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Agonist-Activated *Bombyx* Corazonin Receptor Is Internalized via an Arrestin-Dependent and Clathrin-Independent Pathway

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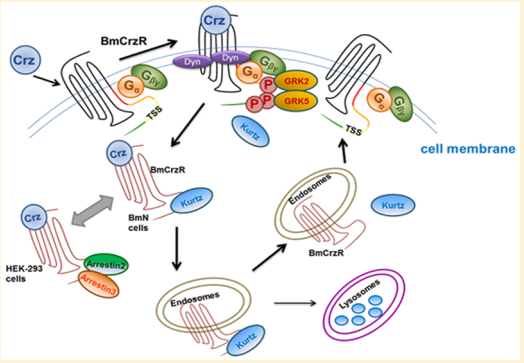
Supporting Information

ABSTRACT: Agonist-induced internalization plays a key role in the tight regulation of the extent and duration of G protein-coupled receptor signaling. Previously, we have shown that the *Bombyx* corazonin receptor (BmCrzR) activates both $G_{\alpha q}$- and $G_{\alpha s}$-dependent signaling cascades. However, the molecular mechanisms involved in the regulation of the internalization and desensitization of BmCrzR remain to be elucidated. Here, vectors for expressing BmCrzR fused with enhanced green fluorescent protein (EGFP) at the C-terminal end were used to further characterize BmCrzR internalization. We found that the BmCrzR heterologously expressed in HEK-293 and BmN cells was rapidly internalized from the plasma membrane into the cytoplasm in a concentration- and time-dependent manner via a β-arrestin (Kurtz)-dependent and clathrin-independent pathway in response to agonist challenge. While most of the internalized receptors were recycled to the cell surface via early endosomes, some others were transported to lysosomes for degradation. Assays using RNA interference revealed that both GRK2 and GRK5 were essentially involved in the regulation of BmCrzR phosphorylation and internalization. Further investigations indicated that the identified cluster of Ser/Thr residues (^{411}TSS^{413}) was responsible for GRK-mediated phosphorylation and internalization. This is the first detailed investigation of the internalization and trafficking of *Bombyx* corazonin receptors.



The insect neuropeptide corazonin (Crz), an undecapeptide, was originally isolated from the corpora cardiaca (CC) of the cockroach *Periplaneta americana*, in which it displayed the ability to accelerate the heartbeat.[1] Previous investigations using immunohistology and in situ hybridization confirmed the expression of corazonin in a variety of other insect species.[2] While six modified forms of corazonin have thus far been identified, it is corazonin with Arg as the seventh residue from the N-terminus ([Arg^7]-corazonin), which appears to be the most universal within insect lineages.[3] Although corazonin is highly conserved with respect to its amino acid sequence among insects, a clear pattern in physiological function has not yet been elucidated.[1] Corazonin has been found to regulate diverse functions such as heart contraction rates, stress responses, various metabolic activities, female fecundity, and melanization of the locust cuticle and to act in the initiation of the ecdysis and clock functions in assorted species of insects.[1,3−5] In *Bombyx mori*, corazonin has also been found to function in the reduction of the silkworm spinning rates, cocoon sizes, and the hemolymph ecdysteroid levels while also acting to prolong the pupal period.[6,7] However, the function of corazonin neuropeptides in insects remains to be further investigated.

Corazonin exerts its physiological functions through the corazonin receptor (CrzR). This contains seven transmembrane helices and other motifs characteristic of typical Class A G protein-coupled receptors.[8] The insect CrzR was first identified from the fruit fly *Drosophila melanogaster* in 2002.[9] Subsequently, homologous receptors were also cloned from *Manduca sexta*,[10] *Anopheles gambiae*,[11] and *Musca domestica*.[8] *Drosophila* CrzR was found to be expressed in embryos, larvae, pupae, and adult flies.[9] In *M. domestica*, quantitative real-time polymerase chain reaction (qRT-PCR) analysis revealed a higher level of MdCrzR (*M. domestica* corazonin receptor) expression in the larval salivary glands and a moderate level in the central nervous system. This was different from the

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situation in adults, in which the receptor was expressed in both the head and the body.[8] The CrzR in *A. gambiae* was only demonstrated to be activated by endogenous corazonin, resulting in cAMP accumulation with an EC_{50} value of 4 nM.[11] Our recent studies have shown that the direct interaction of BmCrzR with BmCrz (*Bombyx* corazonin) resulted in intracellular cAMP accumulation, Ca^{2+} mobilization, and ERK1/2 phosphorylation via the $G_{\alpha q}$- and $G_{\alpha s}$-coupled signaling pathways.[7]

Upon activation by an agonist, most vertebrate GPCRs undergo rapid internalization from the cell membrane into the cytoplasm. It is this that is believed to contribute to the regulation of the strength and duration of receptor-mediated cell signaling.[12] However, so far in insects, only the adipokinetic hormone receptor (AKHR)[13] and the pheromone biosynthesis activating neuropeptide receptor (PBANR)[14] have been characterized in terms of internalization in transfected mammalian cell lines. In our previous study, BmCrzR has been shown to undergo a rapid internalization via an arrestin-dependent pathway in both HEK-293 and BmN (a cell line established from an ovarian tissue of the silkworm) cells.[7] In the study presented here, we combine confocal microscope observations with a quantitative enzyme-linked immunosorbent assay (ELISA) to characterize agonist-mediated BmCrzR internalization and trafficking in HEK-293 and BmN cells. Our data reveal that BmCrz evokes a rapid BmCrzR internalization in a concentration- and time-dependent manner via a β-arrestin-dependent and clathrin-independent pathway, and that most of the internalized receptors are recycled to the cell surface via early endosomes upon removal of the agonist. Further investigation demonstrated that the ^{411}TSS^{413} serine/threonine cluster in the C-terminus of BmCrzR is partly responsible for BmCrz-mediated internalization. Our findings provide a mechanistic basis for a better understanding of BmCrzR-mediated signaling and associated physiological functions.

MATERIALS AND METHODS

Materials. Cell culture media, Lipofectamine 2000 and G418, were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Hyclone (Beijing, China). The pEGFP-N1 and pCMV-Flag vectors were purchased from Clontech Laboratories, Inc. (Palo Alto, CA), and Sigma (St. Louis, MO), respectively. The anti-α-tubulin and β-actin antibodies were obtained from Beyotime (Haimen, China). Monoclonal anti-FLAG M2 antibody, monoclonal anti-FLAG M2-FITC antibody, and horseradish peroxidase (HRP)-conjugated anti-mouse IgG were obtained from Sigma-Aldrich. Anti-phospho-ERK1/2, anti-ERK1/2, anti-actin, and anti-β-arrestin1/2 were from Cell Signaling (Danvers, MA). The [Arg^7]-corazonin peptides were bought from Gel Company (Shanghai, China).

Cell Culture and Transfection. The human embryonic kidney cells (HEK-293) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) and 4 mM L-glutamine (Invitrogen) at 37 °C in a humidified incubator containing 5% CO_2. The BmN cells were maintained in TC100 Medium (Sigma) with 10% heat-inactivated fetal bovine serum (Hyclone) at 28 °C. The BmN cells were maintained in TC100 Medium (Sigma). The CrzR cDNA plasmid constructs were transfected, or cotransfected, into HEK-293 cells and BmN cells using Lipofectamine 2000 (Invitrogen) and X-tremeGENE HP (Roche), respectively, according to the manufacturer's instructions. Two days after transfection, selection for stable expression was initiated with the addition of G418 (800 μg/mL).

Molecular Cloning and Plasmid Construction. Corazonin receptor deletion or site mutants were constructed with overlap extension PCR strategies. The corresponding PCR products were inserted into the *Hin*dIII and *Bam*HI sites of pCMV-Flag, pEGFP-N1, pBmEGFP-N1, and pBmIE1-FLAG vectors, named vectors FLAG-BmCrzR, BmCrzR-EGFP, BmCrzR-BmEGFP, and BmFLAG-BmCrzR, respectively. The β-arrestin1-EGFP, β-arrestin2-EGFP, and kurtz-EGFP were generated as previously described.[7,15] The human β-arrestin2 ΔLIEFD (Δ372−376) mutant was obtained by overlap extension PCR and subcloned into the pcDNA 3.1 vector. The deletion mutant was screened for the creation of restriction enzyme sites and checked by sequencing. All the constructs described above were sequenced to verify their sequences and orientations.

A shRNA fragment targeting the CDS of GRK2 was generated using a pair of primers [5′-CCGGCGGCGGTACTTCTACCTGTTCCTCGAGGAACAGGTAGAAGTACCGCCGTTTTTG-3′ (forward primer) and 5′-AATTCAAAAACGGCGGTACTTCTACCTGTTCCTCGAGGAACAGGTAGAAGTACCGCCG-3′ (reverse primer)] and cloned into the plasmid described above as described in the TRC protocols (http://www.broadinstitute.org), and the resulting plasmid was designated as GRK2 shRNA. GRK5 shRNA was constructed with a pair of primers: 5′-CCGGACGAGATGATAGAAACAGAATCTCGAGATTCTGTTTCTATCATCTCGTTTTTG-3′ (forward primer) and 5′-AATTCAAAAAACGAGATGATAGAAACAGAATCTCGAGATTCTGTTTCTATCATCTCGT-3′ (reverse primer). The scrambled shRNA plasmid was obtained from Addgene 1864.

