Novel Function for Receptor Activity-modifying Proteins (RAMPs) in Post-endocytic Receptor Trafficking*

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RAMPs (1–3) are single transmembrane accessory proteins crucial for plasma membrane expression, which also determine receptor phenotype of various G-protein-coupled receptors. For example, adrenomedullin receptors are comprised of RAMP2 or RAMP3 (AM1R and AM2R, respectively) and calcitonin receptor-like receptor (CRLR), while a CRLR heterodimer with RAMP1 yields a calcitonin gene-related peptide receptor. The major aim of this study was to determine the role of RAMPs in receptor trafficking. We hypothesized that a PDZ type I domain present in the C terminus of RAMP3, but not in RAMP1 or RAMP2, leads to protein-protein interactions that determine receptor trafficking. Employing adenylate cyclase assays, radioligand binding, and immunofluorescence microscopy, we observed that in HEK293 cells the CRLR-RAMP complex undergoes agonist-stimulated desensitization and internalization and fails to resensitize (i.e. degradation of the receptor complex). Co-expression of N-ethylmaleimide-sensitive factor (NSF) with the CRLR-RAMP3 complex, but not CRLR-RAMP1 or CRLR-RAMP2 complex, altered receptor trafficking to a recycling pathway. Mutational analysis of RAMP3, by deletion and point mutations, indicated that the PDZ motif of RAMP3 interacts with NSF to cause the change in trafficking. The role of RAMP3 and NSF in AM2R recycling was confirmed in rat mesangial cells, where RNA interference with RAMP3 and NSF in AM2R exerted after agonist-induced internalization remains unknown. These findings provide the first functional difference between the AM1R and AM2R at the level of post-endocytic receptor trafficking. These results indicate a novel function for RAMP3 in the post-endocytic sorting of the AM-R and suggest a broader regulatory role for RAMPs in receptor trafficking.

The recent discovery of receptor activity-modifying proteins (RAMPs)1 has raised new possibilities for modes of regulation of G-protein-coupled receptors (GPCRs). RAMPs were discovered as accessory proteins indispensable to the function of an orphan GPCR, now termed the calcitonin receptor-like receptor (CRLR) (1). Three RAMP isoforms (1–3) have been identified as distinct gene products that yield single transmembrane-spanning proteins. RAMPs are required for the plasma membrane expression, as well as for determination of receptor phenotype for CRLR (selective ligand recognition) (1, 2). Co-expression of RAMP1 with CRLR yields a calcitonin gene-related peptide-1 (CGRP-1) receptor, while coexpression of RAMP2 or RAMP3 with CRLR produces adrenomedullin receptors, AM-1 and AM-2 receptors, respectively (3, 4). AM and CGRP are multifunctional peptides with many overlapping functions, ranging from potent vasodilation to proliferation regulation to regulation of salt and water balance (5). Differential expression of RAMP isoforms has been hypothesized to play a regulatory role in both physiological and pathophysiological disease states. Moreover, the recent identification of RAMP interactions with additional members of the Class II GPCR family and RAMP expression in cell lines lacking CRLR have raised the possibility of novel functions for RAMPs in GPCR regulation (6).

Upon activation, the CRLR-RAMP receptor complex causes cyclic AMP activation in most systems, irrespective of whether the ligand is AM or CGRP. In addition, the receptor complex undergoes desensitization and internalization (via clathrin-mediated endocytosis) in response to a prolonged agonist stimulation (7). Once internalized, the receptor complex either undergoes degradation or recycling, depending on the cell type. In HEK293 cells the CRLR-RAMP complex has been shown to be targeted to the lysosomes for degradation, while in rat mesangial cells, the CRLR-RAMP receptor complex is sorted for dephosphorylation and resensitization (and presumably recycling) as a fully functional receptor (2, 8). The mechanism that regulates the pathway to which the receptor complex is targeted after agonist-induced internalization remains unknown.

