that ERK activation stimulated by the β2AR TYY is mediated largely by β-arrestin isoforms.

The data presented in Figs. 1 and 3 demonstrate that ERK activity stimulated by the β2AR can be resolved into components mediated by either Gi/PKA or β-arrestins 1 and 2, sensitive, respectively, to H-89 and β-arrestin siRNA. Because β2AR TYY is uncoupled from Gi, it would be expected that its ability to activate ERK1/2 might be mediated exclusively by β-arrestins. To confirm this we tested the sensitivity of β2AR TYY-activated ERK to H-89 (Fig. 9, A and B). These experiments are complicated by the fact that activity observed with β2AR TYY-expressing cells also contains a component due to the endogenous β2AR. As seen in Fig. 9, after isoproterenol stimulation of β2AR TYY-expressing cells, only ERK activity stimulated in the first few minutes is sensitive to H-89. From 10 min on, the activity is completely resistant to H-89. This early H-89-sensitive activity is likely due to PKA-dependent ERK stimulated via endogenous β2ARs, whereas the bulk of H-89-resistant activity is attributable to β2AR TYY. Thus in Fig. 9 the curve depicting pERK in the presence of control siRNA and H-89 is likely a representation of the β2AR TYY-stimulated activity. Consistent with this model, this H-89-resistant activity is eliminated by siRNA to either β-arrestin1 or β-arrestin2 (Fig. 9, A and B).

Role of GRKs in β2AR TYY Phosphorylation and Signaling—All the above findings are consistent with the formation of a receptor-β-arrestin complex for the β2AR TYY analogous to the β2AR leading to downstream ERK activation. To dissect the roles of GRK phosphorylation in β-arrestin recruitment to the β2AR TYY and regulation of ERK signaling, we analyzed effects of coexpression of different GRK isoforms on β2AR TYY phosphorylation, β-arrestin recruitment, and ERK activation.

We first determined the agonist-induced phosphorylation of the β2AR and β2AR TYY by 32P metabolic labeling of HEK-293 cells stably expressing the receptors at ~2 pmol/mg of cellular protein. As seen in Fig. 10 (A and B), β2AR TYY is phosphorylated to ~20% of WT levels as determined by quantification of autoradiographs by PhosphorImager. β2AR TYY phosphorylation was not augmented by GRK2 coexpression (Fig. 10, A and B). This is not surprising, because β2AR TYY does not couple to G proteins and thereby release G protein βγ subunits that are known to play a major role in GRK2 membrane targeting. On the other hand, expression of either GRK2 with a membrane-tethering prenylation signal (CAAX) at the carboxyl terminus, or GRK5 that is constitutively localized to the plasma membrane, resulted in a doubling of β2AR TYY phosphorylation (Fig. 10, A and B).

We also determined β2AR TYY phosphorylation on specific GRK phosphorylation sites (Ser-355 and Ser-356) with commercially available antibodies directed specifically against these phospho-serines (35). As shown in Fig. 10C (upper left panel), a weak phosphorylation signal is detected in immunoprecipitates of β2AR TYY upon agonist stimulation. Coexpression of GRK5 or GRK6 but not GRK2 enhances β2AR TYY phosphorylation at these serine residues. However, in the presence of lower concentrations of agonist (100 nM isoproterenol), only GRK6 could augment β2AR TYY phosphorylation at these sites (data not shown). A robust phosphorylation signal was observed in response to agonist in the β2AR immunoprecipitates, and the signal changed minimally with coexpression of different GRK isoforms (Fig. 10C, upper right panel). These data suggest that β2AR TYY is preferentially phosphorylated by GRK 5/6 isoforms in HEK-293 cells upon agonist treatment.