Synthesis of Small Interfering RNAs and siRNA Transfection. All arrestins, clathrin, and the nontargeting control siRNAs were chemically synthesized by Dharmacon RNA Technologies (Lafayette, CO). The relative siRNAs were transfected into HEK-293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were split for the indicated assay to be conducted the following day.

Small Hairpin RNA Lentivirus Production and Viral Infection. Viral production and infection were performed following the TRC protocols [the RNAi Consortium (http://www.broadinstitute.org)]. Briefly, 293T cells were cotransfected with the viral vector-based shRNA described above and/or GRK2/5 variant plasmids together with the lentivirus packaging vectors pMD2.G (Addgene 12259) and psPAX2 (Addgene 12260) using X-treme GENE HP (Roche). Forth-eight and seventy-two hours after transfection, the culture supernatants containing the released viral particles were collected, filtered through 0.45 μm membranes (Millipore SCHVU01RE), and used fresh or stored at 4 °C for <2 days. For viral infection, cells were plated and cultured overnight. Viral supernatants were added with Polybrene (Sigma AL-118) at a final concentration of 8 μg/mL. In most cases, the multiplicity of infection (MOI) was estimated to be between 0.5 and 2. After 6−10 h, the media were replaced with fresh viral-free medium to allow further growth until use. Stable knockdown cells were obtained with 3 μg/mL puromycin (Shanghai, Sangon Biotech) co-incubated for 2 weeks and further validated by Western blotting.

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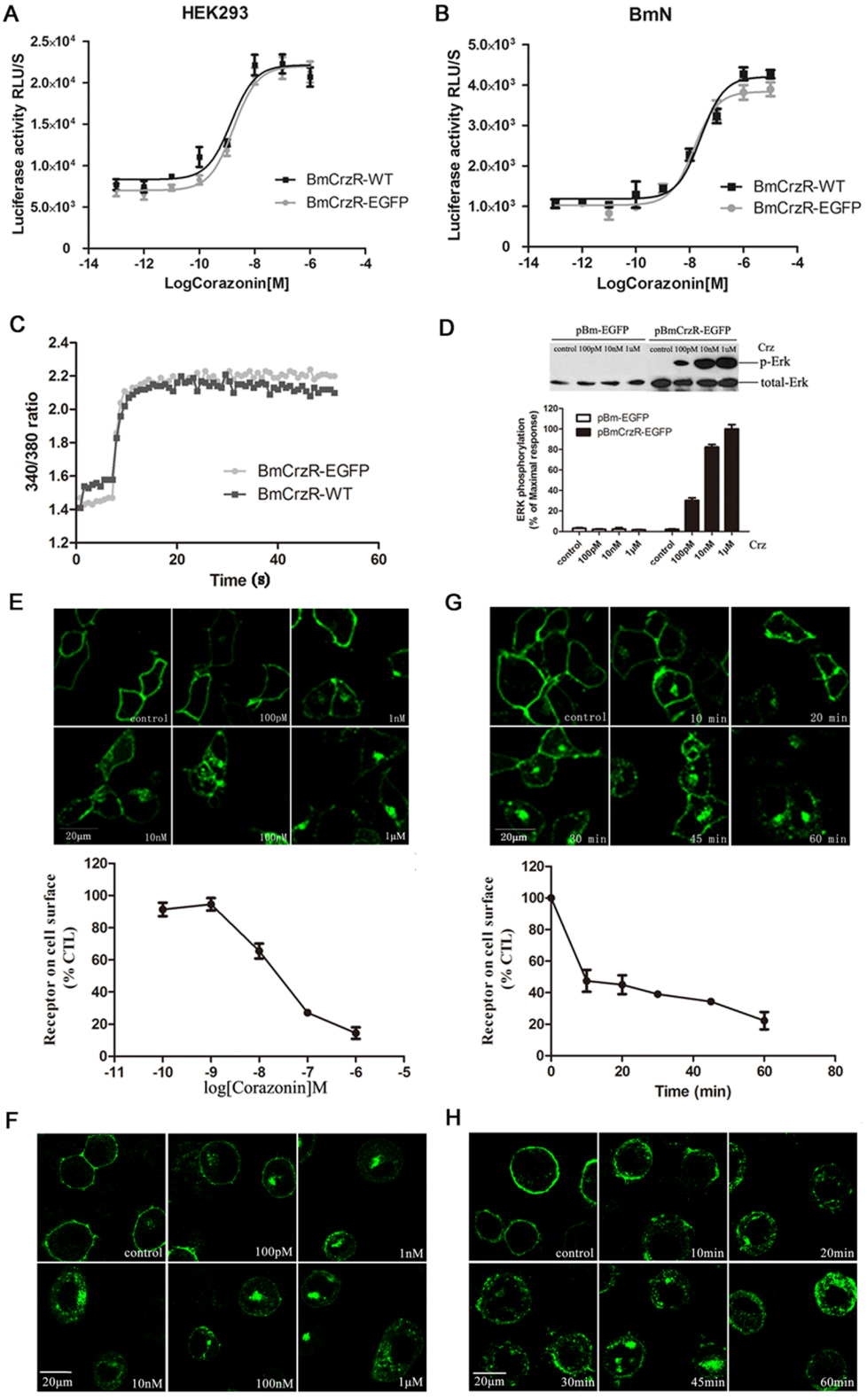

Figure 1. Internalization of corazonin receptor in HEK-293 and BmN cells. (A) HEK-293 and (B) BmN cells stably expressing BmCrzR-EGFP and FLAG-BmCrzR (wild type) were transiently transfected with the firefly luciferase reporter gene system CRE-Luc. Twenty-four to forty-eight hours after transfection, cells were stimulated with different doses of corazonin (Crz). (C) Intracellular Ca^{2+} influx in HEK-293 cells stably expressing BmCrzR-EGFP or Flag-BmCrzR was measured in response to 1 μM Crz peptide using the fluorescent Ca^{2+} indicator fura-2. (D) HEK-293 cells expressing pBm-EGFP vector and BmCrzR-EGFP were challenged with different concentrations of Crz (100 pM to 1 μM) for 5 min. Immunoblots were quantified using a Bio-Rad Quantity One Imaging system. (E) HEK-293 and (F) BmN cells stably expressing BmCrzR-EGFP were activated by the indicated concentrations of Crz for 60 min. (G) HEK-293 and (H) BmN cells stably expressing BmCrzR-EGFP were treated with 1 μM Crz for the indicated time periods (10−60 min). Panels E and G are the result of fluorescence microscopy and an ELISA. Error bars represent SEM for three replicates.

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BmCrzR Localization and Internalization Assay. For the internalization assay, HEK-293 cells stably expressing WT or mutant BmCrzR-EGFP were treated with 1 μM corazonin for 1 h. For measuring receptor localization, HEK-293 cells stably expressing BmCrzR-EGFP were seeded in covered glass-bottom six-well plates. There cells were treated with 1 μM corazonin and either 100 g/mL Alexa Fluor 546-labeled transferrin (red) or 50 nM LysoTracker DND-99 (red) at 37 °C for 60 min. For the recycling assay, HEK-293 cells stably expressing BmCrzR-EGFP were incubated with 100 g/mL cycloheximide, an inhibitor used to block de novo biosynthesized receptors, and 1 μM corazonin for 60 min. After removal of the agonist, the cells were washed three times with PBS and further incubated in the presence of cycloheximide for the indicated time periods. After fixation with 4% paraformaldehyde for 10 min, cells were mounted in mounting reagent (dithiothreitol/PBS/glycerol). Confocal images were recorded on a Zeiss LSM510 microscope with an attached Axiovert 200 microscope and linked to a LSM5 computer system. Excitation was conducted at 488 nm, and fluorescence detection was used with a 505−530 nm bandpass filter.

Measurement of Cell Surface Receptor by an ELISA. For quantification of receptor internalization, an ELISA was performed as described previously, with some modifications.[13] Briefly, 48 h after transfection, cells in 48-well plates were stimulated with the agonist for 60 min, fixed with 4% formaldehyde for 10 min at room temperature, and blocked for 60 min with 1% bovine serum albumin in TBS [20 mM Tris and 150 mM NaCl (pH 7.5)]. The cells were then incubated for 1 h with a 1:3000 dilution of a mouse anti-Flag M2 monoclonal antibody. Cells were then washed four times with TBS followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:3000 in 1% BSA/TBS) for 60 min; 200 μL of HRP substrate (Sigma) was added to each well, and the samples were incubated at 37 °C for 20−30 min. The reactions were stopped by adding an equal volume of 1% SDS, and the sample absorbance was measured at 405 nm using a Bio-Rad microplate reader.

cAMP Accumulation. HEK-293 cells transiently cotransfected with BmCrzR or BmCrzR mutants and pCRE-Luc were grown to 90−95% confluence in DMEM without fetal bovine serum. Cells were then stimulated with different concentrations of corazonin and incubated for 4 h at 37 °C. Luciferase activity was detected using a firefly luciferase kit (Promega, Madison, WI).

Western Blot Analysis. To analyze the knockdown of siRNA-targeted proteins and the phosphorylation of ERK1/2, siRNA-transfected or agonist-stimulated HEK-293 cells in a 24-well plate were lysed using a RIPA buffer (Beyotime). Equal amounts of total cell lysate were size-fractionated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (10 to 12%) and transferred to a PVDF membrane (Millipore). Membranes were blocked in blocking buffer (TBS containing 0.05−0.1% Tween 20 and 5% nonfat dry milk) for 1 h at room temperature and then incubated with rabbit monoclonal anti-phospho-ERK1/2 antibody (Cell Signaling) and anti-rabbit HRP-conjugated secondary antibody (Beyotime) according to the manufacturers' protocols. Total ERK1/2 was assessed as a loading control after p-ERK1/2 chemiluminescence detection using a HRP substrate purchased from Cell Signaling. Clathrin was detected using mouse monoclonal antibody anti-clathrin HC (Santa Cruz Biotechnology, Santa Cruz, CA). β-Arrestin1 and β-arrestin2 were detected using mouse monoclonal anti-arrestin2 and monoclonal anti-arrestin3 (Cell Signaling).

