Resensitization of G protein-coupled receptors (GPCR) following prolonged agonist exposure is critical for restoring the responsiveness of the receptor to subsequent challenges by agonist. The 3′-5′ cyclic AMP-dependent protein kinase (PKA) and serine 312 in the third intracellular loop of the human β1-adrenergic receptor (β1-AR) were both necessary for efficient recycling and resensitization of the agonist-internalized β1-AR (Gardner, L. A., Delos Santos, N. M., Matta, S. G., Whitt, M. A., and Bahouth, S. W. (2004) J. Biol. Chem. 279, 21135–21143). Because PKA is compartmentalized near target substrates by interacting with protein kinase A anchoring proteins (AKAPs), the present study was undertaken to identify the AKAP involved in PKA-mediated phosphorylation of the β1-AR and in its recycling and resensitization. Here, we report that Ht-31 peptide-mediated disruption of PKA/AKAP interactions prevented the recycling and functional resensitization of heterologously expressed β1-AR in HEK-293 cells and endogenously expressed β1-AR in SK-N-MC cells and neonatal rat cortical neurons. Whereas several endogenous AKAPs were identified in HEK-293 cells, small interfering RNA-mediated down-regulation of AKAP79 prevented the recycling of the β1-AR in this cell line. Co-immunoprecipitations and fluorescence resonance energy transfer (FRET) microscopy experiments in HEK-293 cells revealed that the β1-AR, AKAP79, and PKA form a ternary complex at the carboxyl terminus of the β1-AR. This complex was involved in PKA-mediated phosphorylation of the third intracellular loop of the β1-AR because disruption of PKA/AKAP interactions or small interfering RNA-mediated down-regulation of AKAP79 both inhibited this response. Thus, AKAP79 provides PKA to phosphorylate the β1-AR and thereby dictate the recycling and resensitization itineraries of the β1-AR.

The β1-AR is a major receptor for the physiological regulation of cardiac function by the sympathetic nervous system and plays an important role in clinical management of hypertension and heart failure (2). Agonist-mediated activation of the β1-AR results in the generation of intracellular cyclic AMP and in the activation of PKA, which in turn phosphorylates numerous intracellular targets that mediate the familiar effects of β-agonists (3). As a consequence of their activation, the β1-AR and other G protein-coupled receptors (GPCR) can undergo desensitization, which is characterized by attenuation of GPCR signaling intensity (4). The biochemical mechanisms of desensitization are numerous, but appear to be initiated by the phosphorylation of the agonist-occupied GPCR by G protein-coupled receptor kinases (GRK), followed by uncoupling of the GPCR from its cognate G protein by β-arrestins, which culminates in the internalization or sequestration of the GPCR away from its signaling platform (5, 6). Internalization of the GPCR appears to produce different outcomes depending on the type of GPCR and cell line under study. For β1-AR, β2-AR, and other GPCR, internalization is a prerequisite for resensitization because intracellular trafficking and subsequent recycling of resensitized GPCR promotes their insertion into the cell membrane to maintain agonist responsiveness (1, 7–9). For the δ-opioid, other GPCRs, the internalized receptors do not recycle back, instead they are retained intracellularly and later degraded either by lysosomal or proteasomal pathways (10–12).

Recently, PKA and its putative substrate, serine at position 312 (Ser112) in the third intracellular loop (3rd IC) of the human β1-AR, were found to be critical determinants of the ability of the β1-AR to recycle and resensitize (1). Inhibition of PKA or mutagenesis of Ser112 to alanine (S312A) prevented the recycling and resensitization of the agonist-internalized β1-AR (1). PKA-mediated resensitization of the β1-AR is termed “homologous resensitization” because PKA is activated through the recycle and resensitization of the agonist-occupied receptor.
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β1-AR signaling pathway. Therefore, desensitization and resensitization of the β1-AR are orchestrated through interplay between GRK- and PKA-mediated phosphorylation of this receptor. The involvement of kinases such as GRK, PKA, PKC, and phosphatidylinositol 3-kinase in setting of the trafficking itinerary of GPCR and other cell surface proteins is well established (6, 8). For example, PKA-mediated phosphorylation of the cystic fibrosis transmembrane conductance regulator at Ser753 is involved in its recycling, which otherwise is impaired in cystic fibrosis (13). Likewise, PKA is involved in vasopressin-mediated translocation of the water-channel forming protein aquaporin-2 from the intracellular compartment into the plasma membrane, as well as in the trafficking of the AMPA receptor and its insertion into neuronal membranes (14, 15).

PKA is assembled as a tetramer composed of two regulatory (R) and two catalytic (C) subunits. Eukaryotic cells express four isoforms (RI α, β, and RII α, β) of the R subunit and three isoforms (α, β, γ) of the C subunit (16). The RIα and RIIα subunits are ubiquitously expressed, whereas the expression of RIβ and RIIβ is more restricted (16). PKA is a kinase with broad substrate specificity that is involved in numerous biological events (17). The fidelity of PKA-mediated phosphorylation of target proteins is regulated by spatial mechanisms that target PKA toward its substrate and by temporal mechanisms involving phosphodiesterases that degrade cyclic AMP to limit the duration of the biological effects of PKA (18, 19).

The PKA holoenzyme is targeted near potential substrates principally via RII subunit association with AKAPs (20). Each AKAP contains a PKA binding site and a unique subcellular targeting domain that restricts its location within the cell to either unique cellular compartments or specific substrates (20). Many AKAPs serve as organizing centers for signal transduction either by linking upstream signal generators to downstream targets or by recruiting multiple signaling enzymes within signaling hubs (18, 20). In this study, we examined whether the effects of PKA on recycling of the β1-AR were dependent on PKA anchoring and identified AKAP79 as the specific AKAP involved in recycling and resensitization of the β1-AR in HEK-293 cells.

EXPERIMENTAL PROCEDURES

Cell Cultures—Human embryonic kidney 293 (HEK-293) cells and neuroepithelioma SK-N-MC cells were obtained from American Type Culture Collection (Manassas, VA). HEK-293 and SK-N-MC cultures were maintained in DMEM with 10% fetal bovine serum (HyClone, Logan, UT). Cortices from 1-day-old rats were dissociated by papain treatment, triturated through Pasteur pipettes, and suspended in media consisting of minimal essential medium with 10% fetal bovine serum and plated at a density of 10^6 cells/ml (21). After 6 h, cells were washed with Neurobasal media with B27 supplement and 0.5 mM 1-glutamine.

Construction of FLAG-tagged or Myc-tagged β1-AR—To allow rapid assessment of cell surface expression of the β1-AR, the NH2-terminal initiator methionine was replaced either by the FLAG (DYKDDDDK) or Myc (EQKLISEEDL) sequences, resulting in N-FLAG/Myc-tagged WT β1-AR. The 8-amino acid FLAG epitope sequence (between parentheses) was inserted at the NH2 terminus of the β1-AR by the polymerase chain reaction using a sense primer 5'-AAGCTT(ATGGGACT-AACAGGACGACGATCAGAAGCGCGGGGCTCT-GCTTGGGC)-3' and antisense primer 5'-CATGATCTTCA-CACCTTGGATTCGAGGCGAACGCGACGCGG-3' and the antisense primer described earlier. The 1.5-kb β1-AR cDNA flanked with HindIII (5') and EcoRI (3') sites was cloned into the multiple cloning site of mammalian expression vector pcDNA3.1 (Invitrogen). To generate the β1-AR (1–424) construct, the full-length β1-AR cDNA was cut with SmaI and the resulting 1.3-kb cDNA was cloned into pcDNA3.1. Sequences of the epitope-tagged β1-AR were verified by dideoxy sequencing.

