Phosphorylation of the Deubiquitinase USP20 by Protein Kinase A Regulates Post-endocytic Trafficking of β2 Adrenergic Receptors to Autophagosomes during Physiological Stress*

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Background: The mechanisms for recruiting and activating deubiquitinase(s) during GPCR trafficking are unknown.

Results: PKA phosphorylation of USP20 Ser-333 inhibits β2AR interaction as well as deubiquitination and promotes receptor degradation via autolysosomes during physiological stress.

Conclusion: USP20 activity and substrate-specific interaction involves a phosphorylation code.

Significance: We identify a novel role for PKA in USP20 regulation and ubiquitin-dependent sorting of GPCRs.

Ubiquitination by the E3 ligase Nedd4 and deubiquitination by the deubiquitinases USP20 and USP33 have been shown to regulate the lysosomal trafficking and recycling of agonist-activated β2 adrenergic receptors (β2ARs). In this work, we demonstrate that, in cells subjected to physiological stress by nutrient starvation, agonist-activated ubiquitinated β2ARs traffic to autophagosomes to colocalize with the autophagy marker protein LC3-II. Furthermore, this trafficking is synchronized by dynamic posttranslational modifications of USP20 that, in turn, are induced in a β2AR-dependent manner. Upon β2AR activation, a specific isoform of the second messenger cAMP-dependent protein kinase A (PKAα) rapidly phosphorylates USP20 on serine 333 located in its unique insertion domain. This phosphorylation of USP20 correlates with a characteristic SDS-PAGE mobility shift of the protein, blocks its deubiquitinase activity, promotes its dissociation from the activated β2AR complex, and facilitates trafficking of the ubiquitinated β2AR to autophagosomes, which fuse with lysosomes to form autolysosomes where receptors are degraded. Dephosphorylation of USP20 has reciprocal effects and blocks trafficking of the β2AR to autophagosomes while promoting plasma membrane recycling of internalized β2ARs. Our findings reveal a dynamic regulation of USP20 by site-specific phosphorylation as well as the interdependence of signal transduction and trafficking pathways in balancing adrenergic stimulation and maintaining cellular homeostasis.

β-Adrenergic receptor (βAR)3 signaling activates Gαs-coupled adenyl cyclase, leading to the production of cAMP, which activates protein kinase A (PKA), a major signaling kinase that modulates cell responses (1). When continuously or repeatedly subjected to agonist stimulation, βARs cease signal transduction and lose the ability to stimulate G protein effector pathways. This failure to provoke a cellular response is referred to as receptor desensitization (2). Acute desensitization results from a two-step regulatory mechanism. First, G protein-coupled receptor kinases (GRKs) phosphorylate agonist-activated receptors. Second, the phosphorylated receptor recruits and binds to adaptor proteins, β-arrestins (isoforms 1 and 2), which interdict receptor/G protein coupling (3, 4). On the other hand, long-term desensitization of βAR signaling requires degradation of cell surface receptors in the intracellular compartments called lysosomes (5–10). Prolonged desensitization as well as down-regulation of βARs is a hallmark of human heart failure (11, 12). The key factors that promote βAR down-regulation in failing hearts remain unclear.

A posttranslational modification called ubiquitination, discovered originally as a tag for proteasomal degradation, is now widely accepted as a sorting signal during vesicular trafficking of internalized cell surface receptors (10, 13–15). Agonist-stimulated ubiquitination of mammalian GPCRs was first described for the β2AR and the chemokine CXCR4 (16, 17). For these receptors, ubiquitination has been shown to be a tag for post-endocytic lysosomal targeting and receptor degradation. Currently this is one of the main mechanisms required for the regulation of the life cycle of GPCRs and other cell surface receptors (10, 18). Ubiquitination of the β2AR not only requires agonist stimulation but also phosphorylation of the receptor by GRKs and association with β-arrestin2, which functions as an adaptor to link the ubiquitinating enzymatic machinery to the activated receptor complex (16). β-arrestin2 acts as an adaptor for the HECT (homology to E6-AP carboxyl terminus) domain-containing E3 ubiquitin ligase Nedd4, which ubiquitinates the β2AR and regulates lysosomal degradation of the receptor (19, 20). Additionally, the deubiquitinating enzymes (DUBs) USP20 protein-coupled receptor; Iso, isoproterenol; VS, vinyl sulfone; DPBS, Dulbecco’s PBS.

The abbreviations used are: βAR, β-adrenergic receptor; GRK, G protein-coupled receptor kinase; DUB, deubiquitinating enzyme; SUMO, small ubiquitin-like modifier; ANOVA, analysis of variance; Ub, ubiquitin; GPCR, G protein-coupled receptor; Iso, isoproterenol; VS, vinyl sulfone; DPBS, Dulbecco’s PBS.

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and USP33 reverse ubiquitination and divert the internalized β2ARs to a recycling pathway (21, 22). Accordingly, ubiquitination and deubiquitination of the activated β2AR facilitated by specific enzymes define the extent and revival of adrenergic signaling at the plasma membrane.

We discovered a novel mechanism by which the DUB activity of USP20 is regulated by the signaling kinase PKA, which promotes the trafficking of ubiquitinated β2ARs to autophagosomes and, subsequently, to the lysosomes, effecting degradation and prolonged desensitization. Although studies have historically shown GPCR trafficking to lysosomes, there has been little evidence of the trafficking of GPCR complexes into autophagic vesicles. Here we visualize the specific colocalization of internalized β2ARs and the autophagy marker LC3-II (23), and we further demonstrate a link between this colocalization and USP20 phosphorylation and dephosphorylation.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The antibodies used for our studies included anti-FLAG M2 (Sigma, catalog no. F3165), anti-HA 12CA5 (Roche, catalog no. 11666606001), anti-HA probe (Santa Cruz Biotechnology, catalog no. sc-805), anti-ubiquitin FK1 (Enzo Life Sciences, catalog no. BML-PW8805), anti-α-tubulin (Santa Cruz Biotechnology, catalog no. sc-903), anti-PKA used in our studies.