As demonstrated above, β2AR TYY phosphorylation can be augmented by GRK5/6 isoforms but not by GRK2. To assess the relative effects of GRK phosphorylation on subsequent β-arrestin recruitment to the β2AR TYY, we expressed different GRK isoforms in HEK-293 cells
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**β-Arrestins and Adrenergic Signaling**

![Image](https://example.com/image.png)

**FIGURE 10. Phosphorylation of β2AR**

HEK-293 cells stably expressing β2AR**

were transiently transfected with pCDNA3, or the indicated GRK plasmids. Cells were metabolically labeled with 32P, and stimulated for 5 min with 10 μM isoproterenol. β2AR**

and β2AR were immunoprecipitated and separated on SDS-PAGE. Panel A shows a representative autoradiograph. The bar graphs in B are a quantification of receptor phosphorylation shown as a percentage of maximal phosphorylation (WT + Iso = 100%) and represent mean ± S.E. from 3–5 independent experiments. ***, p = 0.002 Mock (−) versus CAAX (+), p = 0.005 Mock (−), versus GRK5 (+) as analyzed by an unpaired t test. C, cells stably expressing FLAG-β2AR**

or FLAG-β2AR were transiently transfected with pCDNA3 or different GRK plasmids and stimulated with 10 μM isoproterenol for 5 min. FLAG immunoprecipitates were probed with a phosphoserine antibody specific for series 355 and 356 in the carboxyl tail of the β2AR (upper panel). The same blots were stripped and reprobed with a FLAG-M2 antibody to detect receptor levels (middle panel). The lowest lanes are lysate blots for detecting the expression of transfected GRK isoforms. For this a mixture of monoclonal antibodies that recognize GRK2/3 and GRK4/5/6 was used (18). The blots are representative of identical results from four independent experiments.

Stably expressing the β2AR**

immunoprecipitated the receptors after chemical cross-linking and determined the amount of bound endogenous β-arrestins by Western blotting. Expression of GRK2 did not cause any increase in β-arrestin binding beyond that observed under mock conditions, upon 5 min of isoproterenol stimulation (Fig. 11A). In contrast, both GRK5 and GRK6 markedly enhanced the recruitment of β-arrestin to the β2AR**

(Fig. 11, A and B). In the presence of GRK5 or GRK6, β-arrestin binding to the receptor increased by at least 2-fold (Fig. 11B). Again, these results closely correlate with the increase in β2AR**

phosphorylation observed after the expression of these GRKs (Fig. 10C). In all cases, no further increase in β-arrestin recruitment was seen at longer times of agonist stimulation (data not shown).

To evaluate if GRK expression altered the translocation patterns of β-arrestin, we performed confocal microscopy by transiently expressing β-arrestin-2-GFP along with GRKs in HEK-293 cells stably expressing either the β2AR or β2AR**

receptors. For the β2AR, GRK2 expression caused greater cytosolic “clearance” of GFP fluorescence and formation of brighter puncta at the plasma membrane after 20 min of 1 μM isoproterenol (Fig. 11C, compare first and second upper panels). Quite unexpectedly, GRK5 or GRK6 led to detection of β-arrestin-GFP in vesicles, indicating a stable Class B-type interaction between the internalized receptor and β-arrestin (Fig. 11C, top row, third and fourth panels). In some experiments, basal recruitment of β-arrestin was observed at the plasma membrane with GRK5/6 but not GRK2 (data not shown). In the case of β2AR**

GRK2 overexpression did not increase the efficiency of β-arrestin translocation. Similar to the WT receptor, GRK5 and -6 promoted the receptor-driven accumulation of β-arrestin in intracellular vesicles, although less robustly than in the case of the WT receptor (Fig. 11C, bottom row, third and fourth panels).

To determine if the enhancing effects of GRK5 and -6 on receptor trafficking and on β-arrestin recruitment to the β2AR**

are associated with increased ERK1/2 activation, we treated cells stably expressing the β2AR**

(1 pmol/mg) with 1 μM isoproterenol for different times and analyzed the cellular lysates by Western blotting for pERK1/2. β2AR**

-stimulated pERK peaked at 5 min of agonist just as for the WT receptor. However, the signal was more sustained and remained fairly stable for 20 min. pERK1/2 levels returned to basal beyond 40 min (data not shown). GRK2 overexpression did not cause significant changes in the pERK activation by β2AR**

(Fig. 12, A and B). In contrast, both GRK5 and -6 significantly augmented the activity especially at earlier time points (Fig. 12, A and C). Additionally, GRK5 also markedly enhanced ERK activation beyond 5 min (Fig. 12, A and C). These data indicate that GRK5 and -6 enhance not only β-arrestin recruitment to β2AR**

and its trafficking but also its ability to activate ERK1/2.

