Accelerated Dephosphorylation of the β_2-Adrenergic Receptor by Mutation of the C-Terminal Lysines: Effects on Ubiquitination, Intracellular Trafficking, and Degradation†

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ABSTRACT: Agonist-mediated ubiquitination regulates some G protein-coupled receptors by targeting them to lysosomes for degradation. Phosphorylation also regulates receptor endocytosis and trafficking to lysosomes. To explore the roles of the two post-translational modifications, we mutated the three C-terminal lysines to arginines in the human β_2-adrenergic receptor (β_2AR) (K348/372/375R). The level of agonist-mediated ubiquitination of the mutant (3K/R) was greatly reduced compared to that of wild-type (WT) β_2AR in whole cells and in cell-free assays. Downregulation of 3K/R also was attenuated compared to that of the WT, whereas internalization and recycling were more similar. During endocytosis, WT and 3K/R appeared in different vesicles and WT, but not 3K/R, was transported to lysosomes. Both were rapidly phosphorylated in agonist-stimulated cells, but upon agonist removal, the rate of dephosphorylation of 3K/R initially was ~5 times faster than that of WT. The increased rate also was observed in a cell-free, soluble assay and, thus, was not due to differences in receptor trafficking. Okadaic acid, a potent phosphatase inhibitor, reduced the level of dephosphorylation and increased the levels of lysosomal targeting and degradation of 3K/R. The reduced level of ubiquitination and rapid dephosphorylation of 3K/R appear to prevent it from being sorted to lysosomes in contrast to the phosphorylated and ubiquitinated WT β_2AR. Our findings indicate that both phosphorylation and ubiquitination are involved in the intracellular sorting of β_2AR between pathways of recycling to the plasma membrane and degradation in lysosomes, and that the rate of dephosphorylation may be another mechanism of regulating the sorting.

Phosphorylation of G protein-coupled receptors (GPCRs)[1] by second-messenger and GPCR kinases (GRKs) modulates receptor function, endocytosis, intracellular trafficking, and degradation (*1*−*4*). Some of the molecular mechanisms involved in the earlier processes such as desensitization and internalization have been identified. Phosphorylation of agonist-occupied receptors by GRKs promotes high-affinity binding of arrestins which uncouple the receptors from their G proteins and attenuate signaling. Arrestins also bind clathrin and adapter protein-2 and recruit many GPCRs to clathrin-coated pits where they are internalized. Less understood are the mechanisms for the intracellular sorting and degradation of GPCRs (*4*−*6*). Most models envision interactions between sorting motifs on the receptors and sorting proteins which target the receptors to different endosomal compartments and fates such as being recycled or degraded.

Recently, ubiquitination has been found to regulate several mammalian GPCRs by targeting them to lysosomes (*7*−*11;* reviewed in ref *12*). Ubiquitin, a 76-amino acid residue polypeptide, is conjugated to acceptor proteins in three enzymatic steps: activation of the terminal carboxyl group of ubiquitin (E1), transfer of the activated polypeptide to the conjugating enzyme (E2), and ligation of the ubiquitin to ε-amino groups of lysines on the acceptor protein (E3) (*13*). Originally, ubiquitination was identified as a mechanism for directing proteins to proteasomes for degradation (*13, 14*). Subsequent studies revealed that many plasma membrane receptors are sorted to lysosomes by ligand-induced ubiquitination (*14*−*16*).

A relationship between phosphorylation and ubiquitination in the intracellular sorting and trafficking of GPCRs has yet to be established (*17*). For the mammalian GPCRs studied so far, phosphorylation is required for ubiquitination and endocytosis, whereas ubiquitination is not required for either phosphorylation or endocytosis (*7, 8*). Recently, human β_1AR in contrast to β_2AR was shown not to undergo agonist-mediated ubiquitination (*18*) which may explain the resis-

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[1] Abbreviations: βAR, β-adrenergic receptor; DMEM, Dulbecco's modified essential medium; DPBS, Dulbecco's phosphate-buffered saline; DUB, deubiquitinating enzyme; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; GPCR, G protein-coupled receptor; GRK, GPCR kinase; GRP, GPCR phosphatase; ^{125}ICYP, [^{125}I]iodocyanopindolol; ISO, isoproterenol; PNS, postnuclear supernatant(s); PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; pNPP, *p*-nitrophenyl phosphate; SEM, standard error of the mean.

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tance of β_1AR to agonist-mediated degradation and downregulation (*19*). βAR chimeras in which the cytoplasmic termini are exchanged were used to establish that the β_2AR C-tail contains the major sites for ubiquitination (*18*). Thus, β_1AR with a β_2AR C-tail is ubiquitinated and degraded, whereas β_2AR with a β_1AR C-tail is not. Previously, it was found that the β_1AR C-tail undergoes less agonist-stimulated phosphorylation than the β_2AR C-tail does (*19, 20*), and β_1AR is more resistant to agonist-mediated internalization than β_2AR (see ref *20* and references cited therein). These findings suggested to us that a mutant β_2AR resistant to ubiquitination but still able to undergo phosphorylation and endocytosis may be useful in deciphering the relative roles of phosphorylation and ubiquitination in receptor trafficking.

In this study, we demonstrate that a mutant β_2AR with the three C-tail lysines replaced with arginines (K348/372/375R or 3K/R) is more resistant to agonist-mediated ubiquitination, sorting to lysosomes, and downregulation. Upon endocytosis, WT and mutant β_2ARs appeared in different populations of cytoplasmic vesicles and WT but not 3K/R appeared in lysosomes. Furthermore, while the C-tail GRK sites of both were rapidly phosphorylated in agonist-stimulated cells, the sites on 3K/R were more rapidly dephosphorylated than those on WT β_2AR, both in whole cells and under cell-free conditions. Inhibition of dephosphorylation by okadaic acid increased the levels of lysosomal targeting and degradation of 3K/R. Thus, both ubiquitination and phosphorylation have roles in the sorting of β_2AR between recycling and degradative pathways.

MATERIALS AND METHODS

Materials. (−)-Isoproterenol (ISO), (−)-propranolol, *N*-ethylmaleimide, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), and *p*-nitrophenol phosphate (pNPP) were from Sigma. Okadaic acid, biotinylated ubiquitin, and lactacystin were from BioMol. Protease inhibitor cocktail was from Roche Applied Science. Peptide *N*-glycosidase F and protein phosphatase 1 (PP1) were from New England Biolabs. PP2A and rabbit anti-PP1α IgG were from Upstate. Rabbit anti-β_2AR and -β_2AR pS(355/356) IgGs, goat anti-PP2A-Aα IgG, mouse anti-ubiquitin IgG_1, and protein A/G Plus agarose were from Santa Cruz Biotechnology. Unconjugated and Alexa Fluor 488-conjugated mouse anti-HA.11 IgG_1 were from Covance. HRP-conjugated streptavidin and IgGs were from Zymed. Cy3-conjugated donkey anti-mouse IgG was from Jackson ImmunoResearch. LysoTracker Red and ProLong were from Molecular Probes. Other reagents were obtained as described previously (*18*−*21*). Plasmid pcDNA3.1-HA-hβ_2AR (*19*) was used to generate mutants with either one (K348R), two (K372/375R), or three (K348/372/375R) lysines replaced with arginines by site-directed mutagenesis. All the mutants were sequenced.

Cell Culture and Transfection. HEK 293 cells from the American type Culture Collection were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Cells were transfected using LipofectAMINE Plus and selected with G418 to produce resistant, uncloned cultures that expressed fairly constant β_2AR levels (3.11 ± 0.22 and 2.55 ± 0.25 pmol/mg of protein, respectively, for WT and 3K/R HA-β_2AR) during the experimental period (*18*). All stably transfected cells were maintained in medium containing 0.2 mg/mL G418. For colocalization of receptors, cells stably expressing 3K/R HA-β_2AR were transiently transfected with pcDNA3.1-β_2AR-GFP (*20*) and used 24−36 h after transfection. Cells used for experiments were grown on polylysine-coated plastic culture ware or glass coverslips.

β_2AR Binding Assays. The assays for determining changes in surface and total cellular receptor levels have been described previously (*21*). For internalization of surface β_2AR, cells grown in 24-well plates were stimulated with 1 μM ISO in serum-free medium containing 25 mM Hepes at 37 °C for up to 30 min, washed twice with ice-cold Dulbecco's phosphate-buffered saline (DPBS), exposed to 5 nM (−)-[^{3}H]CGP-12177 at 4 °C for 1 h, washed as described above, and assayed for ^{3}H and protein. To assess β_2AR recycling, the cells were stimulated with ISO usually for 30 min, rapidly washed twice with warm medium, incubated for increasing times of up to 1 h, and then assayed for surface binding as described above. For downregulation of total β_2AR, cells grown in six-well plates were incubated with 10 μM ISO at 37 °C for up to 24 h, rinsed with ice-cold Ca^{2+}- and Mg^{2+}-free DPBS, and lysed in 1 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1 mM EGTA, and protease inhibitors at 4 °C (lysis buffer). Portions of the lysates were assayed for protein and total binding activity with 250 pM (−)-[^{125}I]iodocyanopindolol (^{125}ICYP) for 1 h at 37 °C. For both binding assays, 10 μM propranolol was used to define nonspecific binding.