Data Analysis. All results were expressed as means ± the standard error of the mean (SEM). Data were analyzed using nonlinear curve fitting (GraphPad Prism version 5.0) to obtain EC_{50} values. Statistical significance was determined using a Student's t test and analysis of variance (ANOVA). Probability values that were less than or equal to 0.05 were considered significant.

RESULTS

Corazonin Induces the Rapid Internalization of BmCrzR. To directly visualize the localization, internalization, and trafficking of BmCrzRs, a vector was constructed to transiently or stably express BmCrzR fused with enhanced green fluorescent protein (EGFP) at the C-terminus (BmCrzR-EGFP) in HEK-293 and BmN cells. The functional signaling of BmCrzR-EGFP was first examined by assaying cAMP accumulation and intracellular Ca^{2+} flux. In both HEK-293 and BmN cells cotransfected with cAMP response element (CRE)-driven firefly luciferase and BmCrzR-EGFP, agonist administration produced a dose-dependent increase in luciferase activity comparable to that of cells expressing the wild-type receptors (Figure 1A,B). Corazonin peptide also stimulated a similar level of intracellular Ca^{2+} concentration increase in BmCrzR-EGFP-expressing HEK-293 cells as compared to that seen with the wild type (Figure 1C). In addition, agonist treatment induced ERK1/2 phosphorylation in HEK-293-BmCrzR-EGFP cells (Figure 1D). These results suggest that the presence of EGFP fused to the C-terminus of BmCrzR does not interfere with the interaction between the receptor and G proteins or subsequent signaling activities. This construct was then used for further characterization of BmCrzR internalization.

Our previous study demonstrated that BmCrz stimulation leads to a rapid internalization of BmCrzR from the cell surface into the cytoplasm.[7] In the study presented here, a kinetic analysis of agonist-mediated receptor internalization was performed. Upon stimulation with BmCrz peptides, BmCrzRs were internalized in a concentration-dependent manner in HEK-293 (Figure 1E) and BmN (Figure 1F) cells. Treatment of cells with 1 μM BmCrz evoked a rapid internalization of BmCrzR-EGFP in HEK-293 (Figure 1G) and BmN (Figure 1H) cells in a time course. After being exposed to BmCrz for 10 min, internalized receptors were distributed throughout the cytoplasm. At 30 min, these were largely clustered in the perinuclear region. In addition, an ELISA was developed using an anti-Flag antibody to quantify cell surface Flag-BmCrzR. Agonist exposure induced receptor internalization within 10 min and resulted in a significant loss of cell surface expression within 1 h in a dose-dependent manner (Figure 1E,F). This was consistent with our confocal microscopy observations.

Agonist-Mediated BmCrzR Internalization Is Arrestin-Dependent. It is generally accepted that upon activation by an agonist, GPCRs are phosphorylated at the C-terminal tail by G protein-coupled receptor kinase (GRK). This is followed by arrestin binding, which results in rapid receptor internalization.[16,17] To assess the role of arrestins in the regulation of BmCrzR internalization, *Bombyx* Kurtz, a novel nonvisual arrestin cloned from *B. mori*, fused with EGFP at the C-terminal end (Bm-kurtz-EGFP), was cotransfected with Flag-tagged BmCrzR into BmN cells. Confocal microscopy revealed that, in the absence of BmCrz, Bm-kurtz was localized primarily

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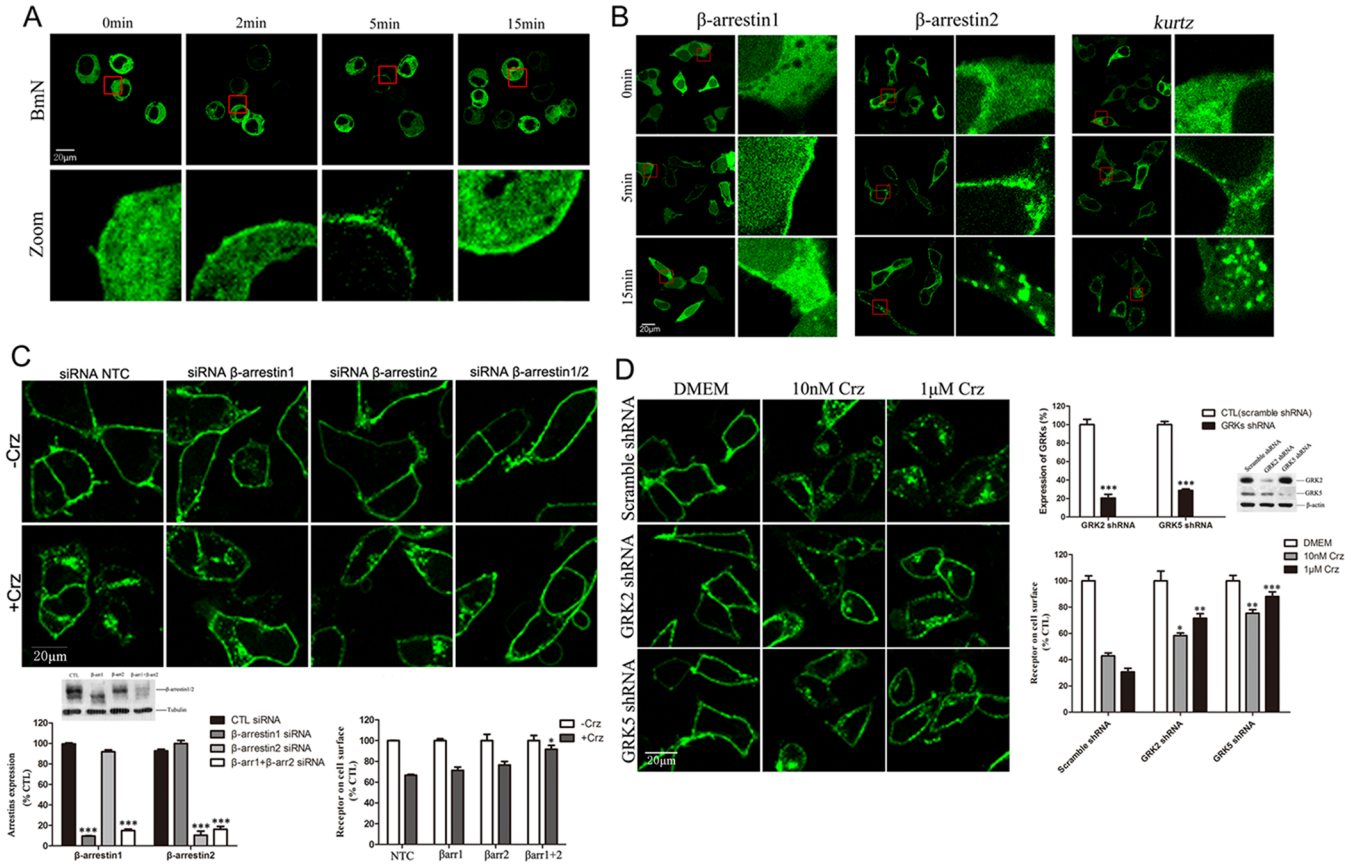

Figure 2. Arrestins and GRKs are involved in BmCrzR internalization. (A) BmN cells were cotransfected with FLAG-BmCrzR and kurtz-EGFP. Kurtz resided in the cytosol prior to corazonin stimulation (PBS) and was translocated to the membrane in response to treatment with 1 μM corazonin at 2 and 5 min. It then returned to the cytosol in an inactive state at ~15 min. (B) HEK-293 cells were cotransfected with FLAG-BmCrzR with β-arrestin1-EGFP, β-arrestin2-EGFP, or kurtz-EGFP. All arrestin isoforms resided in the cytosol prior to Crz stimulation (control) and were translocated to the membrane in response to treatment with 1 μM Crz for 5 min. Some β-arrestin1-EGFP was still bound to receptors on the membrane at 15 min, while β-arrestin2-EGFP and kurtz-EGFP return to the cytosol at 15 min. (C) HEK-293 cells stably expressing BmCrzR-EGFP were transfected with specific β-arrestin1 siRNA, β-arrestin2 siRNA, or both β-arrestin1 and β-arrestin2 siRNA. The nonspecific control siRNA (NTC siRNA) was loaded as the control. Seventy-two hours after transfection, cells were stimulated with 1 μM Crz for 30 min and examined via confocal microscopy and an ELISA. (D) Knockdown of GRK2/5 using a shRNA lentivirus in BmCrzR-EGFP-expressing HEK-293 cells. After being treated with DMEM (control), 10 nM Crz, or 1 μM Crz for 1 h, cells were analyzed using a confocal microscope. An ELISA was performed for measurement of cell surface receptors in BmCrzR-expressing cells treated with shRNA. ELISA data are expressed as a percentage of receptors detected on the surface of agonist-untreated cells expressing BmCrzR. β-Arrestin1/2 or GRK2/5 expression of the knockdown group and NTC or scramble group was analyzed by Western blotting with the indicated antibody. Data in panels C and D were analyzed using ANOVA and a Student's t test (*p < 0.05; **p < 0.01; ***p < 0.001). All pictures and data shown are representative of at least three independent experiments.

in the cytoplasm. After treatment with 1 μM agonist for 5 and 15 min at 28 °C, kurtz had been significantly translocated to the plasma membrane. This suggests that Bm-kurtz plays a major role in the process of regulating BmCrzR internalization (Figure 2A). Further observation demonstrated that, in the absence of BmCrz, β-arrestin1 was localized in both the cytoplasm and nucleus while β-arrestin2 and Bm-kurtz were localized primarily in the cytoplasm. Upon stimulation with 1 μM BmCrz for 5 min at 37 °C, β-arrestin1, β-arrestin2, and Bm-kurtz had been significantly translocated to the plasma membrane. Further incubation with BmCrz for 15 min at 37 °C led to the redistribution of fluorescent vesicles of β-arrestin2 and Bm-kurtz, but not of β-arrestin1, from the plasma membrane to the cytoplasm (Figure 2B). This suggests a tight association of phosphorylated receptors with β-arrestin2 and Bm-kurtz. Our data also strongly suggest that *Bombyx* kurtz functions in a manner more similar to that of mammalian β-arrestin2 in the regulation of BmCrzR internalization than to that of mammalian β-arrestin1.