We used in various assays. Sequences of siRNA oligonucleotides were as follows: control non-targeting sequence, 5′-AAUUCUCGGAAAGUCGUACAGU-3′; PKAα (human), 5′-CGUCCUGACCUUUGAGAUU-3′; and PKAβ (human), 5′-GGUGACUUGGGUUU-3′.

For siRNA experiments, early-passage HEK293 cells on 100-mm dishes that were at 40–50% confluence were transfected with siRNA using Lipofectamine 2000 transfection reagent. Forty-eight hours later, cells were processed for further assays.

Cell Culture and Transfections—HEK293 and COS-7 cells were purchased from the ATCC and maintained in minimal Eagle’s medium (MEM) or DMEM containing 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin at 37 °C in a humidified incubator at 5% CO₂. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) or FuGENE 6 transfection reagent (Roche Applied Science). HEK293 cells stably transfected with FLAG-β2AR and FLAG-β2AR-mYFP used in these studies have been described previously (21). S49 cell lines, WT, and Kin− (which lack activation of the catalytic subunit of PKA) were gifts from Dr. Paul A. Insel (Department of Pharmacology, University of California, San Diego). S49 cells were maintained in DMEM containing 10% heat-inactivated horse serum and 100 μg/ml penicillin/streptomycin in a humidified incubator containing 10% CO₂ at 37 °C. Rat vascular smooth muscle cells were isolated and maintained as described previously (22); animal procedures were approved by the Duke University Institutional Animal Care and Use Committee.

Immunoprecipitation and Immunoblotting—48h post-transfection, cells were starved for 1 h in serum-free medium or in Hanks’ balanced salt solution and stimulated in the same medium with isoproterenol for the desired time. Cells were washed with ice-cold phosphate-buffered saline (pH 7.4) and lysed in an ice-cold lysis buffer containing 50 mM HEPES (pH 7.5), 2 mM EDTA, 250 mM NaCl, 10% (v/v) glycerol, 0.5% Nonidet P-40 and supplemented with phosphahte and protease inhibitors (1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml), aprotonin (5 μg/ml), pepstatin A (1 μg/ml), and benzamidine (100 μM) (Sigma)). The cell lysate was centrifuged at 15,000 × g for 30 min at 4°C. After centrifugation, protein concentrations were determined by Bradford protein assay, and equivalent protein was used for immunoblotting or immunoprecipitation. For immunoprecipitations, soluble cell extracts were mixed with anti-FLAG M2 resin or anti-HA-agarose beads, and then the sample was incubated at 4°C with end-over-end rotation overnight. Immunocomplexes were washed extensively with human HA-USP20 identified by bioinformatics searches to be sites for post-translational modification were mutated using the QuikChange Lightning or multisite-directed mutagenesis kit from Stratagene (La Jolla, CA), and the mutations were confirmed by DNA sequencing. pEGFP-LC3 was provided by Dr. Finkel (Addgene) (24).

siRNA—Double-stranded siRNA oligonucleotides with 21- or 19-nucleotide duplex RNA and two-nucleotide 3′-dTdT overhangs were chemically synthesized (Dharmacon GE Healthcare Life Sciences) in a deprotected desalted form and used in various assays. Sequences of siRNA oligonucleotides were used in various assays. Sequences of siRNA oligonucleotides were as follows: control non-targeting sequence, 5′-AAUUCUCGGAAAGUCGUACAGU-3′; PKAα (human), 5′-CGUCCUGACCUUUGAGAUU-3′; and PKAβ (human), 5′-GGUGACUUGGGUUU-3′.

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USP20 Phosphorylation and β₂AR Trafficking

Nonidet P-40 lysis buffer to remove nonspecific binding, and bound protein was eluted in 1× SDS-PAGE sample buffer. For immunoblotting, protein samples were resolved by 4–20% gradient gels or 10% gels (Invitrogen) and transferred onto a nitrocellulose membrane. Separation of the two USP20 bands required modified gel conditions: 60 min run with higher current (50 mA constant per minigel). Membranes were blocked in TTBS (10 mM Tris (pH 8.0), 150 mM NaCl, and 2% Tween 20) supplemented with 5% (w/v) dried skim milk powder. Primary and secondary antibody incubations were performed in blocking solution, and washes were performed using TTBS. Immunoreactive bands were detected using enhanced chemiluminescence (SuperSignal West Pico Reagent, Pierce). Signals were detected and acquired with a charge-coupled device camera system (Bio-Rad Chemidoc-XRS) and analyzed with Image Lab software (Bio-Rad).

Immunofluorescence Staining and Confocal Imaging—HEK293 cells with stable transfection of FLAG-β₂AR-mYFP or FLAG-β₂AR were transiently transfected with HA-pcDNA3.0, HA-USP20 wild-type, HA-USP20-S333A, HA-USP20-S333D, or pEGFP-LC3. 24 h after transfection, cells were seeded on poly-D-lysine or collagen-coated 35-mm glass bottom plates. 48 h post-transfection, cells were starved for 1 h in serum-free medium or Hanks’ balanced salt solution, stimulated in the