β_2AR Binding Properties, Coupling to Adenylyl Cyclase, and Desensitization. The methods for determining the coupling efficiency for activation of adenylyl cyclase by agonist stimulation of β_2AR have been described previously (*22, 23*). Membranes were prepared from the cells, and portions (≤1 μg of membrane protein) were used in saturation and competition binding assays to determine the maximum receptor levels (B_{max}) and the K_d values for ^{125}ICYP and ISO. For the former, a range of 5−200 pM ^{125}ICYP was used, and at each concentration, the amount of nonspecific binding was determined. The values were calculated by fitting the data to a one-site hyperbolic curve. For the latter, the assays included 30 pM ^{125}ICYP, 100 μM GTP, and from 0.1 nM to 100 μM ISO. The values were calculated by fitting the data to a one-site competition curve. Membranes (5−10 μg) were assayed for adenylyl cyclase activity with six to eight concentrations of ISO, and EC_{50} and V_{max} for ISO were determined by fitting the data to a sigmoidal concentration−response curve (*22*). The parameters determined above were used to calculate the coupling efficiency of the mutant and WT β_2ARs by eq 1:

$$k_1/k_{-1} = (K_d - EC_{50})/(EC_{50}r) \quad (1)$$

where k_1 and k_{-1} are the rate constants for activation and inactivation of adenylyl cyclase, respectively, and r is B_{max}. The ratio of k_1/k_{-1} represents the coupling efficiency. Desensitization was assessed by eq 2:

$$(k_1r)_D/(k_1r)_N = (V_{max})_D(EC_{50})_N/(V_{max})_N(EC_{50})_D \quad (2)$$

The term $(k_1r)_D/(k_1r)_N$ is defined as the fractional activity remaining and is calculated from the EC_{50} and V_{max} values for ISO-stimulated adenylyl cyclase in membranes prepared from naïve (N) and desensitized (D) cells (1 μM ISO for 15 min).

Immunoprecipitations and Immunoblotting. Control and treated cells were washed and lysed in lysis buffer. When ubiquitinated β_2AR was being detected, the lysis buffer contained 10 mM *N*-ethylmaleimide to inhibit deubiquitinating enzymes (DUBs). The lysates were mixed with 5× RIPA buffer, gently rotated for 45 min at 4 °C, and centrifuged at 17000*g* for 20 min. Proteins were immunoprecipitated from the soluble extracts using protein A-agarose beads coated with anti-β_2AR or protein A/G Plus-agarose beads coated with anti-HA antibodies. The beads were rotated overnight at 4 °C, collected by centrifugation, washed four times with RIPA buffer, and heated in SDS sample buffer at 60 °C for 15 min. Proteins from the immunoprecipitates as well as lysates and soluble extracts were separated by SDS−PAGE, transferred to Immobilon-p (Millipore), and probed with HRP-conjugated streptavidin (1:10000) to detect biotinylated β_2AR, mouse anti-HA (1:5000) or anti-ubiquitin (1:2000), or rabbit anti-pS(355/356) (1:5000) or anti-β_2AR (1:80000) followed by HRP-conjugated goat anti-mouse or -rabbit IgG (1:10000), respectively. Proteins were detected by enhanced chemiluminescence and, following exposure to Bio-Max MR or Lite 2 film (Kodak), quantified by scanning densitometry and NIH Image J (*21*). Where indicated, blots probed with anti-pS(355/356) were stripped and probed with anti-β_2AR.

β_2AR Phosphorylation and Dephosphorylation Assays. Cells in six-well plates were stimulated with 1 μM ISO for different periods of time, washed, and lysed as described above except the buffer included phosphatase inhibitors (10 mM NaF, 20 mM sodium pyrophosphate, and 0.1 μM okadaic acid). Portions of the lysates were mixed with 5× solubilization buffer [1× = 0.25% dodecyl β-maltoside, 20 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 2 mM EGTA], gently rotated for 45 min, and centrifuged at 17000*g* for 20 min. The soluble supernatants (50 μg of protein) were treated with peptide *N*-glycosidase F (500 units) for 2 h at 37 °C (*22, 24*), diluted with 4× SDS sample buffer, heated for 15 min at 60 °C, and subjected to Western blotting (*21*) as described above. The deglycosylation step was omitted in later experiments.

For dephosphorylation, the cells were stimulated for 10 min with 1 μM ISO, rinsed once with warm medium, exposed to 10 μM propranolol for increasing amounts of time, and then processed as described above. For cell-free dephosphorylation (*25*), the stimulated cells were washed, scraped into phosphatase assay buffer [50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1% β-mercaptoethanol, and protease inhibitors], and homogenized with seven strokes in a Dounce homogenizer. Portions were incubated at 37 °C for up to 1 h, diluted with 4× SDS sample buffer, and analyzed for phosphorylated and total β_2AR by Western blotting as described above. For some experiments, the homogenates were centrifuged at 600*g* for 5 min to remove cell debris and nuclei. The postnuclear supernatants (PNS) were directly assayed or solubilized in 0.5% Triton X-100, centrifuged for clarification, and then assayed.

Dephosphorylation of Immunoprecipitated β_2ARs by Purified Phosphatases. β_2ARs were extracted in RIPA buffer from cells stimulated with 1 μM ISO for 10 min and immunoprecipitated with anti-HA antibody-coated protein A/G Plus-agarose beads. The receptors were eluted from the beads with 50 mM glycine and 100 mM NaCl (pH 2.5) which was neutralized with 1 M Tris-HCl (pH 8.0). Portions (16 fmol) of the eluted receptors were incubated at 30 °C in 24 μL reaction mixtures containing 50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1% β-ME, 1 mM PMSF, 0.1% NP-40, 40 μg/mL BSA, and 100 milliunits of PP2A or 50 mM Hepes (pH 7.0), 1 mM $MnCl_2$, 0.1 mM EDTA, 5 mM DTT, 1 mM PMSF, 0.025% Tween 20, and 100 milliunits of PP1. The reactions were stopped by adding 8 μL of 4× SDS sample buffer and the mixtures heated at 60 °C for 15 min. Portions of 12 and 2 fmol were analyzed by Western blotting for phosphorylated and total β_2AR, respectively.

Cell-Free Ubiquitination Assay. Portions of PNS (180 μg of cell protein) from cells stimulated with ISO for 5 min were incubated in 150 μL reaction mixtures containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.2 mM dithiothreitol, 10 mM ATP, 2 μM biotinylated ubiquitin, and 10 μM ISO for 60 min at 37 °C. Then, 40 μL of 5× RIPA buffer and 10 μL of 0.2 M *N*-ethylmaleimide were added. The samples were immunoprecipitated with anti-HA antibodies and blotted with HRP-conjugated streptavidin or anti-β_2AR antibodies as described above.

β_2AR Degradation Assay. A biotinylation method was used to assess basal and agonist-mediated β_2AR turnover as previously described (*19*). Briefly, cells grown in six-well plates were rinsed with ice-cold buffer B [40 mM $NaHCO_3$ and 100 mM NaCl (pH 8.6)], incubated with 1 mM sulfo-NHS-LC-biotin in buffer B at 4 °C for 30 min, and rinsed with ice-cold 20 mM glutamine in DPBS. Cells in some of the wells were lysed as described above and the rest warmed to 37 °C in fresh medium in the absence or presence of 0.2 μM okadaic acid for 30 min and 10 μM ISO for an additional 4 h. The lysates were extracted in RIPA buffer, and βARs were immunoprecipitated, detected with HRP-conjugated streptavidin, and quantified as described above.

Confocal Fluorescence Microscopy. Cells were visualized using a Zeiss LSM 510 laser confocal microscope as previously described with minor modifications (*20*). Briefly, cells expressing WT or mutant HA-β_2AR grown on glass coverslips were stained at 4 °C with Alexa Fluor 488-conjugated anti-HA antibody (1 μg/mL), washed with DPBS, and incubated at 37 °C in medium with or without 10 μM ISO for up to 4 h. When used, LysoTracker Red (75 nM) was added 30 min prior to the end of the ISO treatment. Cells were washed and fixed with 4% paraformaldehyde in PBS. The coverslips were mounted on slides with ProLong, and the cells were scanned. The scanned images were processed using Adobe Photoshop 7.0. For cells coexpressing WT β_2AR-GFP and 3K/R HA-β_2AR, the fixed cells were permeabilized and stained with anti-HA and then Cy3-conjugated donkey anti-mouse antibody (*20*).

Data Analysis. Unless otherwise indicated, each experiment was repeated at least three times and each data point within an experiment was determined in triplicate. Data were fitted to curves by nonlinear regression analysis and analyzed for statistical significance with a two-tailed *t* test using Prism 4 or 5 (GraphPad Software). Receptor phosphorylation was expressed directly by the ratio of densitometric units of phosphorylated to that of total receptors or by normalizing the ratios to the percentage of initial phosphorylation as indicated in the figures.