**DISCUSSION**

Our results document that isoproterenol stimulation of the β2AR can induce pERK signals in HEK-293 cells by at least two separate pathways: (i) H-89-sensitive, PKA-dependent G protein-mediated and (ii) H-89-insensitive, PKA-independent β-arrestin-dependent. Furthermore, the H-89-insensitive signals are also unaffected by pertussis toxin indicating that the late ERK activity is independent of Gs/Gi switching. In essence, the G protein-
dependent signals display an early and transient response, whereas the 
β-arrestin-dependent signals are late and sustained. Evidently, the 
β2AR can bind β-arrestin and stimulate pertussis toxin-insensitive ERK in the 
complete absence of cognate Gαi proteins in Gs null fibroblasts. When the 
β2AR is uncoupled from Gs by mutagenesis, it can still signal to ERK in 
response to isoproterenol in an efficient and β-arrestin-dependent manner.

β-Arrestin-dependent ERK1/2 activation is a recently appreciated 
mechanism of signal transduction elicited by 7TMRs (5). For the AT1aR 
and the V2R, this function is exclusively carried out by the 
β-arrestin2 isoform while the 
β-arrestin1 isoform plays an inhibitory role (15, 36). On the other hand, both 
β-arrestin1 and β-arrestin2 are crucial for 
protease-activated receptor type 2-stimulated ERK activation (37, 38). 
Furthermore, it has been shown for the AT1aR, utilizing either a mutant 
receptor or a mutant agonist peptide, that β-arrestin2-dependent 
pERK1/2 signals are stably generated in the absence of G protein 
coupling (12). These data indicate that the adaptor protein β-arrestin2 not 
only scaffolds MAPK components but also functions as an indispensa-
ble signal transducer in response to 7TMR activation.

Both the AT1aR and the V2R are members of a class of receptors that 
display stable interaction with β-arrestins leading to cointernalization 
of receptor-arrestin complexes to be localized on endosomes (16). For 
these receptors as well as for the protease-activated receptor type 2 and 
the Neurokinin1 receptors, β-arrestin has been shown to stably associ-
ate with pERK upon receptor activation (39 – 41). Hence, it may not be 
surprising that β-arrestin can act as an essential intermediate in the ERK 
activation pathway elicited by these receptors. On the other hand, we 
now show that the β2AR, which interacts only transiently with 
β-arrestin-GFP at the plasma membrane and does not form stable receptor-
β-arrestin-GFP complexes on endosomes, can nevertheless lead to ERK 
activation in a β-arrestin-dependent manner. Additionally, β2ARs 
require both β-arrestin1 and -2 for efficient ERK activation, because 
knockdown of either β-arrestin isoform leads to significant inhibition of 
isoproterenol-stimulated pERK. In contrast, both AT1aR and V2R spe-
cifically use β-arrestin2 as the signaling intermediate for the ERK path-
way (15, 36). It remains to be determined whether β-arrestin1 and -2 act 
sequentially or simultaneously in the isoproterenol-dependent ERK

FIGURE 11. Effect of GRK coexpression on β-arrestin recruitment by β2ARTYY. A, HEK-293 cells stably expressing β2ARTYY were transiently transfected with vector or the indicated 
GRK plasmids, stimulated with 10 μM isoproterenol, and the receptor immunoprecipitated after chemical cross-linking with DSP. An anti-β-arrestin antibody was used to detect 
derogenous β-arrestin1 and -2 in the IP and lysates. Shown are representative blots from one of four independent experiments. B, β-arrestin bands in the receptor immunoprecipi-
tates were quantified and plotted as a percentage of maximal signal. *, p < 0.01 (mock versus GRK5; mock versus GRK6) according to one-way ANOVA, Tukey's Multiple Comparison 
Test. C, confocal images show recruitment of β-arrestin2-GFP to β2AR and β2ARTYY without and with co-expression of indicated GRKs. Images represent fixed cells after 20 min of 1 
μM isoproterenol treatment. These data correspond to one of 4 independent experiments performed with similar results.
activation. Alternatively, heterodimerization of both β-arrestin1 and -2 may be necessary for ERK activation. An interesting question remains as to how transient complex formation between a class A receptor and β-arrestin can still induce the longer β-arrestin-dependent ERK activity demonstrated in this report for the β2AR and by Gesty-Palmer et al. (42) for LPA receptors. One possibility is that there is a continuous reformation of these receptor-β-arrestin complexes at steady state, even though they are short-lived.