Construction of Fluorescently Tagged β1-AR, AKAP79, and RIIα Subunit—The coding sequence of the FLAG-tagged WT β1-AR was amplified by PCR using synthetic oligonucleotides to introduce a 5' HindIII site, followed by the coding sequence and then by a 3' BamHI site. The amplification primers for the β1-AR were: forward primer (5'-AAGCTTATGGACTACACAGAGCCAGCATGACGATCAGAAGCGCGGGGCTCTGCTTGGGC)-3' and reverse primer (TGGATCCACCTTGGATTCCGAGGCGGCGGAACGCGGC)-3'. The resulting 1.5-kb HindIII-BamHI cDNA was fused in-frame 5' to the CFP/YFP coding sequence in the pECFP-N1 and pEYFP-N1 vectors (BD Bioscience) to generate NH2-terminal fusions of the β1-AR to CFP and YFP. For AKAP79, the coding sequence of human AKAP79 was amplified by PCR using a forward primer (5'-AAGCTTATGGGAAAACACAAATTTCAAGA)-3' and a reverse primer (3'-TGGATTCTGAGAAGATTGTTTATTT)-3' to generate 1.6-kb HindIII-EcoRI cDNA, which was later fused into the pECFP-N1 and pEYPF-N1 vectors. Expression of the fusion proteins was confirmed by fluorescence microscopy and Western blot analysis. The cloned sequences were verified by DNA sequencing.

Mouse PKA-RIα in the pECFP and pEYFP vectors (22) was provided by Mark Dell’Acqua (University of Colorado HSC, Denver, CO).

Antibodies, siRNA, Peptides, and Additional Reagents—The antibodies against FLAG (M2) and Myc (9E-10) epitopes were purchased from Sigma and Upstate (Charlottesville, VA), respectively. The monoclonal antibodies to human AKAP79 and to the various subunits of PKA were from BD Bioscience. st-Ht31 and st-Ht31-pro peptides were obtained from Promega Corp. The anti-β1-AR (A-20 and V-19) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and the anti-rat AKAP150 antibody was from Upstate (Charlottesville, VA). AKAP79 siRNA (AAggagacagacagaaugu) corresponding to nucleotides 48–66 in human AKAP79 or its scrambled control siRNA (AAggagacacagaauggu) and human gravin siRNA (cgaggcgccgccagacacc) corresponding to nucleotides 161–181 or its scrambled control (ggaggccgccagacacc) were synthesized by Dharmacon Corp. (Lafayette, CO). siRNAs were transfected into a concentration of 50–100 nM into HEK-293 by the Lipofectamine 2000™ transfection reagent (Invitrogen). After 2 days, cell extracts were probed for AKAP79 or gravin expression by Northern and Western blots. To determine the
effect of the siRNAs on recycling of the WT β₁-AR, cells stably expressing the FLAG-tagged β₁-AR were transiently transfected with 100 nM of each duplex siRNA for 2 days, before conducting the confocal recycling assay described below.

**Acid Strip Confocal Recycling Microscopy Protocol**—HEK-293 cells stably expressing the FLAG- or Myc-tagged WT β₁-AR were grown on poly-L-lysine-coated glass coverslips and serum-starved at 37 °C for 1 h in DMEM supplemented with 25 mM HEPES, pH 7.4. The receptors were labeled with fluorescein isothiocyanate-conjugated, anti-FLAG M2 IgG (10 μg/ml) for 1 h at 37 °C. Cells were treated with 10 μM isoproterenol for 30 min at 37 °C to promote agonist-mediated receptor internalization. Then the cells were chilled in 4 °C Tris-buffered saline to stop endocytosis, and exposed to 0.5 M NaCl, 0.2 M acetic acid (pH 3.5) for 4 min on ice to remove antibody bound to extracellular β₁-AR (1, 15, 23). Cultures were quickly rinsed in warm DMEM supplemented with HEPES, then incubated with 100 μM of the β₁-antagonist alprenolol at 37 °C for 10, 20, 30, or 45 min to establish the recycle time. After each time period, the coverslips were rinsed and fixed in 4% paraformaldehyde with 0.1% sodium azide for 1 h at 37 °C. Cellsweretreatedwith10

**Dual Confocal Microscopy**—HEK-293 cells stably expressing the Myc-tagged β₁-AR were transiently transfected with AKAP79-GFP. The cells were treated with 10 μM isoproterenol for 30 min, acid washed, and then exposed to 100 μM alprenolol for 30 min or 1 h. The coverslips were fixed with 4% paraformaldehyde and stained with Cy3 conjugated to 9E10 anti-Myc tag antibody and visualized by dual confocal microscopy (GFP, λ<sub>ex</sub> = 488 nm, λ<sub>em</sub> = 505–530 BP; Cy3, λ<sub>ex</sub> = 543 nm, λ<sub>em</sub> = 560 LP) using LSM-510 multitracking configuration.

**FRET Microscopy**—These experiments were performed on live or fixed cells using sensitized emission and acceptor photobleaching methods, respectively (25, 26). Double stable cell lines expressing AKAP79-CFP and β₁-AR-YFP, AKAP79-YFP and RII PKA-CFP, and β₁-AR-YFP and RII PKA-CFP were established. In some cases, HEK-293 cells were transfected with the desired plasmids using Lipofectamine for 24–36 h. For live cell microscopy, transiently transfected or double-stable cells were plated onto glass-bottom Petri dishes (Mat-Tek, Ashland, MA) for 24 h and imaged live at room temperature. For fixed cell microscopy, cells were plated on poly-L-lysine-covered coverslips for 24 h. The coverslips were washed with PBS, fixed with 4% paraformaldehyde, pH 7.4, and mounted onto glass slides in Fluoromount G mounting media (Electron Microscopy Sciences, Hatfield, PA). Coverslips were sealed with clear nail polish and imaged within 24 h after fixation.

**Sensitized Emission FRET Microscopy**—FRET was recorded using the three-channel sensitized emission mode (27, 28). Donor channel (CFP) was acquired using donor excitation (λ = 458 nm) and donor emission (λ = 475–525 nm) with BP filter. Acceptor channel (YFP) was acquired using acceptor excitation (λ = 514 nm) and emission (λ = 530 nm) with LP filter. FRET was acquired using excitation (λ = 458 nm) and emission (λ = 530 nm) with LP filters. Images were taken from donor, acceptor, and FRET samples. Donor and acceptor images were used to evaluate the cross-talk of signals that is caused by image settings and fluorophore properties. The same acquisition parameters were used for donor, acceptor, and FRET samples (28).

**FRET Calculations**—LSM 510 FRET Macro tool was used to calculate FRET values. FRET is a measure of FRET that is normalized for the concentrations of donor and acceptor fluorophores and therefore represents a fully corrected measure of FRET (27). Quantitative comparisons of different FRET methods has determined that FRET provides the most accurate measure of FRET efficiencies (29). In this method the corrected FRET value for each pixel is calculated and then divided by concentration values for donor and acceptor (27). FRET was calculated on a pixel-by-pixel basis for the entire image and in regions of interest (marked by rectangles) using Equation 1.

\[
\frac{\text{FRET}}{DfD \times A_{f}} \propto \frac{[\text{bound}]}{[\text{total d}] \times [\text{total a}]} \quad \text{(Eq. 1)}
\]

The equation indicates the proportional (∞) relationship between FRET and the concentrations of the interacting and noninteracting species. In the equation [bound] represents the concentration of interacting pairs of donor labeled species and acceptor labeled species. The values for [total d] and [total a] represent the total concentrations (interacting and non-interacting) of the donor and acceptor labeled species, respectively. FRET is proportional to the FRET signal from the specimen. DfD is the donor signal that would take place if no FRET occurred and is therefore proportional to the total concentra-
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...tion of donor. \( \text{Afa} \) is the acceptor signal that would take place if no FRET occurred and is therefore proportional to the total concentration of acceptor.

Donor and acceptor coefficients were determined in the beginning of each experiment and kept the same throughout. Donor, acceptor, and FRET thresholds were set to determine the background value. Threshold values were subtracted from all pixels before FRET calculations. Extreme values were excluded from both, the FRET image as well as data table calculation. FRETN images are presented in pseudocolor mode.

**Acceptor Photobleaching FRET Microscopy**—Changes in the intensity of the donor channel were observed upon complete photobleaching the acceptor (YFP) by a 514-nm argon laser (30). During each photobleaching session, we obtained an image set consisting of time-lapse recordings of donor and acceptor channel intensities. FRET was recorded by examining the quenching of CFP during YFP photobleaching. FRET images were analyzed by the LSM FRET tool version 1.5 (AIM software release 3.2) to calculate the FRET efficiencies using selected area averages for donor CFP before and after bleaching: \[ \text{FRET Efficiency} = \frac{(\text{donor CFP after bleaching} - \text{donor CFP before bleaching})}{\text{donor CFP after bleaching}} \]

Thresholds were set for donor (CFP), acceptor (YFP), and FRET images at the beginning of data collection and the threshold was kept the same for the entire data analysis. FRET efficiencies (%) are presented as mean ± S.E. from 3 to 10 separate acquisition experiments on 5–10 images per experiment.