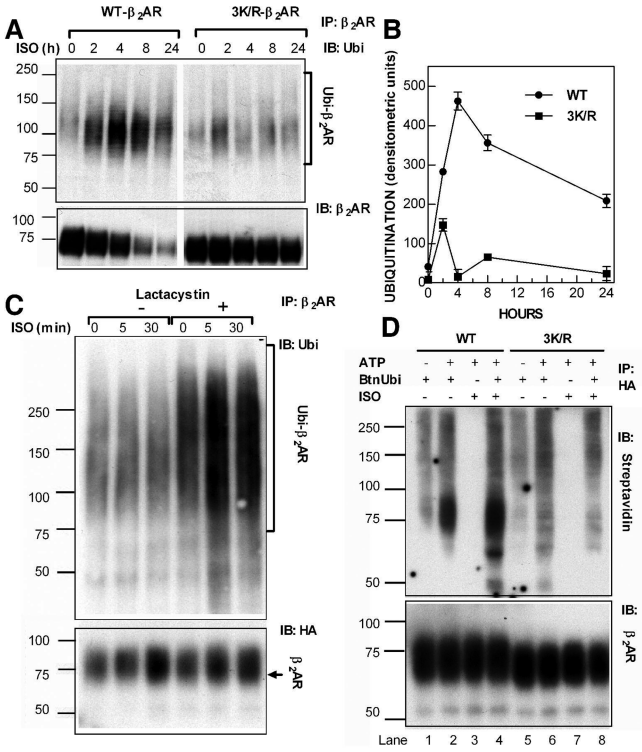


FIGURE 1: Agonist-stimulated ubiquitination of WT and mutant β_2ARs. (A) Western blots of ubiquitinated HA-tagged β_2ARs stably expressed in HEK 293 cells. Cells were stimulated at 37 °C with 10 μM ISO for the indicated times, washed, lysed, and solubilized in RIPA buffer as described in Materials and Methods. β_2ARs were immunoprecipitated using anti-β_2AR antibodies, and the immunoprecipitates were resolved by SDS−PAGE and detected by blotting with mouse monoclonal anti-ubiquitin (top panel) or anti-HA antibodies (bottom panel). The same amount of receptors from each control cell sample and the equivalent amount of protein from the corresponding stimulated cell samples were loaded on the gels. The blot shown is one of three similar blots from separate experiments. (B) Quantification of ubiquitination of WT (●) and 3K/R (■) β_2ARs by scanning densitometry of the blots. Values are expressed as arbitrary densitometric units and are the means ± SEM from three separate experiments. (C) Rapid ubiquitination of WT β_2AR. Details are the same as those described for panel A except cells were incubated in the absence and presence of 10 μM lactacystin for 30 min and then stimulated with 10 μM ISO for the indicated periods of time. (D) Cell-free ubiquitination of WT and 3K/R. Portions of PNS prepared from ISO-stimulated cells were incubated with ISO, ATP, and biotinylated ubiquitin as indicated. β_2AR was immunoprecipitated using anti-HA antibody and subjected to blotting with HRP-conjugated streptavidin (top panel) or anti-β_2AR antibodies (bottom panel). Shown is one of three similar blots.

RESULTS

Agonist-Mediated Ubiquitination and Downregulation of WT and Mutant β_2ARs. In a previous study, it was shown that β_2AR and a β_1AR−β_2AR C-tail chimera undergo agonist-stimulated ubiquitination whereas β_1AR and a β_2AR−β_1AR C-tail chimera do not (*18*). The human β_2AR has 16 lysines, three of which are located in the C-tail (K348, K372, and K375). We constructed the β_2AR mutant 3K/R (K348/372/375R) to investigate the role of the C-tail lysines in ubiquitination. HEK 293 cells stably expressing WT and mutant HA-β_2ARs were stimulated with ISO for increasing times, and the receptors were mmunoprecipitated with anti-β_2AR antibodies and probed with anti-ubiquitin antibodies (*18*). Agonist-stimulated ubiquitination of WT β_2AR was readily observed compared to that of 3K/R (Figure 1A). Quantification of the blots by scanning densitometry showed that the level of ubiquitination of WT β_2AR increased steadily for up to 4 h and then declined slowly (Figure 1B). A small peak of ubiquitinated 3K/R was observed at 2 h. On the basis of analysis by area under the curve, the level of ubiquitination of 3K/R was 16 ± 4.5% of that of WT. While we determined the level of ubiquitination of β_2AR over a 24 h period, it occurred very quickly and was enhanced by proteasomal inhibitors such as lactacystin (Figure 1C) as reported previously (*7, 18*). Although the mechanism is not known, it indicates that deubiquitination is constantly occurring.

We also succeeded in ubiquitinating β_2AR in a cell-free assay containing biotinylated ubiquitin, ATP, ISO, and PNS from cells stimulated with ISO for 5 min (Figure 1D). β_2AR from the reactions was immunoprecipitated with anti-HA antibody and detected on blots with HRP-conjugated streptavidin. PNS from unstimulated cells did not work (data not shown) which is consistent with ubiquitination requiring phosphorylated receptors (*7*). In the absence of ATP and ISO, a faint band was detected in the area where β_2AR is found (lanes 1 and 5). In the presence of ATP, the intensity of this band greatly increased in the samples containing WT β_2AR (lane 2) and even more in samples containing ATP and ISO (lane 4). In contrast, ATP had only a slight effect on the labeling of 3K/R (lane 6) which actually weakened in samples containing ATP and ISO (lane 8). No labeling was detected in the absence of biotinylated ubiquitin (lanes 3 and 7).

It is important to point out that in the experiments described above, only a small fraction of WT β_2AR was ubiquitinated based on the minimal reduction in unmodified receptors detected by anti-β_2AR antibodies (see the bottom panels of Figure 1C,D). In our previous study, we had to overexpose the blots to visualize the ubiquitinated receptors with anti-β_2AR antibodies (*18*). Similar results were obtained in another study on β_2AR (*7*) as well as other GPCRs (*9, 11*).

We also noticed that with time of agonist exposure, immune reactive WT receptor proteins disappeared more rapidly than the 3K/R mutant (Figure 1A, bottom panel). The differences in receptor loss were confirmed by determining the levels of receptor proteins and binding activity in cell lysates (Figure 2). In cells expressing WT β_2AR, prolonged agonist treatment decreased the number of binding sites by 40 ± 1.6% compared to only 12 ± 2.5% for 3K/R-expressing cells (Figure 2B,C). The loss of C-tail immune reactivity paralleled that of binding activity, and both occurred in WT-expressing cells after a distinct lag of 4 h. Due to the small changes in 3K/R-expressing cells, delays were not apparent (Figure 2C). In contrast, the disappearance of HA-tagged receptors was more rapid than loss of binding or the C-tails in WT- and 3K/R-expressing cells. Others had observed that during agonist-mediated downregulation of human HA-β_2AR in HEK cells, anti-HA immunofluorescence disappeared more rapidly than did binding activity (*26*). Loss of an N-terminal FLAG epitope from the delta opioid receptor expressed in HEK cells was 2.5-fold greater than loss of binding activity after agonist treatment for 3 h (*27*). Such results suggest that the N-terminus of a GPCR is more susceptible to proteolysis than the rest of the receptor. Taken together, our data support the thesis that the lysine

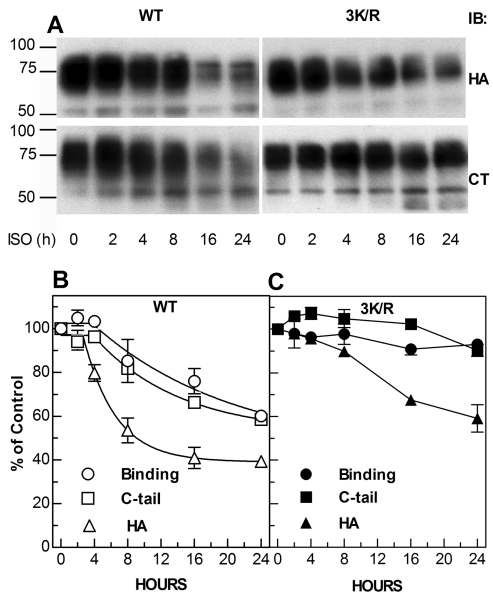


FIGURE 2: Agonist-mediated downregulation of WT and 3K/R receptor binding activity and proteins. Cells were incubated at 37 °C in the absence and presence of 10 μM ISO for the indicated times, washed, and lysed. Portions were assayed for total β_2AR binding sites using ^{125}ICYP or for receptor proteins by Western blotting with anti-HA and anti-β_2AR antibodies as described in Materials and Methods. The blots were quantified by scanning densitometry. (A) Western blots of receptor proteins from ISO-treated cells expressing WT or 3K/R. (B) Effect of agonist treatment on WT β_2AR binding sites (○) and receptor proteins detected by anti-C-tail (□) or anti-HA (△) antibodies. (C) Same as panel B except cells expressing 3K/R were used: binding sites (●), anti-C-tail (■), or anti-HA (▲). Values are the means ± SEM from four to six independent experiments.