To further confirm the function of the arrestins involved in BmCrzR internalization, we used specific siRNAs to reduce the level of expression of β-arrestin1 and β-arrestin2 in BmCrzR-EGFP-expressing HEK-293 cells. The endogenous expression of β-arrestins was effectively and specifically knocked down by the specific siRNA treatment but remained unaffected by nonspecific or control siRNA (Figure 2C). Silencing either β-arrestin1 or β-arrestin2 alone showed no significant effect on BmCrzR internalization. Conversely, knockdown of both β-arrestin1 and β-arrestin2 led to a significant inhibition of agonist-induced internalization of BmCrzR, as analyzed by confocal microscopy and an ELISA (Figure 2C). In addition, our data demonstrated that agonist-bound BmCrzRs remained associated with arrestin throughout internalization, whereas as a control, *Bombyx* adipokinetic hormone receptor (AKHR) maintained translocated arrestin only at the plasma membrane (Figure S1).

Involvement of GRK2 and GRK5 in the Regulation of Agonist-Mediated Internalization of BmCrzR. It is well-

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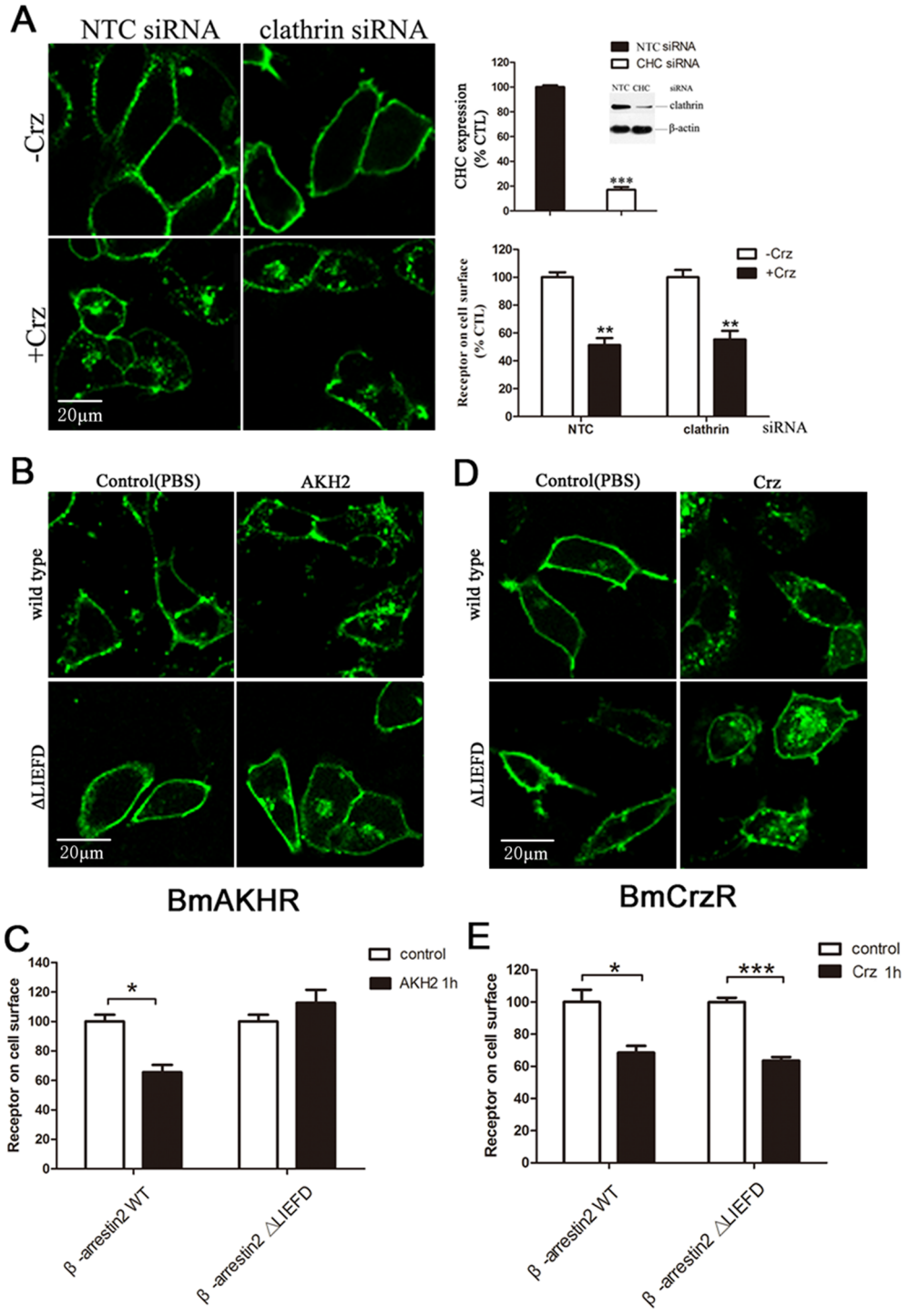

Figure 3. BmCrzR internalized via the clathrin-independent pathway. (A) HEK-293 cells stably expressing BmCrzR were transfected with specific clathrin heavy chain siRNA (CHC) or nonspecific control siRNA (NTC). Seventy-two hours after transfection, cells were stimulated with 1 μM Crz for 30 min and examined via confocal microscopy and an ELISA as described in Materials and Methods. Seventy-two hours after transfection, cells were harvested, and equal amounts of total cellular lysate were separated by 10% SDS−PAGE, transferred to PVDF, and incubated with the indicated antibody. Blots were stripped and reprobed for β-actin for a loading control. Shown is a representative immunoblot from three independent experiments. Internalization of BmAKHR (*Bombyx* Adipokinetic hormone receptor) and BmCrzR influenced by overexpression of β-arrestin2 ΔLIEFD mutants deficient in clathrin binding. HEK-293 cells cotransiently transfected with BmAKHR-EGFP and the wild type (WT) or ΔLIEFD mutant of pcDNA β-arrestin2 and incubated with 1 μM AKH2 for 60 min and examined via (B) confocal microscopy or (C) an ELISA as described in Materials and Methods. HEK-293 cells cotransiently transfected with BmCrzR-EGFP with the wild type (WT) or ΔLIEFD mutant of pcDNA-β-arrestin2 and incubated with 1 μM Crz for 60 min and examined via (D) confocal microscopy or (E) an ELISA as described in Materials and Methods. Data were analyzed using a Student's *t* test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). All pictures and data shown are representative of at least three independent experiments.

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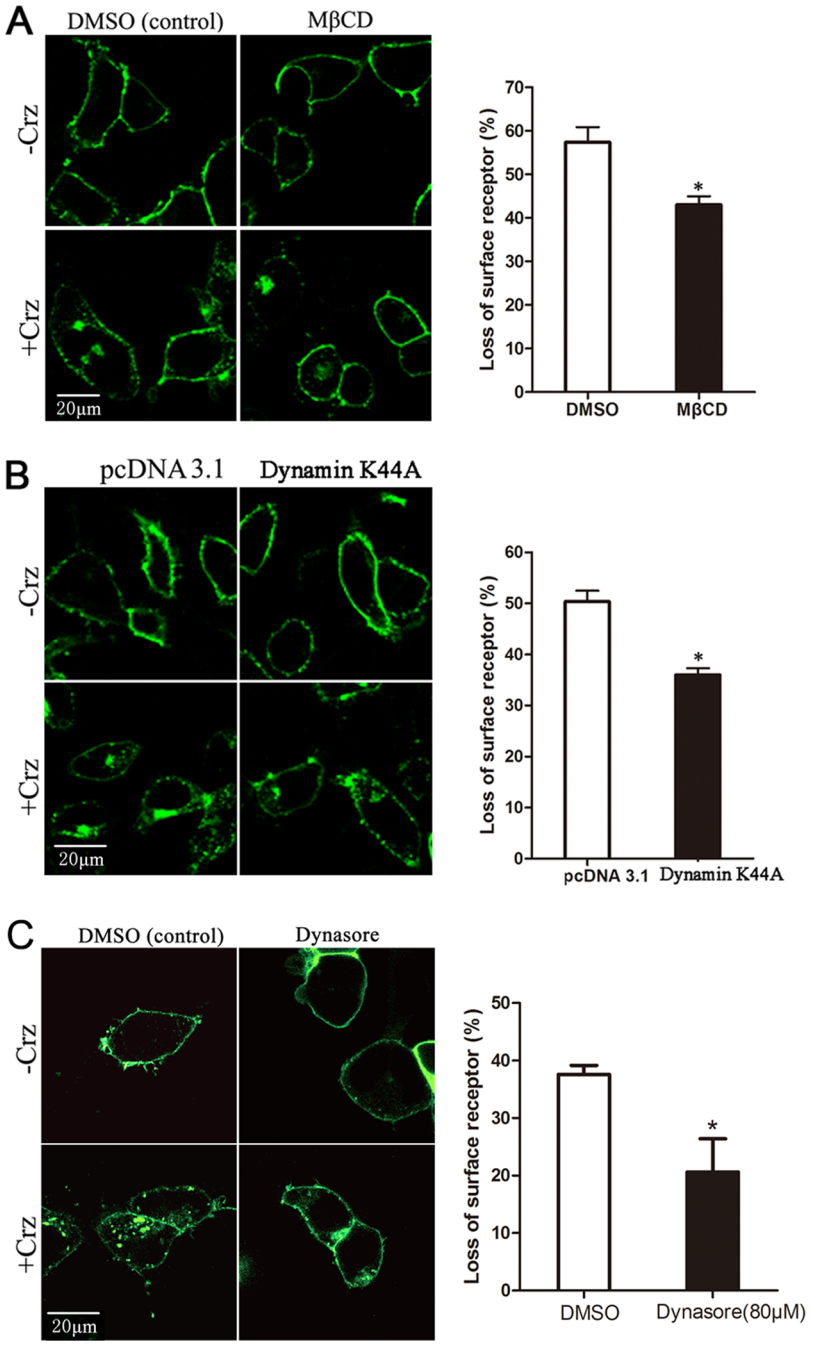