**Effect of Disrupting AKAP-PKA Interactions on the Recycling of the Biotinylated \( \beta_1 \)-AR in Neuronal Cells**—\( \beta_1 \)-AR recycling in human neuroepithelium SK-N-MC cells and in neonatal rat cortical neurons was measured by the loss of internalized biotinylated \( \beta_1 \)-AR. In this assay, the cells were incubated with 50 \( \mu \)M st-Ht31 or st-Ht31-pro for 30 min at 37 °C to inhibit PKA-AKAP interactions. Then cell surface proteins were biotinylated by incubating the cells in ice-cold Hanks' balanced salt solution supplemented with 1.5 mg/ml of sulfo-NHS-SS-biotin (Pierce Biotechnology) for 20 min, followed by quenching of excess biotinylation reagent with glycine. Biotinylated cells were rewarmed to 37 °C in complete culture medium and exposed to isoproterenol for 30 min at 37 °C to induce the internalization of the \( \beta_1 \)-AR, followed by chiling of each culture dish on ice to stop membrane trafficking. The remaining surface biotin was quantitatively cleaved with glutathione cleavage buffer (50 mM glutathione in 75 mM NaCl and 10 mM EDTA containing 1% bovine serum albumin and 0.075 N NaOH) twice for 15 min at 4 °C. Cultures were then warmed to 37 °C in complete culture medium containing 10 \( \mu \)M of the \( \beta_1 \)-antagonist alprenolol and 10 \( \mu \)M of each st-Ht31 peptide for 15, 30, and 60 min to allow the internalized \( \beta_1 \)-AR to recycle back to the cell surface (1). After each time period, the cells were quickly chilled on ice and incubated for a second time with glutathione cleavage buffer to ensure complete cleavage of any newly appearing (recycled) surface biotin. Then the cells were lysed in radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 1% (v/v) Triton X-100 0.1% SDS, 10 mM NaF, 10 mM Na₃-pyrophosphate, and protease inhibitors). Equal amounts of protein from these cells were mixed with 50 \( \mu \)l of bovine serum albumin-blocked ultralink-neutra avidin beads (Pierce Biotechnology) at 4 °C overnight. The resin was collected by centrifugation, washed several times with lysis buffer, and then extracted with 10 \( \mu \)l/100 \( \mu \)g of input protein of 2× Laemml sample buffer with 40 mM dithiothreitol at 37 °C for 40 min. The supernatant was subjected to electrophoresis on SDS-containing 4–12% gels, transferred to nitrocellulose, and probed with 1:250 dilution of the anti-\( \beta_1 \)-AR antibody and visualized with horseradish peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare) using the Super Signal detection reagent (Pierce). The density of the \( M_\text{r} \) 73 kilodaltons \( \beta_1 \)-AR from each condition was quantified using a Chemi DOC XRS densitometer (Bio-Rad) equipped with Quantity One software.

**Co-immunoprecipitations, Immunoblotting, and Overlay Assays**—For co-immunoprecipitations, the cells were washed three times in PBS buffered with 10 mM HEPES, pH 7.5 (H/PBS), then incubated for 30 min at room temperature in cross-linking buffer (H/PBS, 1 mM dithiobis(succinimidyl pro- pionate)) before quenching with H/PBS containing 20 mM glycine. Cells were lysed in RIPA buffer and the insoluble cellular debris was removed by centrifugation at 14,000 \( \times g \), for 15 min at 4 °C. After equalizing protein concentrations across all samples, lysates were added to M2 anti-FLAG- or anti-Myc-agarose beads at 4 °C with gentle rotation overnight. Control experiments were performed by incubating lysates with preimmune IgG at the same concentration at 4 °C overnight. Rat brain extracts were prepared by Dounce homogenization in RIPA buffer, followed by clarifying the lysate by centrifugation at 14,000 \( \times g \), for 30 min at 4 °C. For each milliliter of lysate, 40 \( \mu \)l of anti-\( \beta_1 \)-AR or anti-AKAP79 antibodies were added for ~16 h, then 40 \( \mu \)l of protein G-agarose were added for another 4 h at 4 °C. The immune complexes from all the cells were washed three times in RIPA buffer and eluted from the beads with 2× Laemml sample buffer containing 100 \( \mu \)M dithiothreitol and 10% 2-mercaptoethanol at 37 °C for 45 min. Resolved proteins and lysate representing between 2 and 5% of input were separated by SDS-PAGE under denaturing conditions and electroblotted to nitrocellulose. Identical gels were run and transferred for separate detection of receptor, AKAP79, or the RII subunits of PKA. AKAP79 and RII subunits were detected with the anti-AKAP79 or anti-RII\( \alpha \) and RII\( \beta \) monoclonal antibodies. The \( \beta_1 \)-AR in SK-N-MC cells was detected with the A-20 anti-\( \beta_1 \)-AR antibody and the \( \beta_1 \)-AR in rat brain or neonatal rat cortical neurons was detected with the V-19 anti-\( \beta_1 \)-AR antibody. AKAP150 in rat neonatal cortical neurons was detected by the anti-rat AKAP150 antibody from Upstate (Charlestown, VA).

**Far Western and Pulldown Assays**—The overlay assay between HEK-293 cell extracts and the \(^{32}\text{P}\)-labeled RII\( \alpha \)-subunit of PKA were performed as described (31).

The human \( \beta_1 \)-AR cDNA was digested with Smal and Xhol to isolate the 150-bp carboxyl terminus fragment encoding amino acids between 425 and 477. This fragment was cloned in-frame into the glutathione S-transferase (GST) pGEX-4T-2 vector (GE Healthcare) and amplified in BL-21 Escherichia coli cells. HEK-293 cells that were transfected either with pcDNA 3.1 or Myc-AKAP79 in pcDNA 3.1 were lysed with PBS, 0.2% Triton X-100 supplemented with protease inhibitors. After 16,000 \( \times g \), centrifugation of cell lysates, GST or GST-\( \beta_1 \)-AR-
(424 – 477) fusion proteins were added to aliquots of the supernatants. Twenty μl of glutathione-agarose beads (50% slurry in H2O) were added after mixing for 30 min at 4°C. The mixture was rotated for another 2 h at 4°C. After washing three times with the same lysis buffer, the proteins were eluted from beads with sample buffer (containing 2.5% mercaptoethanol). Eluates were separated on a 4–15% gel and analyzed for AKAP79 by immunoblotting.

Adenylyl Cyclase Assays for β-AR Desensitization and Resensitization—HEK-293 cells stably expressing the WT β1-AR were transiently transfected with 100 nM AKAP79 siRNA or its scrambled control for 2 days. In the experiments using the st-Ht31 peptide, cells expressing the WT β1-AR were exposed to 50 μM st-Ht31 or st-Ht31-pro at 30 min prior to initiating the experiment. For each condition, four identical sets of cells were set up; the first set was used as control for desensitization and the second set for resensitization assays. Cells for desensitization were exposed to 1 mM ascorbic acid (control) or 10 μM isoproterenol for 10 min at 37°C, and then washed with serum-free DMEM supplemented with 10 mM HEPES and processed for the preparation of membranes as described below. The third set was used as the control for resensitization and the fourth set for resensitization assays. Cells for resensitization were exposed either to 1 mM ascorbic acid (control) or 10 μM isoproterenol for 3 h at 37°C and then washed with serum-free DMEM supplemented with 10 mM HEPES. The cells were then incubated with 100 μM alpenrolon for 1 h at 37°C, and then washed with serum-free DMEM supplemented with 10 mM HEPES. Membranes were prepared from all cells by hypotonic lysis of the cells with 20 mM HEPES, pH 7.4, 2 mM MgCl2, 1 mM EDTA, and 1 mM 2-mercaptoethanol supplemented with 10 μg/ml leupeptin and 10 μg/ml aprotinin for 10 min on ice. The cells were transferred into a glass–glass homogenizer and lysed by 30 up and down stokes. Cell lysates were centrifuged at 2,500 × g for 5 min to pellet the nuclei and the supernatant was centrifuged at 15,000 × g for 20 min to pellet the membranes. Then 50 μg of membrane proteins were incubated at 30°C in a final volume of 0.1 ml containing 50 mM Tris-HCl, pH 7.4, 1 mM MgCl2, 10 mM phosphocreatine, 1 mM cyclic AMP, 2 mM 2-mercaptoethanol, 1 mg/ml bovine serum albumin, 0.4 mM EGTA, 2 mg/ml creatine kinase, 0.2 mM ATP containing 1 μCi of [α-32P]ATP, 1 mM GTP and the various concentrations of isoproterenol. The assay was initiated by the addition of membranes and terminated after 10 min (32). The cyclic AMP that formed was isolated by column chromatography and quantified by liquid scintillation counting. Assays were performed in triplicate and replicated 4 times. The Kact ± S.E. for each β1-AR was calculated using the GraphPad Prism 4 program and statistical comparisons were analyzed using GraphPad Prism 4 and Instat programs.