Table 1: Properties of WT and 3K/R Mutant β_2ARs[a]

	WT (mean ± SEM)	3K/R (mean ± SEM)	*n*
naïve cells			
EC_{50} for ISO (nM)	0.647 ± 0.19	0.411 ± 0.091	4
V_{max} for ISO (pmol min^{-1} mg^{-1})	420 ± 39	347 ± 38	4
K_d for ^{125}ICYP (pM)	8.14 ± 0.80	8.50 ± 2.2	4
K_d for ISO (nM)	238 ± 21	215 ± 32	5
B_{max} (fmol/mg of protein)	6850 ± 562	6840 ± 792	4
coupling efficiency	0.070 ± 0.01	0.087 ± 0.021	4
desensitized cells			
shift in EC_{50} (*x*-fold)	9.0 ± 2.1	7.8 ± 1.7	4
V_{max} for ISO (% of control)	62.5 ± 9.5	59.3 ± 4.9	4
fractional activity remaining	0.092 ± 0.01	0.087 ± 0.02	4

[a] Membranes were prepared from naïve and agonist-stimulated (1 μM ISO for 15 min) cells and assayed for binding and adenylyl cyclase activity as described in Materials and Methods. The K_d and B_{max} values for ^{125}ICYP were determined by saturation binding. The K_d for ISO was measured by competition binding in the presence of 100 μM GTP. The EC_{50} and V_{max} values for ISO were determined by stimulation of adenylyl cyclase activity with increasing concentrations of ISO. For all properties, differences between WT and 3K/R receptors were not significant ($P > 0.05$; two-tailed *t* test).

residues in the cytoplasmic tail of β_2AR are the sites of ubiquitination involved in receptor degradation.

Properties of 3K/R Mutant β_2AR. We next showed that the mutations did not impair receptor functions such as ligand binding, adenylyl cyclase activation, and desensitization. The parameters for 3K/R were similar to those for WT β_2AR (Table 1 and Figure S1 of the Supporting Information). These included the binding constants or K_d values for ^{125}ICYP and ISO, the EC_{50} and V_{max} values for ISO-stimulated adenylyl cyclase activity, and the coupling

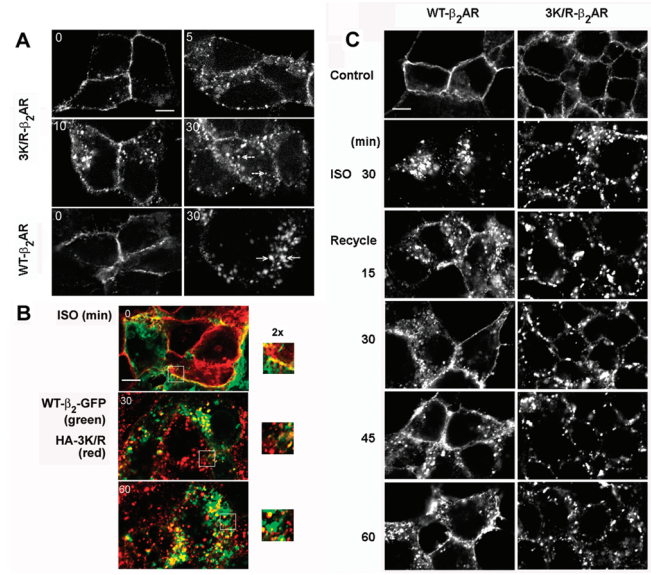


FIGURE 3: Visualization of agonist-stimulated internalization, trafficking, and recycling of WT β_2AR and the 3K/R mutant. (A) Divergence of intracellular trafficking of WT and 3K/R. HEK 293 cells stably expressing WT or 3K/R HA-β_2AR were stained at 4 °C with Alexa Fluor 488-conjugated mouse anti-HA antibody, washed, heated to 37 °C, and stimulated with 1 μM ISO for the indicated periods of time. The cells then were washed, fixed, and visualized by confocal fluorescence microscopy. Arrows point to vesicles containing WT (dashed arrows) and 3K/R (solid arrows). (B) Minimal colocalization of WT and 3K/R during intracellular trafficking. HEK 293 cells stably expressing 3K/R HA-β_2AR and transiently expressing WT β_2AR-GFP were stimulated with 1 μM ISO for the indicated periods of time. The cells were washed, fixed, permeabilized, and stained with mouse anti-HA antibody followed by Cy3-conjugated donkey anti-mouse IgG. The cells then were visualized for WT β_2AR-GFP (green), 3K/R HA-β_2AR (red), and colocalization (yellow). Selected areas were magnified 2-fold (2×). (C) Visualization of β_2AR recycling. Details are the same as those in panel A except after stimulation with 1 μM ISO for 30 min, the cells were washed, incubated in fresh medium for the indicated periods of time, fixed, and visualized. The bar is 5 μm. For each panel, images are from the same experiment and are representative of at least three separate experiments.

efficiencies calculated from these values. In addition, exposing the cells to 1 μM ISO for 15 min resulted in desensitization of 3K/R (Figure S1C,D of the Supporting Information) as evidenced by a 90% reduction in the fractional activity remaining and an almost 8-fold increase in the EC_{50} for ISO-stimulated activity which are similar to the changes observed for WT β_2AR (Table 1).

Endocytosis, Intracellular Trafficking, and Recycling of WT and Mutant β_2ARs. While β_2AR ubiquitination is not required for endocytosis, it is necessary for degradation (data above and ref *7*) and may affect the intracellular trafficking of the receptor. To explore this possibility, HEK 293 cells expressing HA-tagged β_2ARs were stained at 4 °C with Alexa Fluor 488-conjugated anti-HA antibody and incubated at 37 °C for increasing amounts of time with ISO (Figure 3A). Initially, most of the receptors were on the plasma membrane and, upon agonist stimulation, rapidly internalized. WT accumulated in larger, perinuclear vesicles as previously reported (*18, 20*), whereas 3K/R β_2AR appeared in small, peripheral, cytoplasmic vesicles that became more punctate and intense with time. We directly compare the distribution of the two receptors by transiently expressing WT β_2AR-GFP in cells stably expressing HA-3K/R (Figure 3B). We

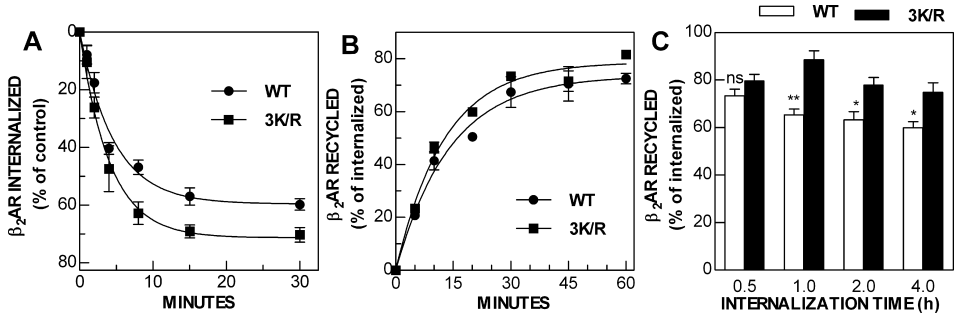


FIGURE 4: Quantification of agonist-stimulated internalization and recycling of WT β_2AR and the 3K/R mutant. (A) Time course of internalization. Cells stably expressing WT (●) or 3K/R (■) β_2AR were treated with 1 μM ISO at 37 °C for the indicated periods of time and assayed for cell surface β_2AR sites using [^{3}H]CGP12177 as described in Materials and Methods. Data represent the means ± SEM of three independent experiments. Maximum levels of internalization of WT and 3K/R were 59.6 ± 2.3 and 71.3 ± 3.0%, respectively, with rate constants (k) of 0.215 ± 0.024 and 0.245 ± 0.032, respectively. (B) Time course of recycling of internalized surface receptors. Cells stably expressing WT (●) or 3K/R (■) were stimulated with 1 μM ISO at 37 °C for 30 min, washed, incubated in fresh medium for increasing periods of time, and assayed for surface binding sites using [^{3}H]CGP12177. Data are expressed as the percent of internalized receptors that were recycled and are the means ± SEM of three independent experiments. The maximum levels of recycling of WT and 3K/R were 73.4 ± 2.8 and 79.6 ± 2.8%, respectively, with rate constants (k) of 0.072 ± 0.008 and 0.077 ± 0.008, respectively. (C) Effect of internalization time on the extent of recycling of internalized receptors. Cells stably expressing WT (white bars) or 3K/R (black bars) were stimulated with 1 μM ISO for the indicated periods of time, washed, incubated for 1 h in fresh medium, and assayed for surface receptors. Values are expressed as the percent of internalized receptors that were recycled and are the means ± SEM of three to five independent experiments. Significance determined by a two-tailed t test: ns, not significant; *, $P < 0.05$; **, $P < 0.01$.

observed some colocalization (yellow) of the immunostained HA-tagged mutant (red) and the GFP-tagged WT (green) at the plasma membrane. After agonist stimulation of the cells, however, both receptors mostly appeared in different vesicles by 30 min and the WT in larger aggregates by 60 min (see the 2× magnification images in Figure 3B). The trafficking of 3K/R is very reminiscent of that of β_1AR which does not undergo agonist-induced ubiquitination, appears in small, peripheral, punctate endosomes after endocytosis, and is resistant to downregulation (*18*−*20*). Using a radioligand binding assay, we quantified the agonist-mediated internalization of WT and 3K/R and found that the rates for both were very similar but 3K/R internalized more than WT (Figure 4A).