Figure 4. BmCrzR internalized via the lipid raft-dependent pathway. (A) HEK-293 cells stably expressing BmCrzR-EGFP were pretreated with or without the lipid raft inhibitor MβCD (10 mM) for 1 h prior to agonist stimulation. Cells were then treated with 1 μM Crz for 30 min and examined via confocal microscopy and an ELISA. (B) HEK-293 cells stably expressing BmCrzR-EGFP were transfected with dynamin-K44A. Forty-eight hours after transfection, cells were treated with 1 μM Crz for 30 min and examined via confocal microscopy and an ELISA. (C) HEK-293 cells stably expressing BmCrzR-EGFP were pretreated with or without the dynamin inhibitor Dynasore (80 μM) for 1 h prior to agonist stimulation. Cells were then treated with 1 μM Crz for 60 min and examined via confocal microscopy and an ELISA. All pictures and data shown are representative of at least three independent experiments. Data were analyzed by using a Student's t test (*p < 0.05; **p < 0.01; ***p < 0.001).

known that G protein-coupled receptor kinase (GRK)-mediated phosphorylation is an essential requirement for agonist-induced internalization of most GPCRs.[18] To assess the role of GRKs in the regulation of BmCrzR internalization, recombinant lentiviruses expressing small hairpin RNAs against GRK2 and GRK5 were constructed. Stably infected HEK-293 cell lines were then established. The endogenous expression of GRKs was effectively silenced by the specific shRNA treatment but remained unaffected in cells treated with nonspecific (scramble) shRNA. Our data, derived from confocal microscopy observation and ELISA quantitative analysis, showed that knockdown of GRK2 or GRK5 alone resulted in a significant inhibition of agonist-mediated internalization of BmCrzR (Figure 2D). Collectively, these results suggest that GRK2 and GRK5 are essential requirements for the agonist-mediated internalization of BmCrzR.

Agonist-Induced Internalization of BmCrzR via a Clathrin-Independent and Dynamin-Dependent Path-

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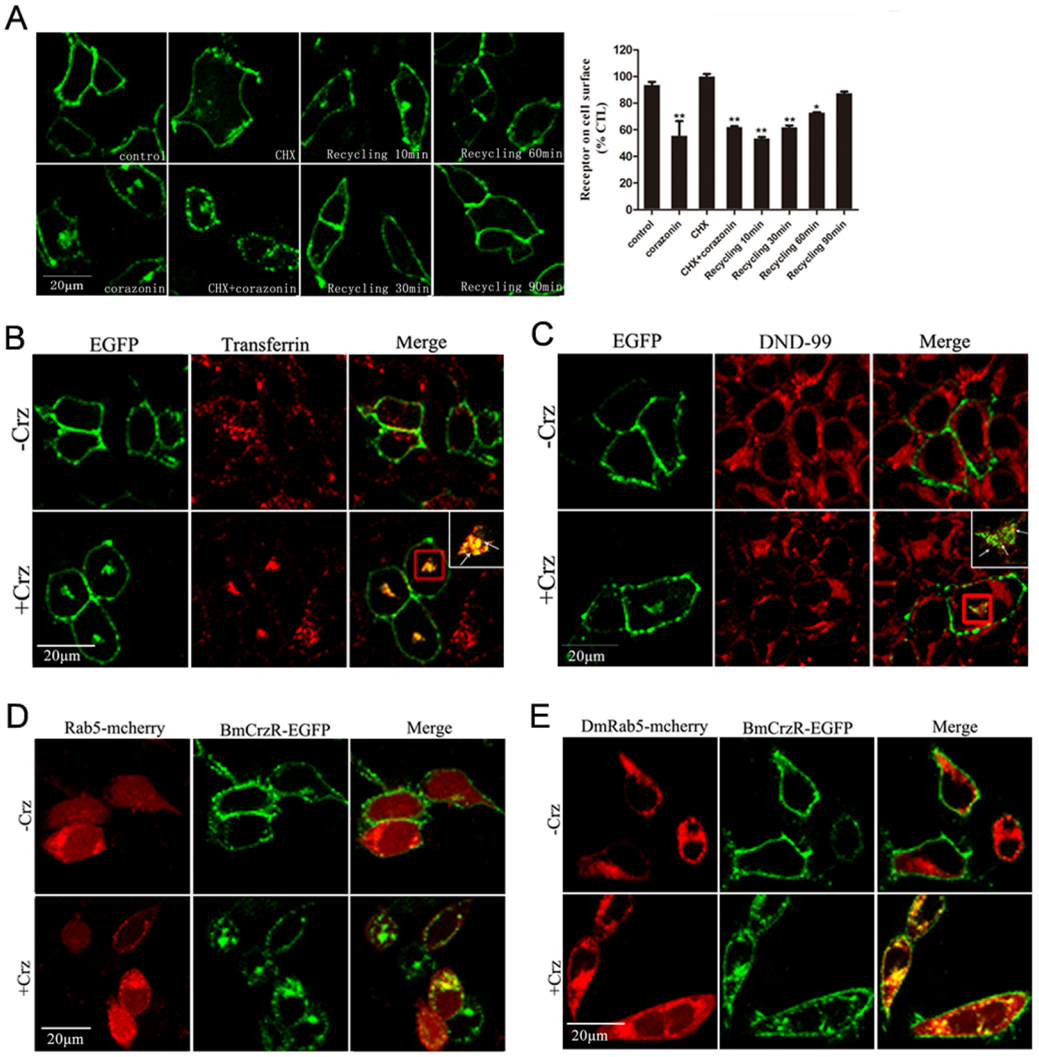

Figure 5. Recycling of internalized BmCrzR in HEK-293 cells. (A) HEK-293 cells expressing BmCrzR-EGFP were treated with 100 μg/mL cycloheximide and 1 μM Crz for 60 min. After the agonist had been thoroughly washed out, the cells were further incubated for the indicated time periods, with or without cycloheximide, and examined via fluorescence microscopy. ELISA data are expressed as a percentage of receptors detected on the surface of agonist-untreated cells expressing BmCrzR. HEK-293 cells expressing BmCrzR-EGFP were incubated with or without 100 nM Crz at 37 °C in the presence of either (B) 100 μg/mL Alexa Fluor 594-labeled transferrin or (C) 50 nM Lysotracker DND-99 for 60 min. Cells were fixed and examined via confocal microscopy as described in Materials and Methods. HEK-293 cells cotransfected with BmCrzR-EGFP and (D) Rab5-mCherry or (E) DmRab5-mCherry were stimulated with or without Crz and then fixed and examined using the confocal microscope. Error bars represent SEM for three replicates. One-way ANOVA with Tukey's post hoc test revealed differences from unstimulated (control) cells (*p < 0.05; **p < 0.01). All data are from at least three independent experiments.

way. To determine the role of clathrin in the agonist-induced BmCrzR internalization, BmCrzR-expressing HEK-293 cells were transfected with specific siRNAs targeted against the clathrin heavy chain to silence the expression of clathrin.[15] The specific siRNA treatment led to a specific and effective knockdown of clathrin expression (Figure 3A). The silencing of clathrin expression resulted in no significant inhibition of agonist-induced BmCrzR internalization, as analyzed by microscopy and an ELISA (Figure 3A).