FIGURE 1. Agonist-stimulated protein mobility shift of USP20. A, HEK293 cells stably transfected with FLAG-β₂AR were serum-starved for 60 min. After starvation, the cells were stimulated with 100 nM isoproterenol for the indicated times and then lysed and immunoblotted with USP20 antibody. B, COS-7 cells were transiently transfected with HA-pcDNA3.0 or HA-USP20, and the endogenous β₂ARs were stimulated with 1 μM isoproterenol for the indicated times, lysed, and immunoblotted with antibody to HA. C, COS-7 cells were transfected as in B, and the endogenous β₂ARs were stimulated with 1 μM epinephrine (Epi) for the indicated times, lysed, and immunoblotted with antibody to HA. D, COS-7 cells were transfected as in B and pretreated with β-blockers (10 μM propranolol and 20 μM ICI-118,551). The cells were then left unstimulated or stimulated with isoproterenol, lysed, and immunoblotted with antibody to HA. E, COS-7 cells were transiently transfected with HA-pcDNA3.0, HA-USP20, or HA-USP20-HA, and the endogenous β₂ARs were stimulated with isoproterenol, lysed, and immunoblotted with antibody to USP20. F, COS-7 cells were transfected as in B and pretreated with MG132 and/or ICI-118,551. The cells were then left unstimulated or stimulated with isoproterenol, lysed, and immunoblotted. G, in vitro translation was carried out using a TNT-coupled reticulocyte lysates system and the indicated plasmids. SDS-PAGE sample buffer was added to the reaction products, and immunoblotting was performed using antibody to HA (lanes 1 and 2). Lanes 3 and 4 show COS-7 cells transfected with HA-USP20, stimulated with isoproterenol, lysed, and immunoblotted with HA antibody. H, COS-7 cells were transiently transfected with HA-USP20, left unstimulated or stimulated with isoproterenol and lysed, and crude cell lysates were labeled with Ub-VS, SUMO-VS, or Nedd8-VS, followed by immunoblotting with HA antibody. I, Ub-VS labeling was performed as in H, but with increasing concentrations as indicated.
same medium with 1 μM isoproterenol, fixed with 5% formaldehyde diluted in Dulbecco’s PBS (DPBS) containing calcium and magnesium, and then washed three times with DPBS. The fixed cells were permeabilized with 0.01% Triton X-100 in DPBS containing 2% bovine serum albumin for 20–30 min and incubated with the appropriate primary antibody overnight at 4 °C. The next day, cells were washed three times with DPBS and incubated with the respective secondary antibody. Imaging was performed on a Zeiss LSM510 laser-scanning microscope using a 100×1.3 oil immersion objective, and the pinhole was set to 1.0 Airy units for single fluorophore imaging. To obtain multichannel acquisition, we utilized the filter settings as multitrack sequential excitation (488, 568, and 633 nm) and emission (515–540 nm, GFP; 585–615 nm, Texas Red; 650 nm, Alexa Fluor 633). All confocal analyses were performed on samples from three to five independent experiments. In each experiment, several cells or groups of cells were analyzed. Image acquisition used the LSM 510 operating software and images were later exported as TIFF files. Further processing (resizing, addition of text, etc.) was performed using Adobe Photoshop software (CS2), and any change in brightness/contrast was applied to the entire image. Pearson’s correlation coefficients for quantification of β2AR/LAMP2 or β2AR/GFP-LC3 colocalizations were performed in ≥20 cells from multiple independent experiments using ImageJ software (National Institutes of Health).

**In Vitro Translation**—In vitro translation was carried out using a TNT quick-coupled transcription/translation system (Promega) along with Transcend™ chemiluminescent translation detection system components (Promega) according to the instructions of the manufacturer. The in vitro synthesized proteins were analyzed by immunoblotting.

**In Vitro Phosphorylation**—Purified protein was mixed without or with recombinant full-length PKA enzyme (EMD Millipore) and incubated at 37 °C for 5 min. The reaction was terminated by the addition of SDS sample buffer and boiling. The samples were resolved on a Tris-glycine 10% polyacrylamide gel (Invitrogen), and protein bands were detected by immunoblotting.

**Receptor Degradation**—The β-adrenergic receptor degradation assay was performed with radiolabeled [125I](-) iodocyanopindolol using methods described previously (21). Following preincubation without or with agonist, cells were washed with ice cold phosphate-buffered saline and collected in MEM sup-

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**FIGURE 2. Analyses of PKA phosphorylation sites in USP20.** A, COS-7 cells were transiently transfected with HA-pcDNA3.0 or HA-USP20 and left unstimulated or stimulated with isoproterenol. HA-USP20 was immunoprecipitated with HA-agarose beads, and the presence of the phosphorylated form in the immunoprecipitates (IP) was detected with a Ser(P)/Thr(P)-specific antibody. B, the functional domains of USP20 are indicated. Potential PKA phosphorylation sites within the human USP20 sequence as predicted by bioinformatics are indicated. zf-UBP, zinc finger ubiquitin-specific protease domain; DUSP, domain in ubiquitin-specific protease. C–E, COS-7 cells were transfected with the HA-USP20 wild type or indicated mutant plasmids, stimulated with isoproterenol, and lysed, and cell lysates were immunoblotted with HA antibody. F, COS-7 cells were transfected with the HA-USP20–12 PKA phosphorylation mutant in which serine 333 was restored (A333S) or the HA-USP20–12 phosphomutant (all 12 PKA phosphorylation sites mutated). Transfected cells were stimulated with isoproterenol, lysed, and immunoblotted with HA antibody.
implemented with 10 mM HEPES (pH7.5) and 5 mM MgCl2. Cells were resuspended thoroughly using a 1-ml U-100 insulin syringe, and protein concentration was determined using the Bradford assay. Whole cell membranes were diluted in assay buffer (50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 12.5 mM MgCl2, and 1 mM ascorbic acid) and added to deep 96-well plates in triplicates. [125I]-((/H11002) iodocyanopindolol was added at a final concentration of 200–400 pM in the presence or absence of the hydrophobic antagonist propranolol (100 M, to define nonspecific binding) in assay buffer, added to the samples, and then incubated at 37 °C for 90 min. Radiolabeled membranes were collected on a Brandel harvester (Whatman GF/B glass filters briefly wetted with distilled water) and quickly washed three times with ice-cold wash buffer (50 mM Tris-HCl (pH 7.4), 2 mM EDTA, and 12.5 mM MgCl2). The filters were placed in plastic tubes and counted in a counter. The receptor density (total specific [125I]-((/H11002) iodocyanopindolol binding sites) was determined after 24 h of ISO treatment and expressed as the percent of receptor number assessed in nonstimulated cells.