Factors influencing the sorting of receptors in the early endosomes are largely unknown, but some of the critical players are beginning to be identified for the GPCRs. It has been shown in other GPCR systems that interactions with PSD-95/Discs-large/ZO-1 homology (PDZ) domain proteins are responsible for altering the receptor-targeting after internalization (9–11). The life cycle of the β2-adrenergic receptor (β2-AR) was reported to be altered in the presence of a protein termed N-ethylmaleimide-sensitive factor; AMPA, α-amino-3-hydroxy-5-methylisoxazolepropionate; SNARE, soluble NSF attachment protein receptor; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; dPBS, Dulbecco’s phosphate-buffered saline; d-siRNA, diced small interfering RNA; RMC, rat mesangial cell; GST, glutathione S-transferase; NEM, N-ethylmaleimide; AM (or ADM), adrenomedullin; AC, adenylyl cyclase; GFP, green fluorescent protein; EGFP, enhanced GFP.
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FIG. 1. Effect of N-ethylmaleimide, an NSF inhibitor, on resensitization of cAMP accumulation in RMCs. The 1-h pretreatment time point shows desensitization of cAMP accumulation response in cells with or without treatment with NEM. The 2-h recovery time point shows resensitization is inhibited in cells treated with NEM, as compared with untreated RMCs. This indicates a role for NSF in the resensitization observed in RMC cells. RMC cells seeded in 24-well plates were pretreated with 10 nM rAM for 1 h were washed extensively to remove residual agonist and treated with 50 μM NEM for 45 s. Following NEM treatment, cells were washed repeatedly and incubated in serum-free medium with 5 μg/ml cycloheximide for indicated times. After recovery time, RMC cells were re-challenged with 100 nM rAM for 15 min, and plates were frozen. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer’s instructions. cAMP levels in rat mesangial cells were calculated using a standard curve ranging from 10 to 10⁴ fmol of cAMP. Each experiment was done in duplicate and repeated at least three times. Data are expressed as percent maximal response, % forskolin. *, p < 0.05; n ≥ 3 experiments.

ethylmaleimide-sensitive factor (NSF) (11). It has been shown that the β₂-AR interacts with NSF via a PDZ type I motif (-DSLL) at its extreme C terminus. In addition, binding of NSF to the Glu2 subunits of the α-amino-3-hydroxy-5-methylisoxazolepropionate (AMPA) receptor was also demonstrated to be crucial for the recycling of the AMPA receptor (12, 13). NSF is a hexameric ATPase that plays a chaperoning role for soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) in the majority of membrane fusion events in a cell, but when targeting membrane receptors for recycling, NSF acts independently of the SNARE complex to promote rapid resensitization of the receptors at the plasma membrane (14–16).

Similar to the C terminus of β₂-AR, human RAMP3 C terminus has a type-I PDZ motif (-DTLL motif). CRLR, RAMP1, or RAMP2 do not, however, contain any PDZ motifs. We hypothesized that RAMP3, via its interaction with NSF, regulates the trafficking of the CRLR-RAMP3 complex. We show here that while CRLR-RAMP1 and CRLR-RAMP2 complexes do not interact with NSF, CRLR-RAMP3 complex interacts with NSF via the PDZ motif of RAMP3. Moreover, we demonstrate that overexpression of NSF in HEK293 cells alters the life cycle of CRLR-RAMP3 complex from a degradative to recycling pathway via interactions of the PDZ motif of RAMP3 and NSF. These findings demonstrate that RAMP3, in addition to determining the receptor phenotype and allowing receptor membrane expression, is also significantly involved with the regulation of the turnover of the CRLR-RAMP complex.

EXPERIMENTAL PROCEDURES

Materials—Adrenomedullin was purchased from Bachem Bioscience, Inc. (King of Prussia, PA). [3H]-Labeled adrenomedullin was purchased from Amersham Biosciences. N-Ethylmaleimide was purchased from Sigma. Cell culture media, fetal bovine serum, penicillin/streptomycin, and trypsin-EDTA were purchased from Invitrogen. RAMP3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and NSF antibody was from Calbiochem. Anti-mouse Cy3 and anti-rabbit Cy5 secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). All other reagents were of highest quality available.