In addition, a β-arrestin2 mutant (β-arrestin 2 ΔLIEFD) that was selectively defective in its interaction with clathrin[19,20] was generated to confirm the role of clathrin in agonist-mediated BmCrzR internalization. Results from both microscopy and an ELISA showed that overexpression of the β-arrestin2 ΔLIEFD mutant significantly blocked agonist-induced internalization of AKHR, which has been shown to undergo internalization via both arrestin- and clathrin-dependent pathways (Figure 3B,C).[13] However, the agonist-induced internalization of BmCrzR was not altered by overexpression of the β-arrestin2 mutant (Figure 3D,E). Taken together, our data suggest that the internalization of BmCrzR is unlikely to be clathrin-dependent. In contrast, treatment of cells with methyl-β-cyclodextrin (MβCD), a specific inhibitor for the caveolar pathway,[21] showed a significant inhibitory effect on agonist-induced BmCrzR internalization (Figure 4A). However, as the control, the ligand-dependent internalization of GPR40 is not altered by MβCD (Figure S2). It is therefore more likely that the caveolar pathway is involved in agonist-induced BmCrzR internalization.

To further assess whether dynamin plays a role in BmCrzR internalization, BmCrzR-expressing cells were transfected with the dynamin-1 dominant negative mutant K44A (dynamin-K44A) and analyzed 48 h after transfection. Furthermore, BmCrzR-EGFP-expressing cells were pretreated with or

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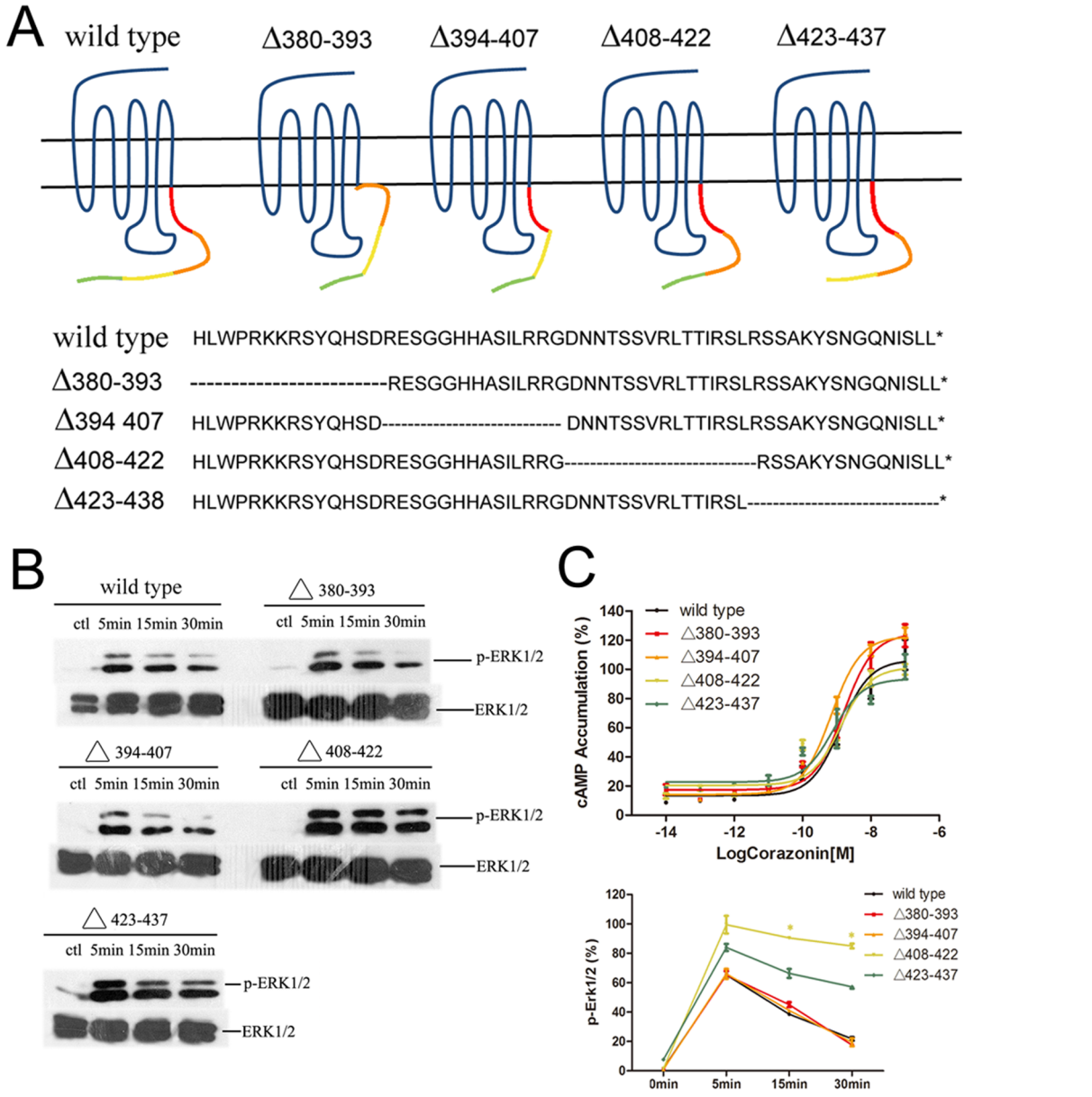

Figure 6. Functional characterization of C-terminal domain deletion mutants of BmCrzR. (A) Composition of truncation mutants. The C-terminus was divided into four fragments with different colors. The overall composition of individual BmCrzR mutants is shown schematically. The deleted amino acids are indicated with a dash (−). (B) Activation of ERK1/2 in HEK-293 cells expressing BmCrzR or BmCrzR mutants through a challenge with 10 nM Crz for 5, 15, and 30 min. (C) Accumulation of cAMP in HEK-293 cells cotransfected with CRE-Luc and receptors was determined in response to various concentrations of Crz treatment. Data were analyzed using a Student's *t* test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). All data are from at least three independent experiments.

without the dynamin inhibitor Dynasore (80 μM) for 1 h prior to agonist stimulation. Agonist-promoted BmCrzR internalization was effectively attenuated by dynamin-K44A or Dynasore (Figure 4B,C). This indicates that dynamin is involved in this process of BmCrzR internalization.

Internalized BmCrzRs Are Recycled to the Cell Membrane, but Some Are Also Transported to Lysosomes. To investigate whether internalized BmCrzRs are recycled back to the plasma membrane via recycling endosomes or degraded in the lysosomes, BmCrzR-EGFP-expressing cells were treated with 100 μg/mL cycloheximide and stimulated by 1 μM agonist peptide at 37 °C for 60 min. This was followed by the removal of any residual agonist by washing and further incubation in the presence of cycloheximide for the indicated periods of time. Confocal microscopy revealed that pretreatment with cycloheximide had no effect on BmCrzR localization and internalization. Most of the BmCrzR was internalized after treatment with 1 μM BmCrz for 60 min (Figure 5A). However, the internalized receptors were partially recycled to the plasma membrane within 30 min of agonist removal. A further 60 min incubation resulted in significant, but not full, recovery of the internalized BmCrzR to the cell surface, as evaluated by microscopy and an ELISA (Figure 5A).

To further clarify the fate of internalized BmCrzR, BmCrzR-EGFP-expressing HEK-293 cells were co-incubated with corazonin peptide together with the endosome marker Alexa Fluor 546-labeled transferrin or the lysosome marker Lyso-

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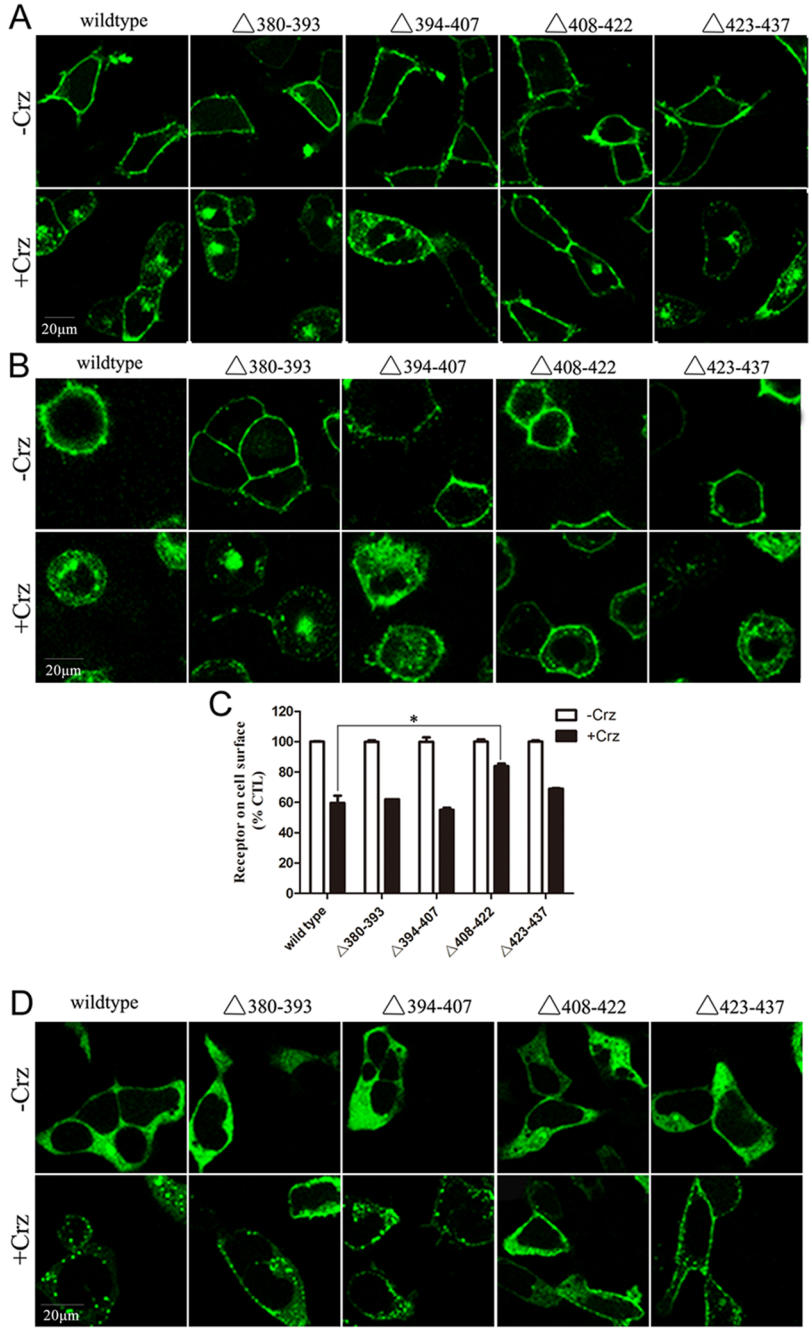