Phosphorylation and Phosphopeptide Mapping of the β1-AR—To determine the effect of disrupting AKAP-PKA interactions on isoproterenol-mediated phosphorylation of the β1-AR, HEK-293 cells expressing the WT β1-AR were pretreated with 50 μM st-Ht31 or 50 μM st-Ht31-pro for 30 min. To determine the effect of down-regulating AKAP79 on isoproterenol-mediated phosphorylation of the β1-AR, HEK-293 cells expressing the WT β1-AR were transiently transfected with 100 nM control or AKAP79 siRNA for 2 days. On the day of the experiment, culture plates were switched to phosphate-free DMEM supplemented with 25 mM HEPES, pH 7.4, for 1 h. The medium was supplemented with 200 μg of 32PO4/ml for 1.5 h to label the ATP pools. The cells were stimulated with either 1 mM ascorbic acid or 10 μM isoproterenol in 1 mM ascorbic acid for 10 min at 37°C. The medium was aspirated and the cells were lysed in RIPA extraction buffer, then centrifuged at 14,000 × g. Equivalent amounts of proteins in each supernatant were incubated with M2 anti-FLAG-agarose beads at 4°C for 5 h. The resin was washed in RIPA buffer, and the proteins were eluted in 2× Laemmli sample buffer supplemented with 40 mM dithiothreitol and resolved by SDS-polyacrylamide gel electrophoresis in 10% gels. The gels were transferred to nitrocellulose in 0.1% sodium vanadate to minimize the dephosphorylation of the receptor during transfer. The amounts of 32P-incorporated into the β1-AR were determined by densitometric scanning of the blots with Packard Instamager™. The bands corresponding to phosphorylated β1-AR protein on the filter were cut out, and submersed in 0.5 ml of 70% (v/v) formic acid containing 100 mg/ml of cyanogen bromide (Science Lab Chemicals, Kingswood, TX) for 1.5 h at room temperature (33). At the end of the digestion, the samples were dried in a SpeedVac concentrator. The dried peptides were dissolved in Tricine sample buffer and subjected to electrophoresis on 16% acrylamide gels in Tricine cathode buffer. The molecular weight of the phosphorylated peptides was determined by electrophoresing alongside the proteolyzed sample a set of pre-stained polypeptides ranging from 26,600 to 1,060 daltons (Sigma, C6210). At the end of the run the gel was electroblotted to nitrocellulose and the filters were counted by the Instantimager, then exposed to an x-ray film overnight (34).

RESULTS

PKA Anchoring Is Essential for Recycling and Resensitization of the Human β1-AR—To test whether PKA/AKAP interactions are necessary for PKA-mediated recycling of the agonist-internalized β1-AR, the association between PKA and AKAP was perturbed with the st-Ht31 peptide. st-Ht31 is a cell-permeable peptide that contains the critical RII-binding domain common to all AKAPs and globally disrupts PKA-AKAP interactions, whereas the st-Ht31-proline does not disrupt their association and is used as a control (31, 35). Pretreatment of cells expressing the WT β1-AR with 50 μM st-Ht31 or st-Ht31-pro did not interfere with the membranous distribution of the β1-AR in control cells (Fig. 1A, panels a, g, and m). Isoproterenol promoted the internalization of the β1-AR from clusters of HEK-293 cells and from individual cells pretreated with st-Ht31 (Fig. 1A, panels b, h, and n). In the untreated or st-Ht31-pro-treated cells, the β1-AR recycled normally with a t1⁄2 of 18 ± 4 min (Fig. 1, A, panels c-f and o-r, and B). Pretreatment of st-Ht31, however, prevented the efficient recycling of the β1-AR (Fig. 1A, panels l–l, and B) and the receptors were distributed intracellularly even 4 h after the removal of isoproterenol (data not shown). Therefore, PKA anchoring is required for recycling of the β1-AR.

To investigate if AKAP-mediated anchoring of PKA exerted an effect on desensitization of the β1-AR, the effect of st-Ht31...
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Effect of destabilizing AKAP/PKA interactions on recycling of the agonist-internalized $\beta_1$-AR. A, HEK293 cells stably expressing the FLAG-tagged WT $\beta_1$-AR were cultured on glass slides and prelabeled for 1 h with fluorescein isothiocyanate anti-FLAG antibody. The cells were incubated with buffer (panel A) with 50 $\mu$M st-Ht31 (panel B) or stHt31-pro peptides (panel C) for 30 min. These cells were then exposed to 10 $\mu$M isoproterenol for 30 min, then acid washed to strip the fluorescent label from non-internalized receptors. After adding alprenolol (100 $\mu$M) and 20 $\mu$M of each peptide, the cells were washed at the indicated times and visualized by confocal microscopy. Each scale bar represents 5 $\mu$m.

B, the pixels inside a 300-nm boundary in isoproterenol/acid-washed cells were set arbitrarily to 100% to indicate 100% internalization and the ratios in alprenolol-treated cells were calculated and expressed as % for each time period. The $t_{0.5}$ for recycling was calculated by fitting the relevant data to a single exponential function of time from $y = y_0 + A(1 - e^{-t/t_{0.5}})$, where $y_0$ and $A$ are constants. C, cells expressing WT $\beta_1$-AR were pretreated with 50 $\mu$M st-Ht31 or st-Ht31-pro for 30 min, then exposed to buffer or 10 $\mu$M isoproterenol for either 10 min or 3 h at 37 °C. For the 3-h condition, the buffer or isoproterenol were replaced with 100 $\mu$M of the $\beta$-adrenergic antagonist propranolol for 1.5 h. The ratios for the specific activity of adenylyl cyclase in membranes exposed to isoproterenol to those exposed to forskolin were used to determine the percentile of maximal adenylyl cyclase activity in each type of membrane. These experiments were replicated ($n = 4$) each in triplicate to determine the S.E.

or st-Ht31-pro on adenylyl cyclase activity in response to acute isoproterenol treatment was determined. In this assay, rapid desensitization of adenylyl cyclase in membranes prepared from cells pre-exposed to st-Ht31 or st-Ht31-pro was observed after 10 min of exposing the cells to isoproterenol, indicating that the cell-permeable peptides did not affect short-term desensitization (Fig. 1C). To determine the effect of these peptides on resensitization, the cells were exposed to isoproterenol, in the presence of the peptides, for 3 h to desensitize and internalize the WT $\beta_1$-AR. Then the agonist was removed, and 10
μM alprenolol and 10 μM of each peptide was added to initiate recycling of the β1-AR for the next 1.5 h. In cells exposed to st-Ht31-pro, the β1-AR recovered from desensitization such that isoproterenol-stimulated adenyl cyclase activity was comparable with control “nondesensitized” cells (Fig. 1C). In contrast, following exposure to st-Ht31, the activation of adenyl cyclase by the β1-AR remained significantly reduced, indicative of persistently desensitized receptors (Fig. 1C). Thus, disruption of PKA-AKAP complexes interfered with functional resensitization of the β1-AR, indicating that proper PKA anchoring is also essential for β1-AR resensitization.