When the agonist was removed after stimulation for 30 min, the internalized β_2ARs recycled back to the plasma membrane (Figure 3C). The recycled 3K/R and to a lesser extent WT β_2AR appeared in punctate structures. This may be a consequence of the prebound anti-HA antibody aggregating during endocytosis and recycling. After internalization for 30 min, the recycling of WT and that of 3K/R β_2ARs were not significantly different (Figure 4B). When we increased the internalization time to 4 h, the level of recycling of WT decreased significantly with time from 73.3 ± 2.8 to 59.9 ± 2.6% ($\rho < 0.02$), whereas the level of recycling of 3K/R decreased from 79.6 ± 2.8 to 74.8 ± 4.0% which was not significant (Figure 4C).

To determine whether newly synthesized receptors were contributing to the recovery of surface receptors, we measured the rate of receptor synthesis (Figure S2 of the Supporting Information). The cells were treated with EEDQ which irreversibly inactivated β_2AR by >95% at 300 μM (panel A). The treated cells were washed and placed in fresh medium. After a 2 h delay, the number of binding sites began to increase at near-linear rates of 120 ± 6 and 114 ± 9 fmol h^{-1} (mg of protein)$^{-1}$ for WT and 3K/R, respectively (panel B). These values represent ~4% of the initial receptors, and thus, total recovery would require 25 h after the 2 h lag. In contrast, after agonist treatment for 2 h, 63.3 ± 3.5 and 77.9 ± 3.1% of the original surface receptors, respectively, were recovered during the 1 h recycling period. At most, the newly synthesized β_2AR represented less than 6% of the recycled receptors.

Effect of the 3K/R Mutations on Phosphorylation of β_2AR. As ubiquitination of β_2AR requires GRK-catalyzed phosphorylation (*7*), we compared the phosphorylation of the WT and 3K/R receptors using antibodies to phosphoserines 355 and 356 in the C-tail (*24*). Although the human β_2AR C-tail has 11 serine/threonine residues that are potential GRK sites, only serines 355, 356, 364, and 411 have been implicated in whole cell phosphorylation and receptor regulation studies (*22, 28, 29*). Mass spectrometric analysis indicated that agonist-mediated phosphorylation of the β_2AR C-tail occurs only in the proximal region (residues 339−369) (*30*). Vaughan et al. (*29*) showed that phosphorylation of the three serines in that sequence, 355, 356, and 364, is necessary for normal desensitization, arrestin binding, and internalization.

We confirmed the specificity of the anti-phosphoserine antibodies by comparing their reactivity to those of both glycosylated and deglycosylated β_2AR from control and agonist-stimulated cells (*22, 24*) with antibodies to the β_2AR C-terminal peptide (Figure S3 of the Supporting Information). The latter recognized glycosylated (64−91 kDa; lanes 1 and 2) and deglycosylated (45 and 42 kDa; lanes 3 and 4) forms of β_2AR from control and agonist-stimulated cells. Anti-pS(355,356) detected the same proteins but only from agonist-stimulated cells (lanes 6 and 8). The levels detected in control cells (lanes 5 and 7) ranged from 0 to 2% of those in agonist-stimulated cells. Agonist-stimulated phosphorylation of S(355,356) in both WT and 3K/R β_2ARs was very rapid and similar up to 2 min, after which the levels of phosphorylated WT remained stable even at 2 h and that of 3K/R decreased over the same time period (Figure 5).

Effect of the 3K/R Mutations on Dephosphorylation of β_2AR. The decrease in the level of phosphorylated 3K/R in the continued presence of agonist may be due to the increased level of dephosphorylation which is believed to be necessary for the recovery of phosphorylated and desensitized GPCRs. After dephosphorylation, the resensitized receptors are recycled back to the plasma membrane (*31*−*34*). β_2AR is

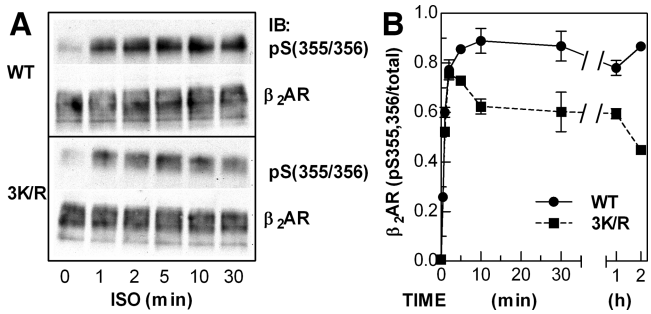


FIGURE 5: Time course of phosphorylation of GRK-specific serines 355 and 356 in WT and 3K/R β_2ARs. Cells stably expressing WT or 3K/R β_2AR were stimulated with 1 μM ISO for the indicated periods of time, washed, and lysed in ice-cold buffer containing protease and phosphatase inhibitors as described in Materials and Methods. The lysates were solubilized, resolved by SDS−PAGE, and immunoblotted with anti-pS(355,356) antibodies. The blots were stripped and probed with anti-β_2AR antibodies. (A) A representative blot is shown. (B) The blots were analyzed by scanning densitometry to quantify the phosphorylation of S(355,356) in WT (●) or 3K/R (■). Each data point represents the ratio of densitometric units of phosphorylated to that of total receptors. Values are the means ± SEM of three separate experiments.

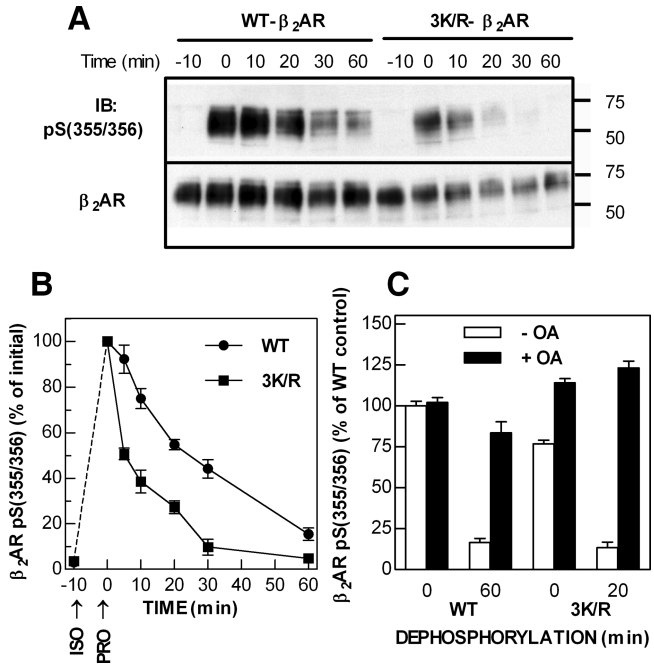


FIGURE 6: Differences in dephosphorylation of WT and 3K/R mutant β_2ARs. (A) Time course of dephosphorylation of pS(355,356) in β_2AR in intact cells. Cells were stimulated with 1 μM ISO for 10 min, washed, exposed to 10 μM propranolol (PRO) for the indicated periods of time, and lysed. Phosphorylated and total receptors were detected by Western blotting as described in the legend of Figure 5. A representative blot is shown. (B) Quantification of dephosphorylation of WT (●) and 3K/R (■) β_2ARs by scanning densitometry of the blots. Values are normalized for total receptors, are expressed as a percentage of initial phosphorylation (−−−), and are the means ± SEM of seven separate experiments. (C) Effect of a phosphatase inhibitor on dephosphorylation. Cells were incubated for 1 h in the absence (white bars) and presence (black bars) of 0.8 μM okadaic acid and stimulated for 10 min with ISO. Cells were either collected (0 min) or washed, exposed to propranolol with and without 0.8 μM okadaic acid for 60 (WT) or 20 (3K/R) min, and analyzed for phosphorylated and total receptors by Western blotting. Values are the means ± SEM of three separate experiments and are presented as a percentage of initial phosphorylation of WT.

reported to be dephosphorylated in acidic endosomes enriched in GRP, a PP2A-like GPCR phosphatase (*33*−*35*). Recently, however, dephosphorylation of β_2AR by a PP1-like phosphatase has been observed in the plasma membrane in the absence of internalization (*25, 36*). Thus, the distinct intracellular trafficking of the 3K/R mutant possibly may influence its dephosphorylation. The latter was determined by stimulating the cells with ISO for 10 min, washing them to remove the agonist, and incubating them for increasing periods of time in the presence of the antagonist propranolol. Phosphoserines 355 and 356 initially were dephosphorylated almost 5-fold faster in 3K/R than in WT β_2AR (Figure 6A,B).

Okadaic acid, a potent inhibitor of protein phosphatases PP2A and GRP (*33*) as well as PP1, blocked the dephosphorylation of WT and 3K/R (Figure 6C). While treatment with okadaic acid had no effect on the initial (0 min) level of phosphorylated WT, it increased the initial level of phosphorylated 3K/R to that of WT which is consistent with the dephosphorylation of 3K/R observed in continuously stimulated cells.

An Increased Level of Dephosphorylation of 3K/R Is Associated with the Receptor and Not Its Trafficking. The data so far are consistent with a model in which the divergent intracellular trafficking of WT β_2AR and the 3K/R mutant accounted for their different rates of dephosphorylation. We also considered the possibilities that replacing the C-terminal lysines with arginines causes a conformational change that makes the phosphoserines more susceptible to phosphatases or that the lysines are involved in regulating the dephosphorylation of the phosphoserines through either the conjugated ubiquitins or a scaffolding protein (*37*). To eliminate some of these possibilities, we performed cell-free dephosphorylation assays. Portions of homogenates or PNS from ISO-stimulated cells were incubated for increasing periods of time at 37 °C and then assayed for phosphorylated β_2AR. Cell-free dephosphorylation of 3K/R was more rapid than that of WT β_2AR (Figure 7A), and the initial increase in rate was similar to that observed in intact cells. Okadaic acid inhibited cell-free dephosphorylation of both receptors with similar potency [$IC_{50} \sim 50$ nM (Figure 7B)].