Figure 7. Effects of C-terminal deletions on internalization of BmCrzR in HEK-293 cells. (A) HEK-293 cells transiently transfected with the wild type (WT) or mutant deletion of BmCrzR and incubated with 1 μM Crz for 60 min and then examined via confocal microscopy as described in Materials and Methods. (B) BmN cells tansfected with WT or mutant deletion of BmCrzR and incubated with 1 μM Crz for 60 min and then examined via confocal microscopy. (C) HEK-293 cells transiently transfected with wild-type or deletion mutant FLAG-BmCrzR were used to assess cell surface receptor levels by an ELISA. Receptors were incubated with 1 μM Crz for 60 min. The ratio of internalized receptors was normalized by the wild type. (D) Crz-induced subcellular redistribution of kurtz-EGFP in HEK-293 cells expressing WT or deletion mutant BmCrzR. Cells were treated with 1 μM Crz for 5 min and then imaged on a confocal microscope. All pictures and data are representative of at least three independent experiments. One-way ANOVA with Tukey's post hoc test revealed differences from wild-type (control) cells (*$p < 0.05$).

Tracker Red DND-99. Analysis by confocal microscopy showed that extensive colocalization of transferrin and BmCrzR-EGFP had occurred (Figure 5B). However, some colocalization of internalized BmCrzR-EGFP with Lyso-tracker DND-99, a marker for late endosomes and lysosomes, was also noted (Figure 5C). Next, the cells were pretreated with lysosome inhibitor Bafilomycin A1 (200 nm) for 1 h prior to the recycling process. Internalized receptors were still partially recycled to the plasma membrane within 30 min of agonist removal, but further incubation resulted in almost full recovery of internalized receptors to the cell surface (Figure S3); in the cells without Bafilomycin A1 treatment, the internalized receptors remained partially inside the cytoplasm (Figure 5A). In addition, BmCrzR-EGFP was co-expressed with mCherry-tagged Ras-associated protein-5 (Rab5) or mCherry-tagged *D. melanogaster* Ras-associated protein-5 (DmRab5).[22]

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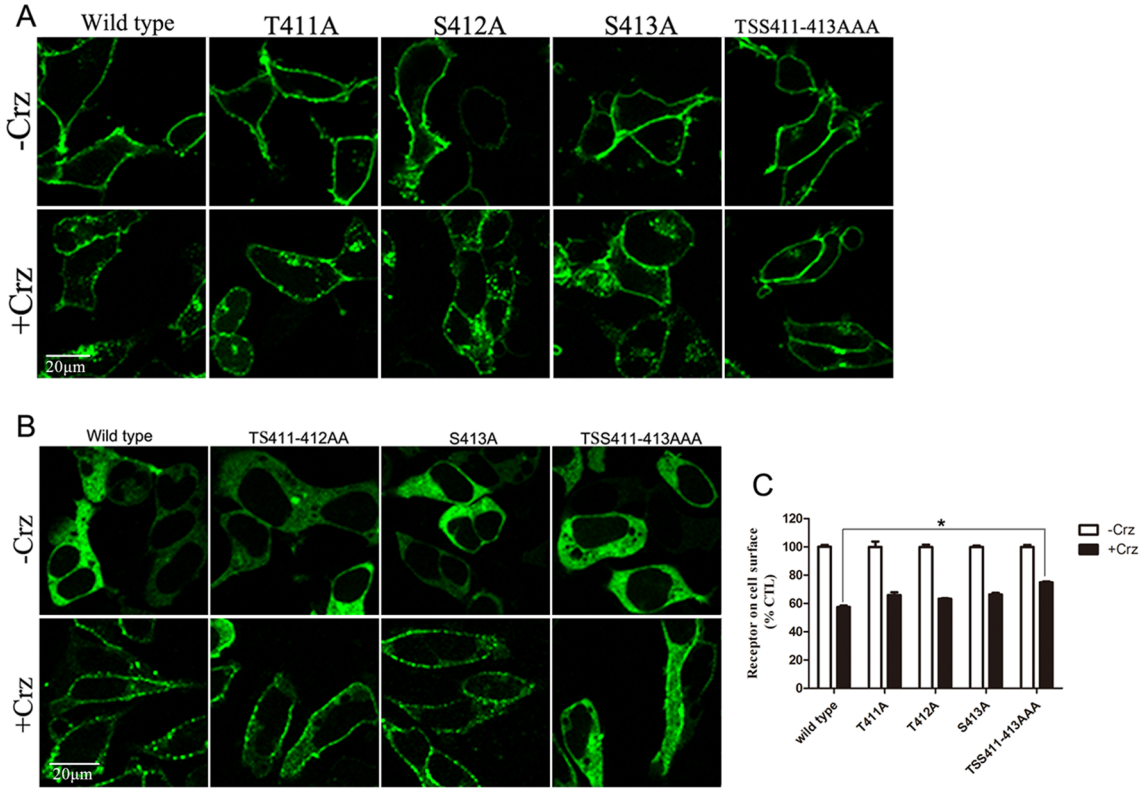

Figure 8. Effects of alanine substitutions on the internalization of BmCrzR in HEK-293 cells. (A) Internalization of wild-type BmCrzR and its site mutants induced by 1 μM Crz for 60 min. (B) Kurtz recruitment of wild-type BmCrzR and its site mutants induced by 1 μM Crz for 5 min. (C) HEK-293 cells transiently transfected with wild-type FLAG-BmCrzR or its site mutants were used to assess cell surface receptor levels and total cell receptor levels by an ELISA. Receptors were incubated with 1 μM Crz for 60 min. Pictures shown are representative of at least three independent experiments. Data were analyzed by using the one-way ANOVA with a Tukey's post hoc test that revealed differences from the wild type (control) (*$p < 0.05$).

As shown in panels D and E of Figure 5, in the presence of the agonist, the internalized BmCrzRs were extensively colocalized with both Rab5 and DmRab5. Taken together, these results suggest that internalized BmCrzRs are transported to Rab5-positive early endosomes. Most are then recycled back to the cell surface, but some of internalized receptors are also transported to lysosomes for degradation.

Distinct Domains and Residues within the C-Terminus of BmCrzR Are Responsible for Phosphorylation and Internalization. Domains and residues within the C-terminus of GPCRs are generally thought to regulate receptor desensitization, internalization, and intracellular trafficking by facilitating association of the receptors with various cellular proteins.[23,24] To evaluate the role of the C-terminus in the regulation of internalization and signaling of BmCrzR, a series of mutants with C-terminal deletions of four segments of the 58 total residues were generated (Figure 6A). Functional assays reveal that all four mutants showed the potential to activate cAMP/CREB Ca^{2+} (data not shown) and ERK1/2 signaling pathways in a manner comparable to that of wild-type BmCrzR. However, mutant Δ408−422 exhibited a higher activity during ERK1/2 signaling. This suggests that Δ408−422 exhibits a disability in desensitization (Figure 6B,C). Deletion mutants Δ380−393, Δ394−407, and Δ423−437 showed the capability to be internalized to an extent comparable to that of the wild type (Figure 7A,B), whereas the Δ408−422 mutant exhibited a significant decrease in the level of BmCrz-mediated internalization in HEK-293 and BmN cells. These confocal microscopy observations were confirmed by quantitative ELISA analysis (Figure 7C). Furthermore, results derived from experiments in the cotransfection of wild-type FLAG-BmCrzR and the C-terminal deletions with kurtz-EGFP showed that mutant Δ408−422, of which internalization was blocked, also lost the ability to recruit Bm-kurtz (Figure 7D). Collectively, these results suggest that some serine and threonine residues within this deleted domain are likely responsible for phosphorylation and β-arrestin recruitment.

To further identify the key residues within the sequence from Asp^{408} to Leu^{422}, responsible for BmCrzR internalization, alanine-substituted mutants were generated and expressed in HEK-293 cells. No site-directed mutants exhibited any differences in cell surface expression and signal transduction from those of wild-type BmCrzR (Figure 8B). Further investigation by confocal microscopy and ELISA analysis demonstrated that alanine-substituted mutants S287A, T411A, T412A, and T413A (data not shown) showed the same activity in agonist-induced internalization as the wild type, while the mutant with the replacement of three amino acids, ^{411}TSS^{413}, with alanines exhibited a partial, but significant, decrease in the level of receptor internalization compared with that of wild-type BmCrzR (Figure 8A,C). This was also confirmed by the arrestin translocation assay (Figure 8B). Altogether, our findings strongly suggest that the serine/threonine cluster ^{411}TSS^{413} is likely to play an essential part in phosphorylation and arrestin recruitment for receptor desensitization and internalization. However, other possible phosphorylation sites mediated by GRKs or PKC may exist for BmCrzR internalization.