AKAP79 Is Involved in Recycling of the Agonist-internalized β1-AR in HEK-293 Cells—The character of the individual AKAPs and PKA subunits that are expressed in HEK-293 cells is unknown. As a first step toward characterization of the AKAP involved in PKA-mediated resensitization of the β1-AR in HEK-293 cells, the AKAPs that were most prominently expressed in this cell line were identified by RII overlay assays. Interaction of 32P-labeled RIIα subunit of PKA and extracts prepared from HEK-293 cells identified three clusters that interacted with the RIIα subunit (Fig. 2A). These clusters migrated with a calculated molecular mass value (in kilodaltons) between 75 and 82, 145 and 155, and 240 and 260. To identify the specific AKAP corresponding to each cluster, extracts prepared from HEK-293 cells were probed with antibodies to AKAPs whose molecular weights were within the Mₐ ranges of each cluster. These immunoblots identified the faster migrating cluster as AKAP79, the cluster with the intermediate Mₐ as AKAP149, and the slowest migrating cluster as AKAP250 (gravin) (Fig. 2B). Using antibodies directed against the various PKA subunits, the 41-kDa catalytic α-subunit and the 48-kDa regulatory RIα, RIα, and RIβ subunits of PKA were detected in HEK-293 cells (data not shown).

We reasoned that if one of these AKAPs is functionally involved in recycling of the agonist internalized β1-AR then its selective down-regulation would interfere with receptor recycling. A specific and effective method for down-regulating the expression of selected genes is RNA interference using short interfering double-stranded RNA (siRNA) (36). Therefore, we generated siRNAs against the membranous AKAPs, namely AKAP79 and gravin, and determined their effect on endogenous AKAP expression by Western blotting. In addition, we tested the effect of the AKAP79 siRNA on the fluorescence of transiently transfected GFP-AKAP79 into naïve HEK-293 cells (Fig. 2, C and D). The AKAP79 siRNA reduced the levels of AKAP79 or the fluorescence of GFP-AKAP79 by >85%, whereas the scrambled duplex siRNA had no such effect (Fig. 2, C and D). The siRNA reduced gravin protein expression by >80% (data not shown).

The effects of these siRNAs on recycling of the agonist-internalized β1-AR were determined by confocal recycling assays (1). HEK-293 cells stably expressing the FLAG-tagged β1-AR were transiently transfected with 100 nM control or active siRNA for 2 days. We determined by [3H]CGP-12177 binding that these siRNAs did not affect the density of cell surface β1-AR or had an appreciable effect on basal adenyl cyclase activity (data not shown). Isoproterenol induced the internalization of the β1-AR, and the rate and magnitude of its seques-
β1-AR that was internalized in response to isoproterenol. The data in Fig. 4D indicate that the Ht31 peptides did not markedly affect internalization of the β1-AR in either cell type. To determine the effect of PKA-AKAP interactions on recycling of the agonist-internalized β1-AR, isoproterenol was replaced with the β-antagonist alprenolol to inhibit β1-AR internalization. Then the internalized β1-AR was allowed to recycle by warming the cells (at 37 °C) for an additional 15, 30, or 60 min (Fig. 4D, lanes 3–5 and 8–10). After each time period, the cells were cooled to 4 °C and cleaved for the second time to ensure cleavage of any newly appearing surface biotin. Thus, the loss of biotin from the second cleavage step indexed recycling of the β1-AR. The data reveal that by 60 min more than 90% of the biotin was lost from β1-AR in cells pre-exposed to st-Ht31-pro, reflecting membrane recycling of β1-AR and subsequent biotin cleavage (Fig. 4D, lanes 3–5). In contrast, the internalized (bio-
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(A) Flag-AKAP79/myc β₁-AR
(B) YFP-AKAP79/Flag β₁-AR
(C) Flag β₁-AR/Gravin

(D) YFP-AKAP79/Flag β₁-AR
(E) YFP-AKAP79/Flag β₁-AR

(F) Flag β₁-AR + Scram. siRNA
(G) Flag β₁-AR + Scram. siRNA

(H) Flag β₁-AR + AKAP79 siRNA
(I) Flag β₁-AR + AKAP79 siRNA

(J) GST-β₁-AR C-tail Pull down (42μg/600μL; 1.94 μM)

FIGURE 3. Role of AKAP79 in the targeting of PKA to the β₁-AR microdomain: analysis by co-immunoprecipitation. A–C, HEK-293 cells were co-transfected with the indicated constructs of AKAP79, gravin, and WT β₁-AR. In all experiments, equal amounts of lysates were incubated anti-tag IgG beads and with normal mouse IgG-conjugated to agarose beads to control for nonspecific antigen/antibody interactions. The immunoprecipitates were resolved on SDSPAGE, blotted, and probed with the indicated antibodies. Lysates represent 2% of the total extract; whereas immunoprecipitations represent 30% of the total volume. D and E, HEK-293 cells stably expressing YFP-AKAP79 were co-transfected with FLAG-WT β₁-AR for 2 days. In all experiments, equal amounts of lysates from untransfected cells (lysate1) or cells that were transfected with the β₁-AR (lysate2) were incubated anti-tag IgG beads and with normal mouse IgG-conjugated to agarose beads to control for nonspecific antigen-antibody interactions. F–I, HEK-293 cells stably expressing the FLAG-WT β₁-AR were transiently transfected with 100 nM scrambled siRNA or AKAP79 siRNA for 2 days followed by immunoprecipitation and blotting as indicated. J–L, HEK-293 cells were transiently transfected with the epitope-tagged β₁-AR-(1–424) and AKAP79 for 2 days, followed by immunoprecipitation and blotting (IB) as indicated.

tinylated) β₁-AR in SK-N-MC or neonatal rat cortical neurons that were pretreated with st-Ht31 was not changed even after 1 h from the removal of isoproterenol, reflecting their internal distribution (Fig. 4D, compare lanes 9 and 10 with lanes 4 and 5). These data indicate that AKAP/PKA interactions were also required for recycling of the endogenous β₁-AR in neurons.

To verify whether AKAP79 is involved in targeting PKA to the β₁-AR, HEK-293 cells stably expressing FLAG-β₁-AR were treated with AKAP79 siRNA or with its scrambled control (Fig. 3, F–I). RIIα and RIIβ subunits were effectively co-immunoprecipitated with β₁-AR in cells treated with control siRNA (Fig. 3, F and G). However, siRNA mediated suppression of AKAP79 markedly reduced the amounts of RIIα and RIIβ subunits co-
immunoprecipitated with the β₁-AR (Fig. 3, H and I). Collectively, these results indicate that endogenous AKAP79 is required for targeting PKA to the β₁-AR.

Most of the binding sites for β₁-AR-interacting proteins are contained within the 3rd IC and the carboxyl-terminal tail of the receptor (40, 41). As a first step toward determining the locus for association between β₁-AR and AKAP79, the carboxyl-terminal 53 amino acids between 425 and 477 in the β₁-AR were fused to GST and bacterial lysates were purified by affinity chromatography. HEK-293 cells transiently expressing pcDNA 3.1 or AKAP79 in pcDNA 3.1 were lysed and subjected to pull down using GST-β₁-AR-(425–477) as bait. As shown in Fig. 3f, GST-β₁-AR-(425–477) could pull down AKAP79 from control and AKAP79 expressing cells. In follow up experiments, cells expressing AKAP79 were transfected with the carboxyl-terminal truncated β₁-AR (β₁-AR-(1–424)), in which the last 53 amino acids in the carboxyl-terminal tail were deleted and subjected to immunoprecipitation. Reciprocal immunoprecipitation of either β₁-AR-(1–424) or AKAP79 did not lead to effective association of the AKAP or β₁-AR, respectively, as was previously observed for the full-length WT β₁-AR (Fig. 3, K and L). Therefore, it appears that the carboxyl-terminal tail of the β₁-AR harbors the principal determinants for AKAP79 association.