Experimental Repeats and Statistical Analyses—All Western blots and confocal experiments show representative data from one of three or more independent experiments. All quantifications shown are mean ± S.E. from at least three independent experiments. To determine significance, results were compared with control condition by one-way ANOVA followed by Bonferroni post hoc test for all bar graphs with more than two groups. All statistical analyses were performed using Prism software (version 6, GraphPad). p < 0.05 at a 95% confidence level was considered significant.

RESULTS

BAR Agonists Induce Dynamic Posttranslational Modifications of USP20—Agonist-activation of βARs in HEK293 cells or in COS-7 cells alters the electrophoretic mobility of endogenous βARs and transfected HA-USP20, or indicated HA-USP20 mutant plasmids. Endogenous βARs were stimulated without or with 1 μM isoproterenol, lysed, and immunoblotted with antibody to HA. E, COS-7 cells were transiently transfected with HA-pcDNA3.0, HA-USP20, or HA-USP20-S333A and then were then stimulated with isoproterenol or left unstimulated. HA-USP20 was immunoprecipitated with HA-agarose beads, and the presence of phosphorylated forms in the immunoprecipitates (IP) was detected with a Ser(P)/Thr(P)-specific antibody (top panel). The levels of USP20 were detected in both immunoprecipitates (center panel) and lysates (bottom panel) using HA antibody.

FIGURE 3. Analyses of GRK phosphorylation sites in USP20. A and B, schematic representation of human USP20 showing putative phosphorylation target residues by GRKs that were mutated to alanine to generate HA-USP20 mutants as indicated. zf-UBP, zinc finger ubiquitin-specific protease domain; DUSP, domain in ubiquitin-specific protease. C and D, COS-7 cells were transiently transfected with HA-pcDNA3.0, HA-USP20, or indicated HA-USP20 mutant plasmids. Endogenous βARs were stimulated without or with 1 μM isoproterenol, lysed, and immunoblotted with antibody to HA. E, COS-7 cells were transiently transfected with HA-pcDNA3.0, HA-USP20, or HA-USP20-S333A and then were then stimulated with isoproterenol or left unstimulated. HA-USP20 was immunoprecipitated with HA-agarose beads, and the presence of phosphorylated forms in the immunoprecipitates (IP) was detected with a Ser(P)/Thr(P)-specific antibody (top panel). The levels of USP20 were detected in both immunoprecipitates (center panel) and lysates (bottom panel) using HA antibody.
USP20 (26, 27). Only modified Ub reversed the mobility of the endogenous USP20 lower band and converted it into the upper band present in quiescent cells (Fig. 1, H and I). As discussed in the following sections, this mobility shift of USP20 is correlated with its site-specific phosphorylation and deubiquitination of the β2AR.

Biochemical analyses revealed that USP20 is phosphorylated. When immunoprecipitated HA-USP20 was immunoblotted with a generic anti-Ser(P)/Thr(P) antibody, both upper and lower bands were detected as phosphorylated species (Fig. 2A). Bioinformatics analyses of USP20 predicted many serines and threonines as phosphorylation sites targeted by different kinases, of which we prioritized a list of sites specific for either PKA or GRK (Figs. 2B and 3, A and B), mainly because these kinases are rapidly activated after β2AR stimulation. Mutation of 10 or 12 of the PKA sites in USP20 to alanines, surprisingly blocked the agonist-induced mobility shift of USP20. However, a seven-site mutant construct still displayed the shift (Fig. 2B and C). Subsequent single-site mutagenesis targeting the three residues that are mutated in the 10-site but not the seven-site mutant revealed that phosphorylation of serine 333 is critical for the agonist-induced mobility shift of USP20. Mutation of serine 333 to alanine prevented the shift, whereas mutation to aspartate caused a constitutive shift (Fig. 2D and E). Furthermore, restoration of serine 333 into the 12 PKA site mutant construct (USP20 A333S) recovered the agonist-induced mobility shift of USP20 (Fig. 2F). Mutation of predicted GRK phosphorylation sites had no effect on USP20 protein mobility shift (Fig. 3A–D). Notably, elimination of phosphorylation at serine 333 had little effect on the overall phosphorylation status of USP20, suggesting that this mutation did not compromise USP20 protein folding or structure (Fig. 3E).

To determine whether serine 333 in USP20 is indeed phosphorylated in vivo and whether this is provoked by β2AR agonists, we generated a custom anti-Ser(P)-333 antibody (pUSP20) using the 12-amino acid phosphorylation domain (Asp-Arg-Lys-Phe-Ser(P)-Trp-Gly-Glu-Glu-Arg-Thr-Asn) as the peptide antigen. This antibody detected Iso-induced phosphorylation of endogenous USP20 and exogenously expressed HA-USP20 (Fig. 4A and B). On the other hand, the pUSP20

![FIGURE 4. Phosphorylation of USP20 at serine 333 is induced by isoproterenol and forskolin.](image-url)
USP20 Phosphorylation and β2AR Trafficking

antibody did not detect HA-USP20 S333A or only weakly detected HA-USP20 S333D, although all of these constructs were expressed at similar levels as the wild-type HA-USP20 (Fig. 4C). βAR antagonists blocked the agonist-induced serine 333 phosphorylation just as they blocked its mobility shift (Figs. 1D and 4D). Furthermore, conditions that are known to induce membrane recycling of internalized receptors, namely removal and repeated washouts of agonists (21, 28), not only dephosphorylated USP20 serine 333 but also reversed the agonist-induced mobility shift of USP20 (Fig. 4E).