To rule out the possibility that during the 10 min period of agonist stimulation, the receptors become compartmentalized and remain so during the cell-free assay, we treated the PNS with Triton X-100 and used the soluble extracts for cell-free dephosphorylation (Figure 7C). Again, 3K/R was dephosphorylated more rapidly than WT β_2AR was. This experiment indicated that the disparity in dephosphorylation is due to differences in the receptors and not in their compartmentalization. As an increased rate of dephosphorylation may be due to changes in protein phosphatase activity, we compared control and agonist-stimulated cells for phosphatase activity and levels of PP1 and PP2A (Figure S4 of the Supporting Information). The total phosphatase activity was not significantly different in WT- and 3K/R-expressing cells with or without ISO stimulation (panel A). Western blotting with antibodies specific for PP1 or PP2A also revealed no major differences in their levels in control and stimulated cells (panels B−D). We directly compare the dephosphorylation of WT and 3K/R by PP1 and PP2A by immunoprecipitating phosphorylated receptors from stimulated cells, eluting the receptors, and incubating them with

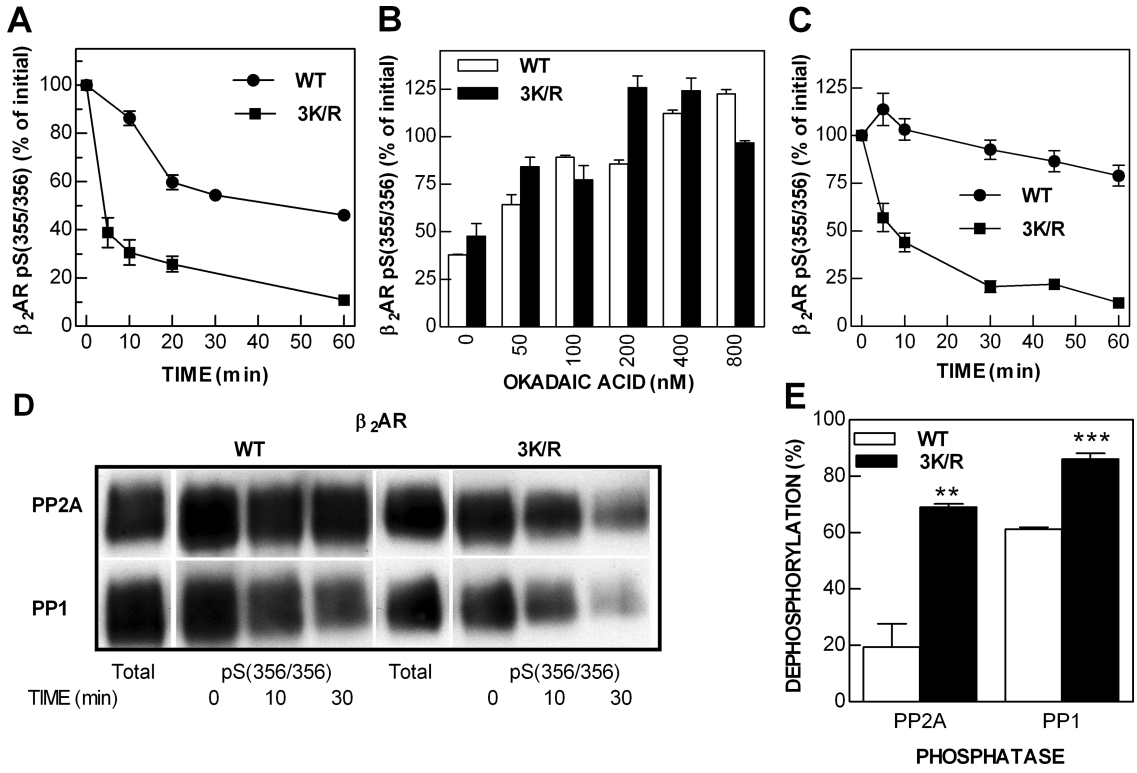


FIGURE 7: Dephosphorylation of β_2ARs in a cell-free system. (A) Time course of cell-free dephosphorylation of WT (●) and 3K/R (■) β_2ARs. Cells stimulated with 1 μM ISO for 10 min were homogenized, and portions were incubated at 37 °C for the indicated periods of time and analyzed sequentially for pS(355,356) and total β_2AR by Western blotting and densitometry as described in Materials and Methods. Values are normalized for total receptors, are expressed as a percentage of initial phosphorylation, and are the means ± SEM of three separate experiments. (B) Effect of okadaic acid on cell-free dephosphorylation. Experiments were performed as described for panel A except the indicated concentrations of okadaic acid were present and the reaction mixtures containing WT (white bars) were incubated for 60 min and those containing 3K/R (black bars) for 20 min. Values are the means ± SEM of three separate experiments. (C) Dephosphorylation of soluble receptors. Experiments were performed as described for panel A except each homogenate was centrifuged at 600*g* for 5 min to obtain a PNS. The latter was solubilized with 0.5% Triton X-100, and the soluble receptors were assayed. Values are the means ± SEM of two separate experiments, each conducted in duplicate. (D) Dephosphorylation of immunoprecipitated β_2ARs by purified phosphatases. Cells expressing WT or 3K/R β_2AR were stimulated with 1 μM ISO for 10 min, washed, and lysed. The receptors were extracted from the lysates, immunoprecipitated using anti-HA antibodies, and eluted from the immunoprecipitates as described in Materials and Methods. Portions of the eluted receptors were incubated with 100 milliunits of the catalytic subunits of purified PP1 or PP2A at 30 °C for the times indicated and were analyzed for phosphorylated (0, 10, and 30 min) and total receptors by Western blotting. Shown is one of three similar blots. (E) The blot shown in panel D and two similar blots were quantified by scanning densitometry. The values (means ± SEM) represent the percentage of dephosphorylation normalized for total receptors after incubation for 30 min with the purified phosphatases. Significance determined by a two-tailed *t* test: **, $P < 0.01$; ***, $P < 0.001$.

purified phosphatases. 3K/R was dephosphorylated more extensively than WT β_2AR was by both phosphatases (Figure 7D,E).

Role for Lysines 372 and 375 but Not Lysine 348. We generated the single (K348R) and double mutants (K372/375R) to determine whether the mutation of all three lysine residues in the C-tail of β_2AR is required for the differences. We chose the two mutations as the lysines bracket the cluster of three serines involved in GRK-catalyzed phosphorylation and subsequent receptor regulation (*22, 29*). When cells expressing the mutants were assayed for dephosphorylation and agonist-mediated downregulation, we found that the single mutant K348R which is proximal to the serine cluster behaved like WT β_2AR whereas the double mutant K372/375R which is distal to the cluster was similar to 3K/R (Figure 8).

Inhibition of Dephosphorylation Increases the Levels of Degradation and Lysosomal Targeting of 3K/R. To determine whether the increased rate of dephosphorylation of 3K/R contributed to the reduction in the level of downregulation, we used a biotinylation procedure to assess receptor turnover. Receptor degradation is detected earlier by this method than by binding activity or Western blotting as any internal or newly synthesized receptors are not detected. The cells were biotinylated, incubated in the absence and presence of 200 nM okadaic acid for 30 min, and then incubated in the absence and presence of ISO for 4 h. The receptors were immunoprecipitated, and the biotinylated receptors were detected with a streptavidin overlay (Figure 9). The basal turnover was 31% for WT compared to 20% for 3K/R, whereas the turnover in the agonist-stimulated cells was 74% compared to 37%. Thus, 43 and 17%, respectively, can be attributed to the effect of the agonist. In the cells treated with okadaic acid and agonist, the turnover of WT was not significantly changed whereas the turnover of 3K/R increased to that of WT β_2AR. Okadaic acid even had a significant effect on the basal turnover of 3K/R. It appears that dephosphorylation impairs the degradation of the receptor and conversely phosphorylation enhances it.

The increased rate of turnover of biotin-labeled β_2AR in cells exposed to agonist for 4 h appears to be inconsistent with absence of downregulation at this time. Most likely, the biotin group is being removed prior to further degradation of the receptor protein. The biotinylation reagent labels cell

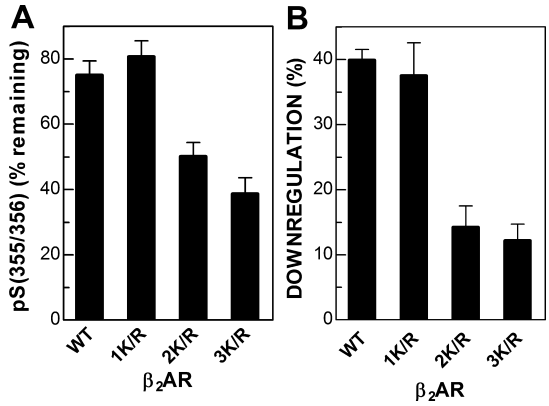


FIGURE 8: Agonist-mediated dephosphorylation and downregulation of mutant β_2ARs with one, two, or three C-tail lysines replaced with arginine. (A) HEK 293 cells stably expressing HA-β_2AR (WT) and K348R HA-β_2AR (1K/R), K372/375R HA-β_2AR (2K/R), and K348/372/375R HA-β_2AR (3K/R) were stimulated with ISO for 10 min and then with propranolol for 10 min and assayed for dephosphorylation of pS(355,356) as described in the legend of Figure 5. Data are normalized for total receptors, are expressed as the percentage of initial phosphorylation, and represent the means ± SEM of three to eight separate experiments. (B) Cells were exposed to 10 μM ISO for 24 h and assayed for total binding sites with ^{125}ICYP as described in the legend of Figure 2. Values are expressed as the percentage of binding sites lost and are the means ± SEM of four to six separate experiments.