Characterization of the Association between the β₁-AR, AKAP79, and PKA by FRET Microscopy—Confocal video microscopy confirmed the membranous distribution of AKAP79 in HEK-293 cells (Fig. 5A). To determine whether AKAP79 and β₁-AR were colocalized, GFP-AKAP79 was expressed with FLAG-β₁-AR and their distribution was determined by dual-labeling confocal microscopy. GFP-AKAP79 (green) co-localized with the Cy3-labeled β₁-AR (red), as indexed by their combined yellow fluorescence (Fig. 5B). Exposing these cells to isoproterenol caused selective internalization of the β₁-AR, without altering the membranous distribution of AKAP79. Within 45 min after the initiation of recycling, the β₁-AR trafficked back into the cell membrane and were co-localized with AKAP79. These data show that colocalization between AKAP79 and β₁-AR is reversible and that AKAP79 is not appreciably internalized.

FRET microscopy was used to supplement the data obtained by immunoprecipitation and determine the relative strength of protein/protein interactions between the β₁-AR, AKAP79, and PKA. FRET relies on the transfer of energy from an excited donor (CFP) to an acceptor (YFP) if the two-tagged proteins are in very close proximity (<50Å) (22). Prior to the initiation of FRET, the localization, internalization, and recycling of β₁-AR-YFP- or CFP chimera in HEK-293 cells were determined by confocal microscopy (Fig. 5C). These experiments show that the β₁-AR-YFP is expressed in the membrane, and that the receptor is internalized in response to isoproterenol (Fig. 5C, panels a and b). Removal of isoproterenol caused rapid recycling of β₁-AR-YFP with kinetics similar to those of the WT β₁-AR (Fig. 5C, panels c-f).

To determine the magnitude of FRET between the β₁-AR and AKAP79, the WT β₁-AR-CFP and AKAP79-YFP were transiently transfected into HEK-293 and imaged in live cells.
AKAP79-mediated Recycling of the β₁-AR

(A) On-line appendix 3-D movie: AKAP79 is membrane targeted.

(B) Time after removal of isoproterenol

|   | No ISO | ISO 30 min | ALP 15 min | ALP 45 min |
|---|--------|------------|------------|------------|
| 0 | a      | b          | c          | d          |
| 10| e      | f          | g          | h          |
| 20| i      | j          | k          | l          |
| 30| m      | n          | o          | p          |
| 45| q      | r          | s          | t          |

FIGURE 5. The wild-type β₁-AR colocalizes with AKAP79 on the membrane. A, AKAP79 is membrane-targeted protein (supplemental movie 1). B, a stable cell line of FLAG-tagged WT β₁-AR was transfected with GFP-AKAP79. Two days later, the cells were labeled with Cy3 anti-FLAG antibody to label the β₁-AR. The cells were exposed to 10 μM isoproterenol (ISO) for 30 min, acid washed, and treated with 100 μM alprenolol for 30 min and 1 h. Dual confocal images of AKAP79 (green) and β₁-AR (red) were taken at each time point. C, cells expressing WT β₁-AR-YFP were exposed to 10 μM isoproterenol for 20 min to internalize the β₁-AR, then the recycling of the receptor was visualized by confocal microscopy as described in the legend of Fig. 1.

The phosphorylation of the WT β₁-AR was markedly increased (6 ± 1-fold) upon exposing the cells to isoproterenol (Fig. 8A). Agonist-mediated phosphorylation of the β₁-AR is mediated by GRK and PKA (5). The preferred substrates for phosphorylation by PKA are serine/threonine residues that are preceded by RX- or RRX- (where X is any amino acid and R is arginine), whereas the preferred substrates for phosphorylation by GRK are serine/threonine residues that are preceded by an acidic amino acid (43–46). The sequence around Ser^{312} in the 3rd IC (SS123) prevented the recycling and resensitization of the β₁-AR (1). AKAP79 is also involved in recycling and resensitization of the β₁-AR, but its mechanism of action is not known. A potential mechanism that might be involved is that the targeting of PKA to the β₁-AR by AKAP79 is required for the phosphorylation of the β₁-AR by PKA. Therefore, we determined if PKA phosphorylates the β₁-AR and if AKAP79 was required.

To confirm FRET measurements, the FRET experiments were repeated using the acceptor photobleaching method to determine FRET efficiencies in fixed cells (Fig. 7 and Table 1). FRET efficiencies (%) were very reproducible across several experiments (n = 8) for AKAP79-CFP and WT β₁-AR-YFP (8 ± 2%), and for AKAP79-YFP paired with RII-CFP (17 ± 1.2%) (Table 1). FRET data that were obtained by two different methods demonstrated that direct interactions between the β₁-AR, AKAP79, and the RII subunit of PKA occurred in live and fixed HEK-293 cells.

Role AKAP79-PKA Interactions in Agonist-mediated Phosphorylation of the β₁-AR—The activation of the β₁-AR signaling pathway by isoproterenol is associated with the generation of cyclic AMP that binds to the R subunit of PKA and releases the C subunit to phosphorylate target proteins. Inhibition of PKA or mutagenesis of its putative substrate Ser^{312} to alanine in the 3rd IC (SS123) prevented the recycling and resensitization of the β₁-AR (1). AKAP79 is also involved in recycling and resensitization of the β₁-AR, but its mechanism of action is not known. A potential mechanism that might be involved is that the targeting of PKA to the β₁-AR by AKAP79 is required for the phosphorylation of the β₁-AR by PKA. Therefore, we determined if PKA phosphorylates the β₁-AR and if AKAP79 was required.

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AKAP79-mediated Recycling of the \( \beta_1 \)-AR

| Donor YFP | Acceptor CFP | CFP/YFP Overlay | FRETN |
|-----------|--------------|-----------------|-------|
| WT \( \beta_1 \)-AR-YFP AKAP79-CFP | | | |
| AKAP79-YFP AKAP79-CFP Negative control | | | |
| AKAP79-YFP RII-PKA-CFP | | | |
| WT \( \beta_1 \)-AR-YFP RII-PKA-CFP | | | |
| WT \( \beta_1 \)-AR-YFP WT \( \beta_1 \)-AR-CFP Negative control | | | |
| WT \( \beta_1 \)-AR-YFP RII-PKA-CFP AKAP79 siRNA | | | |

**TABLE 1**

Normalized FRET efficiencies in % as recorded by the sensitized emission method (FRETN) in live cells or by the acceptor photobleaching method in fixed cells

FRET efficiency was calculated using area averages for donor (D) before and after bleaching. FRET = \((D \text{ before} - D \text{ after}) / D \text{ before})\). Donor (D) and acceptor (A) threshold values were subtracted from all pixels before FRET calculation. The mean values of FRET efficiencies calculated for at least 10 specific regions of interest in each FRET pair from seven experiments were collected and analyzed with FRET tool software for LSM510 version 3.2.

| CFP donor | FRETN | Acceptor photobleaching |
|-----------|-------|-----------------------|
| RII-PKA   | 22 ± 3| 17 ± 5                |
| AKAP79    | 12 ± 1.1| No FRET                |
| \( \beta_1 \)-AR | No FRET| 10 ± 2 | No FRET  |

**FIGURE 6.** Sensitized emission FRET microscopy between AKAP79, WT \( \beta_1 \)-AR, and the PKA RII\( \alpha \) subunit. A, cells co-expressing the WT \( \beta_1 \)-AR-YFP and AKAP79-CFP were used. B, live cells, expressing AKAP79-YFP and RII-CFP were imaged at room temperature in glass-bottom Petri dishes. C, direct binding of PKA RIIu-CFP to WT \( \beta_1 \)-AR-YFP. D, FRET between PKA RII-CFP and the WT \( \beta_1 \)-AR-YFP was not observed in HEK-293 cells when AKAP79 was knocked down with siRNA. Negative controls were performed with AKAP79 (A and B) and WT \( \beta_1 \)-AR (C), labeled with both fluorescent tags. FRETN is present in pseudocolor. Normalized FRETN values were calculated using LSM510 Macro 1.5 FRET software using Equation 1 as described under "Experimental Procedures." FRETN values for the various constructs are presented in Table 1.

To determine whether Ser\(^{312} \) is a potential substrate for phosphorylation by PKA, the phosphorylation of S312A in response to isoproterenol was determined. The phosphorylation of S312A increased by \( \sim 5\) fold in response to isoproterenol. mPKI did not reduce the phosphorylation of S312A, suggesting that Ser\(^{312} \) was a potential substrate for PKA (Fig. 8A).