For both the AT1αR and the V2R, β-arrestin2-dependent ERK activation has been shown to be insensitive to inhibitors of second messenger-dependent kinases (12, 15). Similarly, β-arrestin-dependent pERK stimulated by the β2AR is completely insensitive to the PKA inhibitor, H-89 (Figs. 3D and 9). H-89 has also been reported to act as a β receptor blocker and as an activator of cAMP in alternate systems (43, 44). In our assays, we do find that H-89 displays antagonistic properties at lower (<0.5 μM) isoproterenol concentrations at the 5-min time point (data not shown). On the other hand, H-89 did not lead to any cAMP generation in our assays.

As demonstrated by the experiments performed with β2AR<sup>TTY</sup>, isoproterenol-stimulated β-arrestin-dependent ERK1/2 activation can proceed in the total absence of G protein activation or cAMP generation. This mutant is not phosphorylated by PKA and hence is not coupled to G<sub>i</sub>. Nonetheless, β2AR<sup>TTY</sup> does recruit β-arrestin upon agonist stimulation and activates pERK in a more sustained manner than the pERK generated by the wild-type β2AR (compare the 10-min time point of Figs. 1, 3, 8, and 9). It is possible that, in the case of WT receptor, the G-protein-dependent pathway exerts a suppressive effect on the β-arrestin-dependent pathway. Hence, in the absence of such suppressive mechanisms, we are able to detect persistent ERK activation by the β2AR<sup>TTY</sup>.

The activity of the β2AR<sup>TTY</sup> mutant raises several structural issues. First, three simultaneous point mutations at Thr-68, Tyr-132, and Tyr-219 eliminate the G protein binding site but apparently preserve the sites for GRK5/6 and β-arrestin binding. One possibility is that these mutations directly disturb G protein binding at the transmembrane-cyttoplasmic boundary but leave the ligand-dependent conformational switch intact so that binding sites in the cytoplasmic part of the loops are also intact. In addition, the G protein interaction could also be disrupted allosterically. In that case, the activated β2AR<sup>TTY</sup> cytoplasmic loops would be in an intermediate state sufficient for some, but not all, interactions. Second, the rational, ET-based design of the β2AR<sup>TTY</sup> has implications for the engineering of other 7TMs and proteins. Here, the choice of residues to target for mutation was based on an ET analysis of diverse rhodopsin-like receptors to identify functionally important residues common to all. The choice of which side chains to mutate aimed to exclude known substitutions at cognate residues among the same diverse selection of 7TMs. Neither strategy is specific to the adrenergic receptor. Future studies will test whether cognate mutations in other rhodopsin-like 7TMs will produce similar results.

A striking feature of β2AR<sup>TTY</sup> is the apparent paradox of weak receptor phosphorylation, combined with moderate β-arrestin recruitment and the robust ERK activation. How can a receptor that binds relatively little β-arrestin still robustly engage a β-arrestin-dependent signaling pathway? Several prior and current observations serve to explain these phenomena. Recent RNA interference studies show that GRK2 mediates most of the phosphorylation and desensitization of 7TMs. At least a decade of research demonstrates that agonist-induced β2AR phosphorylation is carried out by GRK2 and is facilitated by G protein β<sub>y</sub> subunits under both in vivo and in vitro conditions. Moreover, membrane targeting of GRK2/3 requires their binding to the G protein β<sub>y</sub> subunits (45–50). As seen in Fig. 10, GRK2 coexpression does not augment β2AR<sup>TTY</sup> phosphorylation, but the membrane targeted GRK2-CAAX does so. Taken together, we conclude that, due to the absence of activated β<sub>y</sub> subunits, GRK2 is unable to phosphorylate the β2AR<sup>TTY</sup>.