Forskolin, which directly activates adenyl cyclase and increases cellular cAMP, also induced USP20 serine 333 phosphoxygenation and mobility shift, whereas the inactive analog dideoxy forskolin did not lead to these effects (Fig. 4, F and G). Previous studies have shown that cAMP signaling is impaired in mutant S49 lymphoma cells, which express a defective PKA regulatory subunit (29, 30). Correlating with the cAMP dependence, Iso-induced seryl 333 phosphorylation of USP20 is detectable in wild-type S49 lymphoma cells but not in PKA-defective cells (Fig. 5, A and B). Most cells including HEK293 cells, typically express the α and β isoforms of the PKA catalytic subunit. To define whether one or both these isoforms can phosphorylate USP20, we resorted to siRNA transfections to down-regulate individual PKA subunits. USP20 serine 333 phosphorylation was blocked upon siRNA-mediated knockdown of PKAα in HEK293 cells, whereas knockdown of PKAβ had no significant effect (Fig. 5, C and D). We further confirmed that USP20 is a direct substrate for PKA phosphorylation by performing in vitro phosphorylation assays with purified proteins. As shown in Fig. 5E, purified PKAα phosphorylated WT and serine 333 put back 12 phosphosite mutant (USP20 A333S) but not S333A or S333D constructs. On the basis of these data, we conclude that, upon βAR agonist-stimulation, activated PKAα rapidly phosphorylates USP20 specifically at serine 333 and that USP20 dephosphorylation could correlate with agonist removal and receptor recycling.

β2AR Deubiquitination by USP20 Is Regulated by Phosphorylation of Serine 333—We demonstrated previously that overexpressed USP20 reverses agonist-induced ubiquitination and lysosomal trafficking of the β2AR, whereas a USP20 mutant lacking deubiquitinase activity does not promote these effects (21). To ascertain whether serine 333 phosphorylation affects the deubiquitinase functions of USP20, we overexpressed WT, S333A, or S333D constructs in HEK293 cells stably transfected with FLAG-β2AR and analyzed isoproterenol-induced ubiquitination of the receptor. In the absence of exogenous USP20, isoproterenol stimulation produced robust β2AR ubiquitination. However, either wild-type USP20 or S333A overexpression led to deubiquitination of the receptor (Fig. 6, A and B). In contrast, S333D overexpression did not lead to receptor deubiquitination. Overexpression of USP20 wild-type or S333A significantly decreased the colocalization of β2ARs with the lysosomal marker protein LAMP2 and also significantly blocked degradation of β2ARs, as assessed by radioligand binding (Fig. 6, C−E). In contrast, overexpression of the USP20 S333D mutant, which did not reverse ubiquitination of the β2AR (Fig. 6, A and B) did not block either lysosomal trafficking or degradation of the β2AR (Fig. 6, C−E).

Concurrent to these effects, siRNA mediated knockdown of PKAα, which blocks USP20 serine 333 phosphorylation, also blocked (i.e. reversed) β2AR ubiquitination (Fig. 7, A and B). Down-regulation of PKAα also significantly decreased colocalization of β2AR and the lysosomal marker protein LAMP2 (Fig. 7, C and D). Accordingly, serine 333 phosphorylation blocks USP20 deubiquitinase activity, whereas dephosphorylation facilitates it. β2AR lysosomal trafficking is regulated by ubiquitin tags on the receptor (21, 22, 31), and the conditions that prevented β2AR ubiquitination, namely, (a) overexpression of USP20 wild-type and S333A constructs and (b) down-regulation of PKAα, significantly decreased colocalization of β2AR and the lysosomal marker protein LAMP2.

The above data suggest that the DUB activity of USP20 is closely linked to its phosphorylation status at serine 333. This site is located in the central domain of USP20 comprising a stretch of about 200 amino acids referred to as the "insertion
domain,” which is a variable region found in a subset of the USP family proteins (32). Interestingly, USP20 differs considerably from its close homolog USP33 within this variable domain. The activation mechanisms of DUBs are not fully understood. However, it has been proposed that DUB activity is initiated by substrate binding (33). To identify putative interaction domains in USP20 involved in binding the β₂AR, we generated USP20 deletion mutants spanning each of the protein’s domains and tested them in coimmunoprecipitation assays. As shown in Fig. 8A and B, β₂AR is able to associate with each of these USP20 subdomain mutants, including the central phosphorylation domain (amino acids 269–390), although to a lesser degree than the full-length protein. To verify whether USP20 phosphorylation within the variable domain affects its interaction with the β₂AR, we generated the deletion construct USP20 269–390 with S333A and wild-type constructs displayed association with the β₂AR, but S333D showed a significantly decreased interaction. These binding data strongly suggest that seryl 333-phosphorylated USP20 has lesser affinity for the substrate β₂AR than the dephosphorylated form and that this decrease in substrate affinity might be an added cause for the lack of DUB activity of USP20 S333D toward the β₂AR.