To provide stronger evidence that Ser\(^{312} \) was potentially phosphorylated by PKA, the WT \( \beta_1 \)-AR and S312A were phosphorylated in response to isoproterenol followed by cleavage of each \(^{32}\)P-labeled receptor with cyanogen bromide (33, 34). The human \( \beta_1 \)-AR is cleaved at methionine residues by cyanogen bromide into peptides that are 4, 9, 33, 44, 69, 91, and 147 amino acids in length. Of these, the 91-amino acid peptide (between amino acids 240 and 330) contains the entire third intracellular loop and the 147-amino acid peptide (between 331 and 477) contains the entire carboxyl-terminal tail. Cyanogen bromide cleavage of the \(^{32}\)P-labeled WT \( \beta_1 \)-AR isolated from cells that were exposed to isoproterenol generated two phosphopeptides of molecular mass = 10 and 15 kDa (Fig. 8B). The size of the 10-kDa peptide corresponds to the expected molecular mass of the 3rd IC-derived phosphopeptide and the 15-kDa peptide corresponded to the expected molecular mass of the carboxyl terminus-derived phosphopeptide. The % of the total cpm of \(^{32}\)P incorporated into the 10-kDa band derived from the WT \( \beta_1 \)-AR was 27 ± 5%, which is in agreement with the mPKI data in Fig. 7A. Cleavage of the phosphorylated S312A with cyanogen bromide indicated that 94 ± 6% of the \(^{32}\)P was incorporated into the 15-kDa peptide and \( \sim 6 \pm 3\) % was incorporated into the 10-kDa peptide.

Pretreatment of cells expressing the WT \( \beta_1 \)-AR with mPKI reduced the phosphorylation of the 10-kDa peptide from 30% to \( \sim 7\)%, in agreement with the assumption that this peptide contained the putative PKA-phosphorylatable serine in the 3rd IC. The percentage of the total cpm phosphorylated serines in the human \( \beta_1 \)-AR.

The percentage of \(^{32}\)P incorporated into the 10-kDa peptide derived from
FIGURE 7. Acceptor photobleaching analysis of FRET interactions between AKAP79, WT β₁-AR, and the PKA RII subunit. By observing donor (CFP) channel intensity changes upon bleaching of the acceptor (YFP), we obtained an image set consisting of a time lapse recording of donor and acceptor channels with acceptor photobleaching carried out in between. FRET was recorded by examining the quenching of CFP during acceptor (YFP) photobleaching by the 514-nm argon laser line. Lambda stacks were acquired at various time points before and after photobleaching of YFP. Upon acceptor (YFP) photobleaching, donor (CFP) intensity increased significantly (compare donor before bleach and merged images after bleach). The color ruler shows the relationship between the pseudo FRET color and the corresponding FRET efficiency reported in Table 1.
S312A was ~7% in the absence or presence of mPKI, whereas mPKI reduced the percentage of \(^{32}\)P incorporated into the WT \(\beta_1\)-AR from 27 to 7%. Therefore, phosphorylation of the 3rd IC in the WT \(\beta_1\)-AR in mPKI-treated cells was similar to the phosphorylation of the 3rd IC in the S312A mutant in untreated cells.

The next series of experiments were designed to determine whether AKAP/PKA interactions or AKAP79 are involved in the PKA component of isoproterenol-mediated phosphorylation of the WT \(\beta_1\)-AR (Fig. 9A). HEK-293 cells were transiently transfected with the FLAG-tagged \(\beta_1\)-AR along with either scrambled control or AKAP79 siRNA. These, and cells expressing the FLAG-WT \(\beta_1\)-AR were metabolically labeled with 200 \(\mu\)Ci of \(^{32}\)PO\(_4\) for 1.5 h. Cells stably expressing the FLAG-\(\beta_1\)-AR along with either st-Ht31 or the scrambled siRNA generated the 15-kDa phosphopeptide only (Fig. 9B, lanes 3, 4, 7, and 8). Therefore, inhibition of AKAP-PKA interactions with st-Ht31 or with AKAP79 siRNA both inhibited the phosphorylation of the peptide derived from the 3rd IC, which contains the putative PKA substrate. These reagents also prevented the recycling of the agonist-internalized \(\beta_1\)-AR, indicating that AKAP79-anchored PKA was involved in resensitization of the \(\beta_1\)-AR and its signaling pathway.

DISCUSSION

Prolonged or repeated activation of many GPCRs induces rapid desensitization followed by a period during which the receptor is either resensitized or degraded (4, 7, 12). Desensitization of GPCR is a consequence of GRK-mediated phosphorylation of the agonist-occupied receptor and subsequent binding of \(\beta\)-arrestins, which together promote rapid desensitization and internalization of the GPCR (4–6). Resensitization refers to the phenomena that repopulate the plasma membrane with GPCRs and concomitantly restore signaling efficacy and specificity (7–9). For many GPCRs, it has been established that internalization is a prerequisite for resensitization as well as for degradation, but our understanding of the motifs in GPCRs, which impart these distinct outcomes, remains incomplete (8, 9).

To date, the best characterized motif controlling the fate of the internalized GPCR is the PDZ type 1 ligand in the COOH-terminal tail of the GPCR that when mutated inhibits recycling and resensitization of the GPCR (47–51). Recently, PKA and its substrate Ser\(^{312}\) in the 3rd IC of the human \(\beta_1\)-AR were found to

FIGURE 8. Effect of inhibiting PKA on isoproterenol-mediated phosphorylation of the \(\beta_1\)-AR. A, cells expressing the FLAG-tagged WT \(\beta_1\)-AR (lanes 1–4) or the Flag-tagged S312A (lanes 5–8) were metabolically labeled with \(^{32}\)PO\(_4\) then incubated where indicated with 1 \(\mu\)M of myristoylated PKI for 30 min (lanes 2, 4, 6, and 8). The cells were exposed to buffer (ascorbic acid 1% (AA) (lanes 1, 2, 5, and 6) or 10 \(\mu\)M isoproterenol for 10 min (lanes 3, 4, 7, and 8). The \(\beta_1\)-AR was immunoprecipitated using anti-Flag IgG-agarose and subjected to SDS-PAGE and autoradiography. B, the immunoprecipitated \(\beta_1\)-AR were subjected to SDS-PAGE, followed by electrophoretion to nitrocellulose. The bands corresponding to the \(^{32}\)P-labeled \(\beta_1\)-AR were cut out and incubated with 100 \(\mu\)g/ml CNBr in 70% formic acid for 1.5 h. The lyophilized peptides were electrophoresed on 16% Tricine-SDS gels. The gels were exposed to x-ray film for 1 day to generate the image in the figure and then counted electronically. In lane 2, the % cpms in the 10- versus the 15-kDa band were 30.7% (3,108 to 10,104 cpms, respectively). In lane 3, the % cpms in the 10- versus the 15-kDa band were 8% (253 to 3,282, respectively). In lane 5, the % cpms in the 10- versus the 15-kDa band were 8% (236 to 2,821 cpms, respectively). The cpm in lanes 1 and 4 could not be accurately determined.
be novel signals that are involved in recycling and resensitization of this GPCR (1).