Cell Culture and Transfection Protocols—Rat mesangial cells were obtained from ATCC and are maintained in DMEM 1640 media containing 15% FBS and 1% penicillin-streptomycin. HEK293T cells (obtained from ATCC) are maintained in DMEM low glucose media containing 10% FBS, 1% penicillin-streptomycin. Rat-2 fibroblast cells (obtained from ATCC) are maintained in DMEM high glucose media containing 10% FBS, 1% penicillin-streptomycin. Transfection of HEK293T and Rat-2 fibroblast cells was performed using Lipofectamine Plus protocol (Invitrogen). Cells were transfected with the DNA and Lipofectamine Plus as per manufacturer’s protocol. Cells were collected for assays after 48 h of transfection.

RAMP Cloning and Expression—Full-length cDNA of human RAMP1, -2, and -3 and bovine CRLR were described before (17, 18). CRLR, cloned into N1-EGFP and also in pcDNA3.1 expression vectors, was used for transfection in HEK293T cells.

Desensitization and Resensitization Assays—48 h post-transfection cells were pretreated with or without 10 nM rADM in DMEM containing 0.2% BSA for indicated time periods (up to 4 h). After agonist exposure, cells were washed three times with Dulbecco’s phosphate-buffered saline (dPBS, Invitrogen) containing 0.2% BSA and either frozen for membrane preparation for adenylate cyclase assays or used immediately for intact-cell radioligand binding. For receptor resensitization assays, after agonist exposure, cells were washed and incubated for various time periods in DMEM containing 0.2% BSA and 5 μg/ml cycloheximide to allow receptor recovery.

Receptor Binding—Competition radioligand binding assays were performed as described by Aiyar et al. (19) and as established in our laboratory. HEK293T cells were transfected and ~200,000 cells/well seeded in poly-d-lysine precoated 24-well plates (BD Biosciences). 48 h post-transfection cells were treated for desensitization or resensitization assays as described above. After agonist exposure, cells were washed three times with dPBS buffer containing 0.2% BSA then incubated with increasing concentrations (1 pm to 100 nm) of competing ligand and 175–250 pm [125I]-labeled rADM for 30 min at 37 °C. After incubation, plates were washed three times with ice-cold assay buffer and the reactions were terminated by the addition of 2 ml of NaOH. Cells were then harvested, and associated radioligand activity was counted on a γ-counter. All binding assays were performed in duplicate, with each experiment repeated at least three times. Nonspecific binding was determined in the presence of 100 nM of unlabeled rADM. Analysis of all binding data were performed by computer-assisted nonlinear least square fitting using GraphPad PRIZM (GraphPad Software, San Diego, CA).

Adenylate Cyclase Assays—Cyclase activity was done as described before with slight modifications (18). Cells were harvested from P100 or P60 plates and homogenized in Tris-HCl (10 mM), EDTA (10 mM) buffer. Membranes were prepared by homogenization and centrifugation in Tris-HCl (50 mM), MgCl (10 mM) buffer. Final concentration of 20 μg of protein/assay tube was obtained. Membranes were incubated for 15 min at 30 °C with appropriate concentrations of drugs and assay mix containing ATP regeneration system and [γ-32P]ATP. After the reaction was stopped (with stop solution containing [3H]cAMP) contents of the assay tubes were passed through Dowex and subsequently through alumina columns to separate the degradation products of ATP, by washing the Dowex with water and alumina with imidazole. Elution profile was done to determine the amount of water and imidazole needed to wash and elute the products. Product eluted from alumina column was counted for the presence of [3H]cAMP and [γ-32P]cAMP in a β-counter. Each experiment was done in triplicate and repeated at least three times. Data are expressed as percent maximal response, % forskolin.

cAMP Accumulation Assays—Rat mesangial cells were seeded on a 24-well plate until reaching 80–90% confluence, then incubated in serum-free medium overnight before experiment. Resensitization experiments were carried out as described under “Experimental Procedures,” with cells pretreated with 10 nM rAM and subsequently challenged with 100 nM rAM in the presence of 200 μM 3-isobutyl-1-methylxanthine. Determination of the cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer’s instructions. cAMP levels in transfected and untransfected cells were calculated using a standard curve ranging from 10 to 10⁴ fmol of cAMP. Each experiment was done in duplicate and repeated at least three times. Data are expressed as percent maximal response, % forskolin.