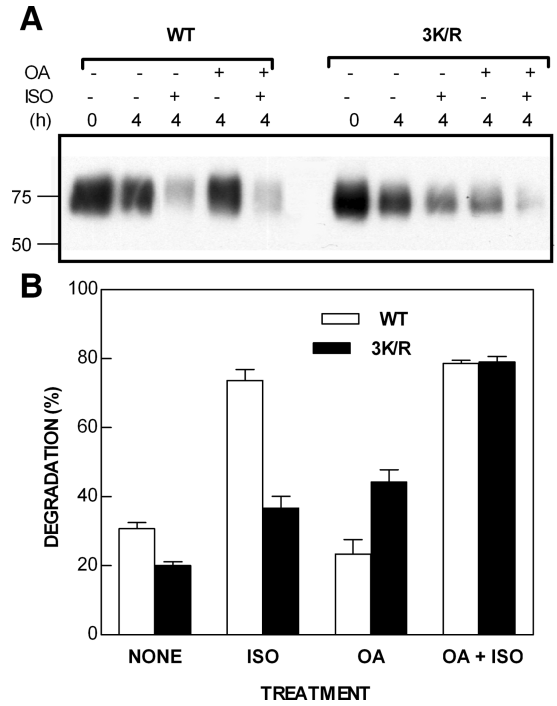


FIGURE 9: Effect of inhibiting receptor dephosphorylation on degradation of WT and 3K/R β_2ARs. (A) Detection of degradation of biotinylated β_2AR with a streptavidin overlay. Cells were surface biotinylated as described in Materials and Methods and incubated for 30 min in the absence and presence of 200 nM okadaic acid. The cells were collected (0 h) in one instance; in another instance, 10 μM ISO was added where indicated and the cells were incubated for an additional 4 h. The cells were washed, lysed, and solubilized in RIPA buffer. β_2ARs were immunoprecipitated with anti-β_2AR antibodies, resolved by SDS−PAGE, transferred to Immobilon-p membranes (Millipore), and detected by being blotted with HRP-conjugated streptavidin. (B) Quantification of WT (white bars) or 3K/R (black bars) β_2AR degradation by scanning densitometry of streptavidin overlays. Values are the means ± SEM of three separate experiments.

surface amino groups such as the ε-amino of lysine and the N-terminus of β_2AR. Although the first and third extracellular loops of β_2AR each have a lysine, it is within one residue of a transmembrane region and unlikely to be available to the reagent. As described above, the N-terminus of β_2AR appears to be more susceptible than the C-terminus to proteases that may be present in a late endosomal or prelysosomal compartment to which WT and 3K/R both traffic. The additional loss of the N-terminus of WT as well as the initiation of loss of its C-tail and binding activity may be a result of its trafficking to lysosomes. Using confocal fluorescence microscopy and the lysosomal marker LysoTracker, we observed the colocalization of the marker (red) and WT β_2AR (green) with the time of agonist treatment (Figure 10, without okadaic acid, panels A−C), whereas colocalization of the marker and 3K/R was substantially less even at 4 h (panels D−F). If phosphorylation of 3K/R influences its degradation, then it may affect its trafficking. The inhibition of dephosphorylation by okadaic acid (with okadaic acid) did not appear to change the trafficking of WT (panels G–I). Initially, 3K/R appeared to traffic the same in the okadaic acid-treated cells as (panels J–L); however, by 1 h, some receptors were located in lysosomes (panel K), and by 4 h, there was extensive colocalization of 3K/R and the lysosomal marker in the okadaic acid-treated cells (panel L).

DISCUSSION

On the basis of a previous study indicating that the β_2AR ubiquitination sites resided in the C-tail (*18*), we replaced the three C-tail lysines with arginines to produce a mutant, 3K/R. Our intention was to further explore the roles of ubiquitination and phosphorylation in receptor regulation. We found that 3K/R underwent less agonist-mediated ubiquitination, lysosomal targeting, and degradation compared to the WT receptor. Agonist-stimulated ubiquitination, however, was not totally eliminated in the 3K/R mutant, whereas it is not detected in a mutant with all 16 lysines replaced with arginines (*7*) or in a chimera of β_2AR with a β_1AR C-tail (*18*). One explanation is the presence of endogenous β_2AR in HEK 293 cells which could represent up to ~5% of the receptors in 3K/R-expressing cells. It also may be explained by differences between β_1AR and β_2AR in interacting with arrestins. In addition to their role in desensitization and endocytosis, arrestins are involved in ubiquitination of β_2AR by recruiting ubiquitin ligases to the receptor complex (*7*). The latter requires high-affinity binding of arrestin to GRK-phosphorylated β_2AR and is not observed in cells lacking arrestins or expressing a phosphorylation-defective β_2AR that neither binds arrestins nor is internalized. The β_2AR−β_1AR chimera undergoes less agonist-stimulated phosphorylation and internalization than WT β_2AR (*19*) and interacts more weakly with arrestins (*38*). In contrast, 3K/R was rapidly phosphorylated by GRKs and was desensitized and internalized like WT in agonist-stimulated cells. It is possible that the arrestin-associated ligases conjugate ubiquitin to other cytoplasmic lysines in β_2AR which serve some unknown function. We also observed agonist-dependent ubiquitination of WT, but not 3K/R, in a cell-free assay using biotinylated ubiquitin.

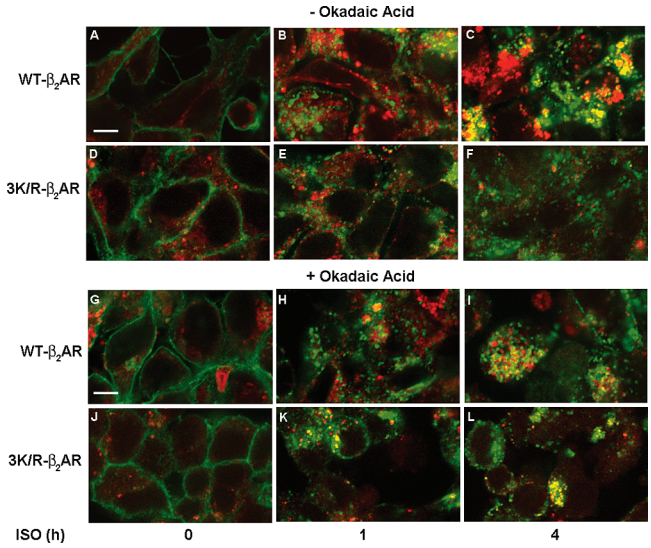


FIGURE 10: Effect of okadaic acid on trafficking of WT and 3K/R β_2ARs in HEK 293 cells. Cells expressing WT (A−C) or mutant (D−F) HA-β_2AR were treated with Alexa Fluor 488-conjugated anti-HA antibody for 1 h in the absence (−okadaic acid) or presence (+okadaic acid) of 500 nM okadaic acid and stimulated with 10 μM ISO for 0 (A, D, G, and J), 2 (B, E, H, and K), and 4 h (C, F, I, and L). During the last 30 min, the cells were exposed to 75 nM LysoTracker Red. The cells then were washed, fixed, and visualized for receptors (green), lysosomal marker (red), and colocalization (yellow). The bar is 5 μm. Images are from the same experiment and are representative of at least three separate experiments.

Although the WT and 3K/R mutant β_2ARs were internalized at similar rates, the two receptors entered distinct endocytic pathways. WT β_2AR was transported to large perinuclear vesicles and then to lysosomes. 3K/R remained in small peripheral vesicles and did not appear in lysosomes. Despite these differences, both receptors were recycled at similar rates. The extent of recycling, however, decreased with an increased time of internalization, more for WT than for 3K/R, and may be due to the increased level of degradation of WT. Using three different assays, we clearly established that agonist-mediated loss of WT β_2AR was more extensive than that of 3K/R. These included loss of total binding sites (3.3-fold), loss of receptor proteins detected by antibodies to the receptor (4.3-fold), and an increased rate of turnover using a biotinylation technique (2.5-fold).