The identification of PKA as a kinase involved in recycling of the human $\beta_1$-AR led us to seek potential intermediates that may target PKA to the $\beta_1$-AR. PKA is targeted to its numerous substrates by members of the AKAP family, which is composed of more than 70 members (18). Blockade of AKAP-PKA interactions with Ht31-derived peptides inhibited the recycling of exogenously and endogenously expressed $\beta_1$-AR, indicating that these interactions were required for recycling of the agonist-internalized $\beta_1$-AR (Figs. 1 and 4). Through the use of siRNA interference, a prominent role for AKAP79 in recycling of the $\beta_1$-AR was established. The involvement of AKAP79 in targeting PKA to the $\beta_1$-AR was further verified by co-immunoprecipitation, which revealed that an AKAP79 in targeting PKA to the $\beta_1$-AR was further verified by co-immunoprecipitation, which revealed that an AKAP79/PKA complex was bound to the carboxyl terminus of the $\beta_1$-AR. This method, however, provides a relatively static view of the $\beta_1$-AR/AKAP79/PKA complex. A more dynamic view was provided by FRET microscopy that localized this ternary complex to the plasma membrane and indicated that the associations between $\beta_1$-AR and AKAP79/PKA occurred at relatively high efficiency. However, it must be emphasized that the strength of the FRET signal is a function of the distance between chromophores, the binding affinity of protein-protein interactions, and chromophore orientation within the complex (21, 25–28). Thus, differences between any of these parameters can affect the intensity of FRET signals between acceptor-donor pairs. For example, the weaker FRET signal that was measured between AKAP79/$\beta_1$-AR versus $\beta_1$-AR/RII was surprising because the data in panels D of Figs. 6 and 7 indicated that the formation of the $\beta_1$-AR/RII complex was mediated by AKAP79. These findings were corroborated in Fig. 3, which showed that the $\beta_1$-AR immunoprecipitants contained larger amounts of RII than AKAP79. We attribute these findings to different stoichiometries and chromophore separations between the $\beta_1$-AR and AKAP79 versus the $\beta_1$-AR and RII. For instance, whereas the PKA holoenzyme binds to AKAP79 with 1:1 stoichiometry, the tetrameric composition of PKA into two R and two C subunits generates a stoichiometry of 2:1 between the RII subunits and AKAP79. In this case, FRET between AKAP79 and $\beta_1$-AR involved a 1:1 stoichiometry, whereas that between the RII subunit and the $\beta_1$-AR involved a 2:1 stoichiometry if the interaction between RII and the $\beta_1$-AR was mediated by AKAP79. The weaker FRET signal between the $\beta_1$-AR and AKAP79 versus the $\beta_1$-AR and RII reflects an anticipated consequence of these stoichiometries and verifies that AKAP79 is the bridging molecule between the $\beta_1$-AR and RII dimer of PKA.

To provide a mechanistic foundation for the effect of AKAP79 on recycling of the $\beta_1$-AR, we explored the role of AKAP79 and its associated scaffold on PKA-mediated phosphorylation of the $\beta_1$-AR. This is a particularly important question because Gardner et al. (1), have determined that PKA and its substrate Ser$^{312}$ in the 3rd IC were involved in recycling and desensitization of the $\beta_1$-AR. However, to show that isoproterenol-mediated phosphorylation of the $\beta_1$-AR involves PKA is challenging because the distribution of PKA versus GRK phosphorylatable serines is ~1:3, respectively, whereas the standard error in phosphorylation experiments is typically between 8 and 12% (1, 34, 44–46). Thus, direct assessment of the PKA component was necessary to unambiguously analyze the effect of AKAP79 on this parameter. Using mPKI, we determined that the magnitude of the PKA component was ~25%, indicating that the expected stoichiometries of PKA- versus GRK-mediated phosphorylation of the $\beta_1$-AR were as expected. Cyanogen bromide cleavage of the phosphorylated $\beta_1$-AR confirmed that PKA-mediated phosphorylation of the $\beta_1$-AR was localized within the 3rd IC and that inhibition of PKA-AKAP interactions or down-regulation of AKAP79 inhibited this parameter, exclusively. We should point out, however, that even though the effect of mPKI was largely because of inhibition of PKA, a minor component was because of inhibition of GRK. As outlined in Fig. 8A, lanes 2 and 6, mPKI reduced basal phosphorylation of the WT $\beta_1$-AR and the PKA null $\beta_1$-AR S312A mutant, which we attribute to indirect inhibition of GRK by mPKI. Phosphorylation of GRK-2 by PKA at Ser$^{385}$ enhances the translocation of the $\beta_1$-AR.
AKAP79-mediated Recycling of the β1-AR

of GRK-2 from the cytosol to the membrane where it phosphorylates the agonist-occupied β-AR (52). Thus, by inhibiting PKA we inadvertently reduced GRK-mediated phosphorylation of the carboxyl terminus of the β1-AR, which was reflected by reduced 32P incorporation in lane 3 of Fig. 8B. The effects of st-Ht31 and AKAP79 siRNA on the phosphorylation of the β1-AR were more pronounced than mPKI, illustrating the importance of AKAP79-mediated targeting of PKA in this phenomenon. These data are similar to the effect of st-Ht31 and the dominant negative AKAP79 (AKAP79-Pro) mutant on the phosphorylation of the β2-AR in response to isoproterenol (53).

The characterization of the β1-AR-AKAP79-PKA scaffold was determined by recycling assays that identified a novel function for AKAP79 as a mediator for the resensitization of this GPCR. This discovery increases the range of functions already attributed to AKAP79. AKAP79/β1-AR interactions are important for facilitating the signaling and phosphorylation of the β2-AR, as well as for regulating the cyclic AMP metabolism by an associated phosphodiesterase (19, 48). A prominent role for AKAP79/β2-AR interactions was exerted on isoproterenol-mediated activation of mitogen-activated protein kinase, which was inhibited by the PKA inhibitor H-89, and by disrupting AKAP-PKA interactions with st-Ht31 or dominant negative AKAP79 (53). In the case of the β1-AR, AKAP79 did not affect the magnitude of cyclic AMP accumulation, sequestration, or mitogen-activated protein kinase activation elicited by isoproterenol, rather it had a marked effect on its recycling and resensitization. The effects produced by PKA and AKAP79 on the β1-AR were biologically similar, suggesting that AKAP79 exerted its effect on the β1-AR largely by PKA-dependent mechanisms. The effects of PKA and AKAP79 on the β2-AR, however, were not the same suggesting that AKAP79 exerted its effects on the β2-AR by PKA-dependent (such as inhibition of mitogen-activated protein kinase) and independent (such as inhibition of the β2-AR sequestration) mechanisms (54). The most plausible cause for the convergence of PKA- and AKAP79-dependent mechanisms in regulating the recycling of the β1-AR is likely because both are integral to Ser312 phosphorylation.

AKAP250 (gravin) is another AKAP that interacted with the β2-AR through its carboxyl-terminal tail (55). AKAP250 exerts numerous effects on the β2-AR that include regulation of internalization, recycling, and recovery of the β2-AR from agonist-induced desensitization (54–56). However, siRNA-mediated suppression of gravin in HEK-293 cells did not disrupt the recycling or the recovery of the β1-AR from agonist-induced desensitization, indicating that the repertoire of AKAPs that bind to each GPCR promote distinct effects on these receptors. The proposed AKAP79-PKA-targeting scaffold is a self-contained signalosome that might produce many effects other than recycling and resensitization of the β1-AR. The diffusion of cyclic AMP generated by the activation of the β1-AR signaling pathway is restricted by spatiotemporal mechanisms that limit its diffusion and reduce its half-life through degradation by phosphodiesterases, which are recruited by β-arrestins to the receptor microdomain (19). The identification of AKAP79 as a binding partner to the β1-AR provides a potential mechanism to ensure faithful signal propagation from the β1-AR to downstream targets that are also linked to AKAP79 such as the L-type Ca2+ channel and the GluR1 AMPA receptor subunit (21, 57).

Although we found that the carboxyl-terminal tail of the β1-AR is necessary for AKAP79 association with the receptor, it remains to be determined whether this is through a direct or indirect interaction. In particular, the carboxyl terminus of the β1-AR between amino acids 474 and 477 contains a PDZ type I ligand that binds to PSD-95, SAP97, as well as four other members of the membrane-associated guanylate kinase (MAGUK) family of scaffolding proteins (37, 57–61). AKAP79 is known to bind to the Src homology 3 and guanylate kinase-like domains of membrane-associated guanylate kinase proteins PSD-95 and SAP97 (62). Like AKAP79, SAP97 interacts with the GluR1 AMPA receptor subunit and the interaction between SAP97 and AKAP79 recruits PKA to ionotropic glutamate receptors (21, 62). Therefore, it is conceivable that multiplexing between a β1-AR-MAGUK-AKAP79 complex might scaffold AMPA receptors and other signaling molecules to the β1-AR microdomain to facilitate their heterologous phosphorylation by the β1-AR signaling pathway as has been observed in hippocampal neurons (58). Therefore, the melange of proteins scaffolded directly or indirectly to the β1-AR or other GPCR produce the range responses that define the pharmacological character of individual GPCR.

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