What are the consequences of this? First, as also shown for the AT1αR and V2R, in the absence of GRK2 phosphorylation, a dramatic reduction is observed in the amount of β-arrestin recruited to the β2AR (Fig. 5). For the AT1αR, it was also demonstrated that depletion of GRK2, actually increased, whereas overexpression of GRK2 decreased, β-arrestin-dependent ERK activation (13). Overall, β-arrestin recruited to GRK2 phosphorylated receptors appears not to be conformationally competent for engaging the ERK cascade (13). Consequently, the absence of GRK2-mediated phosphorylation should actually facilitate β-arrestin-dependent ERK activation by the β2AR<sup>TTY</sup>.

As seen in our <sup>32</sup>P metabolic labeling experiments, β2AR<sup>TTY</sup> is nonetheless phosphorylated to ~20% compared with the WT receptors. This phosphorylation is presumably mediated by the plasma membrane-associated GRK5/6 enzymes. Previous work utilizing inactivating monoclonal antibodies for GRK5/6 and recent RNA interference data indicate that in the
case of several receptors, these enzymes are responsible for ~20% of agonist-induced receptor phosphorylation (15, 18, 51). Furthermore, GRK5/6 expression is crucial for β-arrestin-dependent ERK activation by both the AT1A and the V2R (13, 15). Consistent with these findings, we find that both GRK5 and -6 but not GRK2 augment β2AR\(^{TTY}\) phosphorylation, β-arrestin recruitment, and β-arrestin-dependent ERK activation (Figs. 10–12). Future studies utilizing siRNAs directed against various GRK isoforms should help to decipher the exact contribution of isoform specific phosphorylation of the β2AR and β2AR\(^{TTY}\) in receptor desensitization, β-arrestin binding, and ERK activation. An interesting and unexpected finding was the conversion of a Class A trafficking pattern of the β2AR and β2AR\(^{TTY}\) (β-arrestin at the plasma membrane) to a Class B type (β-arrestin in endosomes) upon coexpression of GRK5/6 but not GRK2. Additionally, overexpression of GRK5 and 6 but not GRK2 augments isoproterenol stimulated ERK activation by the β2AR\(^{TTY}\) especially beyond the 5-min time point when most of the signal is β-arrestin-dependent. Although GRK2 can improve both receptor phosphorylation and β-arrestin recruitment to the β2AR, GRK5/6 serve to create a stable receptor-β-arrestin complex. These data are consistent with the hypothesis of a "barcode" resulting from the phosphorylation of unique residues on the receptor by a specific GRK isoform. Thus, it is proposed that GRK2 phosphorylates a set of preferred residues on a 7TMR such as the AT1A. When β-arrestin binds to a receptor so phosphorylated, it likely undergoes a conformational change facilitating interaction with the endocytic but not the signal transduction machinery. In contrast, GRK5/6 phosphorylate unique residues on the receptor, which now impart signaling functions to the bound β-arrestin. Details of what molecular mechanisms regulate these phosphorylation events and specify which kinases phosphorylate a particular receptor in a particular situation remain to be determined. Previous studies have shown that β2ARs utilize several G protein-dependent pathways to activate ERK in a wide variety of cells, including HEK-293 cells and cardiac myocytes (2, 30, 31, 52). The current study clearly demonstrates the existence of G protein-independent, β-arrestin-dependent ERK activation stimulated by the β2AR in HEK-293 cells. Future studies aimed at identifying specific agonists that activate only the β-arrestin-dependent pathway and the characterization of β2AR\(^{TTY}\) "knock-in" mice should facilitate a greater understanding of the physiological significance of this newly identified pathway. Acknowledgments—We thank Donna Addison and Elizabeth Hall for excellent secretarial assistance.

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