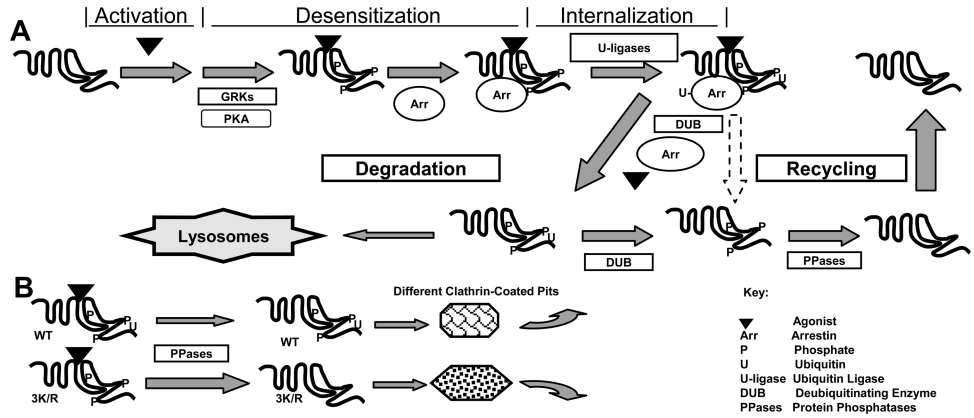
While the level of phosphorylation of serines 355 and 356 of both receptors initially increased in agonist-stimulated cells, the level of the phosphorylated mutant decreased after 2 min and that of the WT was maintained. When the agonist was removed and replaced with an antagonist, 3K/R was more rapidly dephosphorylated than WT β_2AR was. The different rates of dephosphorylation were demonstrated in a cell-free assay using cell homogenates and soluble cell extracts. Finally, using phosphorylated receptors eluted from immunoprecipitates, we found that both purified PP1 and PP2A dephosphorylated 3K/R more effectively than WT. The latter results make it unlikely that the distinct trafficking of 3K/R accounts for its more rapid dephosphorylation. It is more probable that the rapid dephosphorylation of 3K/R contributes to its trafficking and its resistance to degradation. The rapid dephosphorylation of 3K/R is both unexpected and novel as we are unaware of similar effects reported for other GPCRs. Although the PP1 and PP2A levels were similar in control and ISO-stimulated cells expressing WT or 3K/R, it does not rule out the possibility that more protein phosphatase activity associates with 3K/R than with WT.

Our results with okadaic acid further emphasize the role of phosphorylation in receptor trafficking and degradation. Okadaic acid, which inhibited the dephosphorylation of 3K/R, increased the rate of transport of the mutant to lysosomes and its rate of turnover. Our finding suggests that receptor phosphorylation may be sufficient for lysosomal trafficking and degradation. In support of this interpretation, phosphorylation-independent arrestin mutants, which are able to bind to unphosphorylated, agonist-occupied β_2AR, enhance recycling and reduce the level of phosphorylation and degradation of the receptor (*39*). This does not eliminate a role for ubiquitination as 3K/R in okadaic acid-treated cells initially followed the same intracellular route as in control cells but eventually appeared in the lysosomal compartment. Thus, ubiquitination may be important for targeting the receptor to the degradation pathway, whereas phosphorylation may inhibit the receptor from entering the recycling pathway and, by default, ending up in lysosomes.

Our data are consistent with a model of receptor trafficking that involves both phosphorylation and ubiquitination of the C-tail of β_2AR (Scheme 1). The agonist-activated receptors are rapidly desensitized by phosphorylation and arrestin binding. Arrestins bind to clathrin and clathrin adapter protein AP-2, resulting in the recruitment of β_2AR to clathrin-coated pits, and arrestins attract ubiquitin ligases that conjugate ubiquitin to both the arrestins and receptors. Arrestin ubiquitination is transient, and arrestins undergo rapid deubiquitination by DUBs and dissociate from the receptors which are endocytosed (*40*). Receptors with both post-translational modifications are targeted to lysosomes where they are degraded. The ubiquitinated lysines and the phosphoserines may function as a recognition signal for sorting proteins that direct the receptors to lysosomes. The chemokine receptor has such a sequence (*8, 41*). As 3K/R lacks this motif and is rapidly dephosphorylated, it will not be sorted to lysosomes but will be recycled. The ubiquitins also may protect the phosphoserines from phosphatases. As the rate of receptor internalization is much greater than the rate of receptor degradation, only a small fraction of internalized receptors are degraded (*18, 19, 21*) and therefore need to be ubiquitinated. We found that only a small fraction of WT β_2AR was ubiquitinated which is most likely due to the presence of DUBs (*42*).

How do we explain the differences in the rates of dephosphorylation and in trafficking between phosphorylated WT receptors that are not ubiquitinated and phosphorylated 3K/R receptors? The simplest possibility is that the mutations cause a conformational or structural change in the C-tail that makes the phosphoserines more accessible to protein phosphatases. The three lysines in the C-tail overlap the cluster of three serines (S355, S356, and S364) that have been identified as the key GRK phosphorylation sites involved in receptor regulation (*22, 27*). Supporting this possibility is the more rapid dephosphorylation of immunoprecipitated 3K/R than WT by purified phosphatases. Our data for the properties of 3K/R, however, do not support a conformational change. This is the region for GRK-catalyzed phosphorylation and arrestin binding that are prerequisites for desensitization and internalization, processes for which no differences between WT and 3K/R were found. Furthermore,

Scheme 1: Model of the Role of Phosphorylation and Ubiquitination in the Regulation of the β_2AR[a]



[a] (A) Pathway of β_2AR activation, desensitization, internalization, and sorting between recycling and degradation. The receptors are activated by agonist binding and then are phosphorylated by GRKs and PKA. Arrestins bind with high affinity to the GRK sites, and the receptors become desensitized. Arrestins attract ubiquitin ligases to the receptor complexes which ubiquitinate the arrestins and receptors. Arrestins also recruit β_2AR to clathrin-coated pits where the arrestins are deubiquitinated by DUBs and dissociate from the receptors which undergo endocytosis. The fate of WT β_2AR will depend on DUBs and PPases. The receptors that remain phosphorylated and ubiquitinated will be transported through the degradation pathway to lysosomes, while the dephosphorylated and deubiquitinated ones will follow the recycling pathway back to the plasma membrane. The mutant 3K/R receptors lacking the C-tail-conjugated ubiquitins enter the recycling pathway possibly at a different site than the WT receptors (dotted arrow). (B) WT and mutant 3K/R receptors undergo early sorting by entering distinct populations of coated pits. See the text in the Discussion for details.

agonist-mediated activation of Erk1/2 was similar in cells expressing WT or 3K/R β_2ARs.[2] However, a β_2AR with all 16 lysine residues mutated to arginines was reported to bind ligands, stimulate adenylyl cyclase, interact with arrestins, and internalize in a manner similar to that of WT (*7*). Thus, a conformational change may not necessarily affect these properties. To further define the identity of the lysines involved, we separately mutated lysine 348 and lysines 372 and 375 which are proximal and distal, respectively, to the serine cluster. The dephosphorylation and downregulation of K372/375R were similar to those of 3K/R, while those of K348R were similar to those of WT. Elimination of the lysine proximal to the serine cluster indicates that the effect is more localized.

As for differences in intracellular trafficking between WT and 3K/R, sorting may occur early in the internalization process as suggested in Scheme 1B, where WT and 3K/R enter two distinct populations of clathrin-coated pits. Several studies have shown that these distinct populations exist and that different GPCRs including β_2AR enter one type of coated pit and are excluded from the others (*43, 44*). The limited colocalization of the two receptors is consistent with this possibility (Figure 3B). The pre-endocytic sorting of WT and 3K/R β_2ARs may be due to differences in their phosphorylation. Phosphorylated β_2ARs (mainly WT) enter one population of coated pits, whereas dephosphorylated β_2ARs (mostly 3K/R) enter a different population. When the rates of dephosphorylation and internalization are compared, they initially are more similar for 3K/R than for WT. Thus, it is more probable that 3K/R is dephosphorylated before endocytosis, whereas WT dephosphorylation occurs after internalization. Recently, the dephosphorylation of β_2AR at the plasma membrane has been shown in cells where internalization is blocked (*24, 34*). It is also possible but less likely that ubiquitination determines which receptors enter which population of coated pits. After entering the coated pit, the WT receptors may be susceptible to DUBs (*42*) as are arrestins, thus keeping the fraction of ubiquitinated receptors low. Following internalization, the vesicles containing WT cargo will enter the endocytic pathway leading to early sorting endosomes where any ubiquitinated receptors remaining are targeted to lysosomes. The nonubiquitinated WT receptors are dephosphorylated and recycled. The vesicles containing 3K/R cargo are derived from a different population of coated pits, enter a different endocytic pathway, and are directed to the recycling pathway (*6*), a process enhanced by the lack of ubiquitination (*42*). Phosphorylation and ubiquitination of β_2AR appear to reinforce each other to promote the sorting of the receptor to lysosomes and degradation. It is important to point out that not all GPCRs undergo agonist-mediated ubiquitination (*18, 45*) and other lysosomal sorting signals have been identified (*26, 46*). Nevertheless, ubiquitination of GPCRs such as the β_2AR in consort with phosphorylation appears to be an important mechanism for regulating their degradation.

[2] Unpublished data.

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SUPPORTING INFORMATION AVAILABLE

Method for the phosphatase assay, effects of agonist concentration on binding to WT and 3K/R β_2ARs and adenylyl cyclase stimulation (Figure S1), specificity of antiphosphorylated β_2AR antibodies (Figure S2), rates of synthesis of β_2ARs (Figure S3), and levels of phosphatase activity, PP1, and PP2A in control and agonist-stimulated HEK 293 cells expressing WT and 3K/R β_2ARs (Figure S4).

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