**Agonist-activated β₂ARS Traffic to Autophagosomes—**In HEK293 cells, degradation of exogenously expressed FLAG-
β₂ARs is detected as bands of smaller molecular sizes after isoproterenol stimulation for 4–6 h (21, 31). This isoproterenol-induced β₂AR degradation is blocked by inhibitors of lysosomal proteases (leupeptin) as well as by chloroquine and bafilomycin A1, which prevent the fusion between autophagosomes and lysosomes (Fig. 9A). This suggests that agonist-activated β₂ARs traffic via autophagosomes before being degraded in lysosomal compartments of mammalian cells and that this localization in autophagosomes might regulate the timing of receptor degradation in lysosomes. Autophagy is a cellular degradation process in which cytoplasmic components are sequestered into newly formed membrane compartments (autophagosomes) and delivered into lysosomes for degradation (34, 35). Autophagy requires the modification of two proteins, ATG12 and ATG8 (LC3), via enzymatic pathways that are similar to ubiquitin conjugation (36). Modification of ATG12 is required at the initiation steps of the autophagosome, whereas LC3 lipidation (conversion of LC3I to LC3-II by conjugation with phosphatidylethanolamine) marks mature autophagosomes prior to fusion with lysosomes. LC3-II, but not LC3I, is localized to vesicles and serves as a marker for autophagosomes and its stabilization, and detection is facilitated by blocking lysosomal proteases (leupeptin) or by preventing autophagosome-lysosome fusion (chloroquine or bafilomycin A1 incubation (37)).

To define whether β₂ARs traffic via autophagosomes, we next ascertained whether internalized β₂ARs colocalize with LC3-II. After 4 h of isoproterenol stimulation, β₂ARs internalize into late endosomes and lysosomes, whereas coexpressed GFP-LC3 is mostly diffusely distributed with few puncta (i.e., GFP-LC3-II) in each cell (Fig. 9B, top row). The serum-starving condition that was used in the degradation assays can mildly induce autophagy, which is indicated by the presence of LC3-II (Fig. 9A, lanes 1 and 2) and a small increase in colocalization of β₂AR and LC3-II (Fig. 9, B and C). When cells were treated with

**FIGURE 7. Knockdown of PKAα promotes β₂AR deubiquitination and lysosomal trafficking.** A, HEK293 cells with stable FLAG-β₂AR were transiently transfected with siRNAs targeting no mRNA (Ctrl) or PKAα for 48 h; serum-starved for 60 min, and then left unstimulated or stimulated with 1 μM isoproterenol for 15 min. The receptor was immunoprecipitated (IP) with M2 anti-FLAG affinity gel, and the ubiquitinated β₂AR was detected with an anti-ubiquitin antibody, FK1. The amounts of FLAG-tagged β₂AR are shown. Lysates from control or PKAα siRNA-transfected cells were immunoblotted and analyzed using antibodies against pUSP20, PKAα, and β₂-actin. β₂ ubiquitin smears in each lane from the blot in A were quantified, normalized to β₂AR signals, and plotted as bars. Significant differences between control versus PKAα siRNA-transfected cells in isoproterenol-stimulated lanes were determined using one-way ANOVA followed by Bonferroni post hoc test. ***, p < 0.001; Ctrl-Iso versus Ctrl-NS as well as PKAα siRNA samples. Data are mean ± S.E. of five independent experiments. NS, not stimulated. C, HEK293 cells stably expressing FLAG-β₂AR-mYFP were transiently transfected with siRNAs targeting a generic sequence that does not correspond to known mRNA (Ctrl) or PKAα for 48 h, serum-starved for 60 min, and stimulated with vehicle (nonstimulated) or Iso for 6 h. Cells were fixed, permeabilized, and immunostained for LAMP2 (red). Confocal images are shown with FLAG-β₂AR-mYFP in green and LAMP2 in red (Alexa Fluor 594). Scale bar = 10 μm. D, Pearson’s correlation coefficients calculated for β₂AR and LAMP2 colocalization (mean ± S.E.) in the respective cells for isoproterenol-stimulated and non-stimulated conditions. ***, p < 0.001; control versus PKAα siRNA, one-way ANOVA.
both isoproterenol and bafilomycin A1, we not only detected a dramatic redistribution of LC3-II to autophagosomes but also a robust colocalization of LC3-II and internalized \( \beta_2 \)AR (Fig. 9, B and C). A similar pattern of colocalization of \( \beta_2 \)AR and GFP-LC3-II was observed when cells were exposed to both isoproterenol and chloroquine, which blocks both \( \beta_2 \)AR and LC3-II degradation in the lysosomes (Fig. 9, B and C). When we used amino acid deprivation, which is a more potent inducer of autophagy than serum deprivation (23), isoproterenol induced similar \( \beta_2 \)AR degradation (Fig. 9, D), but increased the induction of LC3-II and promoted its robust colocalization with internalized \( \beta_2 \)ARs (Fig. 9, D–F). Under these conditions, addition of chloroquine or bafilomycin A1 did not further increase \( \beta_2 \)AR-LC3-II colocalization, although more LC3-II protein was detectable in immunoblots (Fig. 9, D–F). Interestingly, bafilomycin A1, which enhanced \( \beta_2 \)AR-LC3-II association under serum deprivation (Fig. 9, B and C), reciprocally decreased the colocalization of internalized \( \beta_2 \)ARs with LAMP2, a marker protein for late endosomes and lysosomes (Fig. 10, A and B).

Although bafilomycin A1 is widely used as an inhibitor of autophagy, it is actually a specific inhibitor of the vacuolar type H\(^+\)-ATPase (V-ATPase) in cells and inhibits the acidification and fusion of organelles containing this enzyme, such as lysosomes and endosomes (38). Therefore, both chloroquine and bafilomycin A1 potentially stall intracellular trafficking by inhibiting fusion of late endosomes or multivesicular bodies (which form the trafficking route for the \( \beta_2 \)AR via traditional endocytosis) with lysosomes or block the fusion of autophagosomes with any of these vesicles. If \( \beta_2 \)AR trafficking progressed only through the traditional endocytic route, we would not have