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DISCUSSION

It has been shown that BmCrzR can transduce signals intracellularly via Gs- and Gq-dependent cascades. This can be followed by receptor internalization.[13] While the mechanisms of internalization and desensitization have been previously studied for many GPCRs, including those from humans, mice, and rats, in this study, we provide further detailed characterizations of the agonist-mediated internalization of BmCrzR in HEK-293 and BmN cells through a combination of confocal microscope observations with quantitative ELISA analysis. Investigations using deletion mutants and site-directed mutations at the C-terminus of the receptor helped us to define the motifs and key residues within the C-terminal region responsible for phosphorylation, desensitization, and internalization.

It is generally accepted that upon activation, the C-terminal tail and intracellular loops of GPCRs are rapidly phosphorylated by GRKs or other different protein kinases. This is followed by recruitment of arrestins to the plasma membrane where they bind to the receptor that, in turn, results in receptor internalization.[25,26] Using a β-arrestin-EGFP translocation assay, we revealed that both β-arrestin2-EGFP and β-arrestin1-EGFP were significantly recruited into the plasma membrane. To confirm the BmCrzR internalization in insect cells, we cloned a full-length kurtz cDNA, a novel nonvisual arrestin in insects,[27] from *B. mori* and constructed two expression vectors containing *Bombyx* kurtz fused with EGFP at its C-terminal end for assaying arrestin recruitment in both HEK-293 and BmN cells. *Bombyx* kurtz showed the potential to be recruited to the plasma membrane in both HEK-293 and BmN cells expressing BmCrzR, in a manner comparable to that of human β-arrestin2. *Bombyx* kurtz contains both amino- and carboxy-terminal arrestin domains and is 80, 75, and 79% similar to *Drosophila* kurtz, human β-arrestin2, and β-arrestin1, respectively. Although in sequence, *Bombyx* kurtz lies closer to β-arrestin1, our observation strongly suggests that *Bombyx* kurtz behaves more like human β-arrestin2 in its regulation of BmCrzR internalization. GPCRs can be classified into two different classes based on the interaction of receptor with arrestin following receptor activation. Class A receptors, including β2-adrenoceptor and μ-opioid, display a rapid dissociation from the arrestin complex during the early phase of internalization. Conversely, class B receptors such as vasopressin V2 and neurotensin 1 receptor maintain a stable association with arrestins throughout internalization.[28] Our data demonstrated that BmCrzR belongs to the class B receptors, consistent with the observation by Johnson et al.[29]

Upon agonist activation, most GPCRs are internalized through caveolae-dependent and clathrin/caveolae-independent distinct endocytic pathways.[30,31] Our data demonstrate that agonist-mediated BmCrzR internalization was, if at all, not significantly inhibited by siRNA-targeted knockdown of the clathrin heavy chain. Our further investigation demonstrated that overexpression of β-arrestin2 ΔLIEFD, a dominant-negative construct defective in the interaction with clathrin,[19] resulted in a significant inhibition of agonist-induced internalization of Bombyx AKHR, which undergoes internalization via a clathrin-dependent pathway,[13] but showed no effect on the agonist-mediated internalization of BmCrzR. However, treatment of methyl-β-cyclodextrin (MβCD), a compound that depletes plasma membrane cholesterol and thereby disrupts the structural integrity of caveolae[32−34] or interferes with the formation of lipid rafts at the membrane surface,[35] exhibited inhibitory effects on BmCrzR internalization. A recent study has just confirmed the specificity of MβCD to inhibit the internalization through the caveolar pathway.[21] Previous studies have shown that the endothelin A and B receptors undergo internalization through a β-arrestin-dependent but clathrin-independent endocytic pathway.[36,37] The H1 receptor has been found to be internalized via a clathrin-independent mechanism that most likely involves lipid rafts.[38] Furthermore, our results, derived from overexpression of dominant-negative mutants of dynamin-K44A, indicated that BmCrzR was internalized via a dynamin-dependent pathway. Although the M2 muscarinic acetylcholine receptor appeared to be internalized via a clathrin-independent pathway, nevertheless it remained insensitive to the overexpression of the dynamin-K44A mutant.[39,40] Previous studies have demonstrated that both endocytosis, via caveolae, and lipid rafts are dynamin-dependent.[41,42] It seems more likely that upon activation, BmCrzR undergoes internalization via a clathrin-independent and dynamin-dependent pathway. However, we cannot completely exclude the possible involvement of a clathrin-dependent pathway in the BmCrzR internalization based on our present data, and many of the molecular details involved in the agonist-mediated internalization of BmCrzR remain to be determined.

The internalized receptors are either recycled back to the cytoplasmic membrane via the recycling endosome pathway or led to lysosomes for degradation.[28,43] To investigate the fate of internalized BmCrzR, recycling assays using confocal microscopy and an ELISA were performed. Our results clearly showed that internalized BmCrz receptors were significantly, but not fully, recycled to the cell surface. Treatment with the protein de novo synthesis inhibitor cycloheximide did not affect the recycling of internalized receptors. This suggests that the cell surface recovery of BmCrzR is independent of the de novo synthesis of receptors. This was confirmed by our further observation of the extensive colocalization of BmCrzR-EGFP with the transferrin and Rab5 markers of this endosomal pathway. Moreover, there is evidence that the M2 mAChR is internalized via a clathrin-independent endocytosis (CIE) pathway and then targeted to lysosomes for degradation.[44] Our current data also showed that some of internalized BmCrzRs were colocalized with Lysotracker DND-99. However, it has been suggested that, following internalization via a caveolar endocytic pathway, the cargo is then transferred to the early endosome where it meets clathrin-dependent endocytosis cargo and can then be routed along the degradative pathway to the lysosome or recycled back to the cell surface.[45] Taken together, it is likely that most internalized BmCrzRs are recycled to the plasma membrane via recycling endosomes, but that some were also transported to the lysosomes. Therefore, further investigation will be necessary to clarify the role of PKC in the regulation of agonist-mediated BmCrzR internalization and to elucidate whether the internalized BmCrzR via a clathrin-independent endocytosis pathway is also targeted to lysosomes for degradation.

The C-terminal tail of G protein-coupled receptors has been demonstrated to be essential for receptor internalization and trafficking.[23,24] In this study, an approach combining amino acid deletion with site-directed mutagenesis has been employed to identify structural and functional domains within the BmCrzR C-terminus and to explore their potential roles in receptor phosphorylation, desensitization, and internalization. The results derived from four mutants with deletions of 14

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amino acids each that were distinct from truncated mutants indicated that the sequence from Asp[408] to Leu[422] is required for agonist-mediated phosphorylation and internalization. Further mutagenesis studies with alanine substitution demonstrated that key residues [411]TSS[413] are essential for GRK-mediated phosphorylation and β-arrestin association. In addition, data derived from a functional assay with ERK1/2 activation showed that mutant Δ408−422, defective in agonist-induced internalization, exhibited sustained activation of ERK1/2 from 10 to 30 min compared to the wild type. This is consistent with our previous observation that the cluster of Ser/Thr residues (Ser326, Thr327, and Ser328) in the C-terminal tail of human hydroxy-carboxylic acid receptor 2 is responsible for agonist-induced desensitization.[46] Moreover, siRNA knockdown experiments demonstrated that both GRK2 and GRK5 are involved in agonist-induced phosphorylation of the BmCrzR terminus. Collectively, our findings strongly suggest that Ser/Thr residues [411]TSS[413] in the carboxyl tail of BmCrzR are essential for receptor internalization and desensitization. They serve as targets for agonist-induced phosphorylation by GRK2 and GRK5 and for the association of the β-arrestin and adaptor proteins. Future research will need to focus upon the identification and characterization of phosphorylation sites mediated by PKC in the regulation of receptor signaling, internalization, and trafficking.

In summary, on the basis of our results, we conclude that upon agonist stimulation, BmCrzR undergoes a rapid internalization via a β-arrestin (Kurtz)-dependent and clathrin-independent pathway and that most internalized receptors are recovered to the cell surface through early endosomes while some were otherwise targeted by lysosomes for degradation. Our data provide strong evidence that agonist-mediated receptor activation results in phosphorylation of the C-terminal tail at residue cluster [411]TSS[413], and desensitization. Agonist-induced internalization of GPCRs is a well-characterized phenomenon that is believed to contribute to the tight regulation of signaling and desensitization.[47,48] Our findings provide a mechanistic and structural basis for future *in vitro* and *in vivo* studies of signaling, desensitization, and related physiological processes of the *Bombyx* corazonin receptor.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00250.

BmCrzR associated with arrestin undergoes endocytosis (Figure S1), BmCrzR internalized via a lipid raft-dependent pathway (Figure S2), and recycling of internalized BmCrzR in HEK-293 cells (Figure S3) (PDF)

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Author Contributions

N.Z. and L.S. conceived and coordinated the study. J.Y. wrote the paper. J.Y., Z.S., H.Y., X.J., and Y.C. designed and performed the experiments. N.Z., L.S., and H.H. analyzed the results. L.J. provided technical assistance.

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Notes

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■ ABBREVIATIONS

BmCrz, *Bombyx* corazonin; BmCrzR, *Bombyx* corazonin receptor; GPCR, G protein-coupled receptor; EGFP, enhanced green fluorescent protein; HEK, human embryonic kidney; GRK, G protein-coupled receptor kinase; ERK1/2, extracellular signal-regulated kinase 1/2; CRE, cAMP response element; ER, endoplasmic reticulum; siRNA, small interfering RNA; WT, wild type.

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