**FIGURE 8.** Phosphorylation of serine 333 in the insertion domain of USP20 regulates association with the \( \beta_2 \)AR. **A**, schematic showing the wild-type and truncated forms of USP20. **B**, HEK293 cells stably expressing FLAG-\( \beta_2 \)AR were transiently transfected with full-length or truncated forms of HA-USP20. Forty-eight hours after transfection, cells were starved for 60 min and left unstimulated or stimulated with 1 \( \mu \)M isoproterenol for 15 min. The receptors were isolated with M2 anti-FLAG affinity gel and immunoblotted with antibodies specific to HA (for USP20) and FLAG (M2). **C**, HEK293 cells stably expressing FLAG-\( \beta_2 \)AR were transfected with HA-pcDNA3.0, HA-USP20\(^{269-390}\), HA-USP20\(^{269-390}\)-S333A, or HA-USP20\(^{269-390}\)-S333D and stimulated with 1 \( \mu \)M isoproterenol for 15 min. The receptors were isolated with M2 anti-FLAG affinity gel and immunoblotted with antibodies specific to HA and FLAG (M2). **D**, quantification of USP20\(^{269-390}\)-WT and mutants in the lysates as detected by a HA antibody. *p < 0.05; **p < 0.01; between HA-pcDNA3.0 and others under isoproterenol-stimulated conditions; one-way ANOVA and Bonferroni post hoc test.

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observed a robust colocalization of internalized β2ARs with LC3-II, especially with bafilomycin A1, because most of the receptors would be localized in late endosomes (7), and most of the LC3-II would be associated with autophagosomes (23). Because our assays reveal a robust colocalization of β2ARs and LC3-II, it is likely that β2ARs divert from traditional endocytosis and traffic through autophagosomes when cellular autophagy is induced.

**FIGURE 9.** Agonist-activated β2ARs traffic to autophagosomes. A. HEK293 cells stably transfected with FLAG-β2AR were serum-starved for 60 min. After starvation, the cells were pretreated with 400 nM bafilomycin A1 (Baf) or 25 μM chloroquine (CQ) for 10 min and additionally stimulated without or with 1 μM isoproterenol for 4 h, then lysed and analyzed using antibodies against β2AR, LC3-A, and β-actin. B. HEK293 cells stably expressing FLAG-β2AR were transiently transfected with pEGFP-LC3. Forty-eight hours after transfection, cells were serum-starved for 60 min and then pretreated with 400 nM Baf or 25 μM CQ, followed by stimulation with 1 μM isoproterenol for 4 h. Cells were fixed and stained with anti-FLAG and Alexa Fluor 594 goat anti-rabbit antibodies. Confocal images are shown with FLAG-β2AR in red and GFP-LC3 in green. Scale bar = 10 μm. C. histogram showing Pearson’s correlation coefficients for the colocalization of β2AR (red) and GFP-LC3 (green). Data are mean ± S.E. **, p < 0.01 versus respective nonstimulated (NS) samples and vehicle + Iso, one-way ANOVA, Bonferroni comparison. D–F, the experiments in A–C were repeated with only one modification. Instead of serum deprivation, we incubated the cells in Hanks’ balanced salt solution to achieve amino acid starvation, which induces autophagy (60).

**USP20 Seryl 333 Phosphorylation and β2AR Ubiquitination Promote Receptor Trafficking through Autophagosomes**—We next tested whether USP20 overexpression and its DUB activity would affect β2AR-LC3-II colocalization in autophagic vesicles. We cotransfected GFP-LC3, vector, or USP20 (either wild-type, S333A, or S333D) and determined the localization of each protein in unstimulated and agonist-stimulated cells by confocal microscopy. In the absence of isoproterenol stimulation, we
detect only a small amount of colocalization of the \( \beta_2 \)AR and GFP-LC3-II with bafilomycin A1 (Fig. 11A, Baf, fifth column) or chloroquine (Fig. 11C, CQ, fifth column). However, isoproterenol stimulation along with chloroquine or bafilomycin A1 induced robust colocalization of the internalized \( \beta_2 \)AR and GFP-LC3-II in cells transfected with vector (endogenous USP20) or with S333D (where the DUB activity is inhibited). On the other hand, overexpression of USP20 wild-type or S333A significantly decreased colocalization of GFP-LC3-II and internalized \( \beta_2 \)ARs (Figs. 11, A–D). These data suggest that USP20 activity blocks or reverses \( \beta_2 \)AR trafficking at the autophagosomes en route to the lysosomes.

We also determined whether \( \beta_2 \)AR and LC3-II show protein-protein interaction in addition to localizing in the same subcellular compartment. We used either serum deprivation or amino acid deprivation and immunoprecipitated FLAG-\( \beta_2 \)ARs from cells treated with vehicle or Iso and assessed binding of endogenous LC3I and LC3-II. LC3I displayed minimal binding with the \( \beta_2 \)AR. However, LC3-II displayed an agonist-dependent interaction that was increased dramatically in cells starved of amino acids (Fig. 12, A and B). Addition of either bafilomycin A1 or chloroquine enhanced the association of LC3-II and \( \beta_2 \)ARs under serum deprivation but not under amino acid deprivation (Fig 12, A and B). This is probably because, under amino acid deprivation, the association of \( \beta_2 \)AR and LC3-II reached a maximum level even in the absence of these inhibitors, as also seen in the colocalization assays (Fig. 9, E and F). Additionally, to evaluate whether endogenously expressed \( \beta_2 \)ARs can bind LC3-II, we performed the same coimmunoprecipitation using primary vascular smooth muscle cells by employing methods we have reported previously (22). We detected isoproterenol-induced LC3-II binding with the \( \beta_2 \)AR, which was enhanced by the addition of either bafilomycin A1 or chloroquine (Fig. 12C).

Because the effects of S333A and S333D on \( \beta_2 \)AR-LC3II colocalization (Fig 11) paralleled their effects on \( \beta_2 \)AR ubiquitination (Fig. 6, A and B), we tested whether receptor ubiquitination could serve as a signal for its trafficking to autophagosomes. To address this, we compared wild-type \( \beta_2 \)AR and a \( \beta_2 \)AR mutant that is defective in ubiquitination (\( \beta_2 \)AR-0K) for LC3-II interaction. Previous studies have shown that this mutant receptor is not ubiquitinated by agonist stimulation (16, 22, 31, 39) and does not colocalize with LAMP2 in lysosomes (31). \( \beta_2 \)AR-0K is also impaired in LC3-II interaction (Fig. 12, D and E), suggesting that \( \beta_2 \)AR ubiquitination facilitates receptor trafficking to autophagosomes, and the robust LC3II-\( \beta_2 \)AR colocalization observed with coexpression of S333D is attributed to decreased DUB activity and stabilization of \( \beta_2 \)AR ubiquitination. In this scenario, ubiquitin moieties on the \( \beta_2 \)AR...
could function as “recognition tags” for LC3-II interaction and selective targeting of cargo to autophagosomes (40).

**DISCUSSION**

βARs signal through heterotrimeric G proteins and PKA as well as through β-arrestins via distinct mechanisms and ligand-induced conformations (41–44). β-Arrestins have multifaceted roles and act as adaptors for enzymes that degrade second messengers (desensitization), function as scaffolds for protein kinases (signal transduction), and serve as adaptors for endocytic and ubiquitin pathway components (intracellular trafficking) (45, 46). We discovered an unexpected convergence of the second messenger activated PKA and the β-arrestin-dependent trafficking pathways. Our data suggest that both facilitate the
lysosomal trafficking of agonist-activated β₂AR and that they do so by acting at distinct steps of posttranslational regulation of the β₂AR. As reported previously, β-arrestin promotes ubiquitination of the agonist-bound receptor by escorting the E3 ligase Nedd4 (19, 20), and, as shown in this work, PKA stabilizes ubiquitination of the β₂AR by phosphorylating and deactivating the cognate deubiquitinase USP20. This mode of deactivation of USP20 could be applicable to other GPCR-associated kinases because serine 333 containing a phosphorylation domain is a predicted site of phosphorylation for the AGC kinase sub family of protein kinases, which includes PKA, PKC, and PKG. Serine 333 phosphorylation of USP20 could, therefore, be a general regulatory mechanism in GPCR trafficking, although, so far, only an interaction between USP20 and β₂AR has been documented.

Upon agonist activation, most GPCRs internalize into vesicles called endosomes and may do so by different mechanisms at the plasma membrane (clathrin-coated vesicles, caveolae, uncoated vesicles, etc.) (47). However, upon localizing to endosomes, studies so far predict a direct route for degradation to lysosomes via late endosomes. This work reveals an unexpected navigation of activated receptors via autophagosomes. Autophagy is a distinct pathway of cellular degradation in which specific signals initiate formation of a phagophore that surrounds a variety of cytoplasmic components, including organelles, to form autophagosomes that then fuse with lysosomes to form autolysosomes (36). The colocalization and association of internalized, ubiquitinated β₂AR with the autophagosome-specific marker LC3-II suggests that internalized receptors move through autophagosomes prior to lysosomal degradation.

Recent studies have implicated regulation of autophagy by taste receptors and regulation of neuronal cannabinoid receptors by beclin2, a component of the autophagy pathway, in an autophagy-independent manner (48–50). Our findings illustrate that, in addition to these mechanisms, both autophagy and endocytic trafficking orchestrate the destruction of internalized β₂ARs in a ubiquitin-dependent manner. Because recent studies suggest that selective autophagy, a pathway choreographed by specific protein interactions, is guided by ubiquitin and ubiquitin-binding domains (51), it is likely that USP20 may have unappreciated roles in deubiquitinating components or interactors of its substrate, β₂AR.
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autophagy-associated proteins in addition to its effects of deubiquitinating receptor cargo.

Site-specific phosphorylation of USP20 by PKA within the unique insertion domain is a novel mechanism of “turning off” this deubiquitinating enzyme. In general, DUBs are expressed as fully processed enzymes, and ongoing research is targeted toward understanding how these enzymes, which are synthesized and expressed in their active form, refrain from nonspecific and random deubiquitination of cellular proteins (33). DUBs and their activity have been designated as cryptic in nature, and their activation has been proposed to be triggered upon substrate binding and posttranslational modifications or by protein-protein interaction (52–55). About 85 DUBs are expressed in human cells, and the USP subfamily with about 55 members represents the largest among the five families of DUBs. Each USP may affect a subset of proteins expressed in cells (56) and, so far, a handful of proteins, including the βARs have been identified as physiological substrates of USP20. Nonetheless, it is very likely that, with respect to each of these substrates, USP20 could have specific binding, activation, and deactivation patterns or switches. For the deubiquitination of the β2AR, USP20 phosphorylation by PKA and dephosphorylation by a cellular phosphatase could serve as reversible off/on switches. It remains to be determined whether this phosphatase is scaffolded by β-arrestins, as has been shown for regulating dopamine signaling via the D2 dopamine receptor (57). An imbalance between USP20 phosphorylation/dephosphorylation can perhaps serve as a causative factor toward disease progression, as may be occurring in βAR desensitization in heart failure, where USP20 serine phosphorylation may be irreversible.

That βAR expression is diminished in failing hearts has been known for decades, but the factors or specific trafficking signals that affect βAR degradation in heart failure remain unknown. Our studies suggest that stimulation of cellular autophagy may also provoke β2AR trafficking through autophagosomes. Autophagic activity is augmented in failing hearts (58), but whether this pathway is beneficial or detrimental for cardiomyocyte survival remains controversial (59). Deciphering the relationship between induction of autophagy, trafficking of βARs, and activity of USP20 in healthy versus diseased hearts constitutes an important direction for future research.

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