RNA Interference Analysis—Gene-specific d-siRNA for lucZ (control) and RAMP3 were generated and purified using BLOCK-IT Dicer RNAi kit from Invitrogen. Rat mesangial cells (RMCs) were transfected with d-siRNAs using Lipofectamine 2000 as per manufacturer’s instructions.
FIG. 2. The sorting of the AM2R (CRLR+RAMP3) in HEK293 cells. A, the CRLR/RAMP3 complex does not re-sensitize/recycle in HEK293 cells after agonist-stimulated desensitization/internalization. HEK293 cells transiently transfected with CRLR and RAMP3 were treated for 1 h with AM (10 nM) and then washed and receptor resensitization (adenylate cyclase (AC) activity) and recycling (radioligand binding) were measured after indicated recovery times in the absence of agonist. Adenylate cyclase activity is shown on the left y axis, expressed as percent maximal response (% forskolin stimulation), and radioligand binding is shown on the right y axis, expressed as percent control. *, p < 0.05; n ≥ 3 experiments. B, immunofluorescence microscopy shows failure of CRLR/RAMP3 receptor complex to recycle after agonist-stimulated internalization. HEK293 cells transfected with CRLR-GFP and RAMP3 were pretreated with 10 nM AM for 1 h. After pretreatment with AM, cells were washed and incubated in serum-free medium with 5 μg/ml cycloheximide to allow receptor recycling for indicated times. Cells were fixed and components were visualized using anti-RAMP3 antibody (1:200) and detected with Cy5 secondary antibody (1:200), and CRLR was detected with an EGFP tag; overlays of staining patterns are shown in the far right panels. Images shown are representative of at least 20 fields imaged per experiment from at least three experiments. Bar scales on all images represent 50 μm.

FIG. 3. The role of NSF in the sorting of the AM2R (CRLR+RAMP3) in HEK293 cells. A, NSF causes recycling of AM2R in HEK293 cells. HEK293 cells transiently transfected with CRLR and RAMP3 with or without NSF. At 48 h post-transfection, cells were treated for 1 h with AM (10 nM) and then washed, and receptor recycling was measured after indicated recovery times in the absence of agonist. Receptor recycling was measured by whole-cell competition binding assays using [125I]-rAM as ligand (cold rAM served as the competitor), and the number of binding sites/cell was estimated using the GRAPHPAD PRISM software. “No pretreat” represents samples at maximal radioligand binding that were not preincubated with agonist. “1h pretreat” represents samples pretreated with AM (10 nM) for 1 h, washed as indicated under “Experimental Procedures,” and tested immediately after wash steps for radioligand binding. 1-, 2-, and 4-h recovery samples were pretreated with AM (10 nM) for 1 h, washed, and allowed to recover for indicated times in media without agonist plus 5 μg/ml cycloheximide, then analyzed for radioligand binding. NSF overexpression in cells expressing AM2R caused altered receptor trafficking from degradation to recycling pathway. *, p < 0.05; n ≥ 3 experiments. B, NSF causes resensitization of AM2R in transfected HEK293 cells. HEK293 cells transfected and pretreated with agonist as described in the legend to Fig. 2A. After agonist pretreatments, membranes were extracted, and AC activity in response to 100 nM AM was measured. NSF overexpression with AM2R allowed time-dependent receptor resensitization. Experiments were performed in triplicate and data expressed as percent maximal stimulation (% Forskolin). *, p < 0.05; n ≥ 4.
(Invitrogen). 48 h after transfection cells were frozen for mRNA analysis or used for cAMP accumulation assays or immunofluorescence microscopy.

Quantitative PCR Analysis—Total RNA was isolated from RMCs using TriReagent reagent (Invitrogen). After sodium acetate-ethanol precipitation and several ethanol washes, RNA was used as a template in a quantitative PCR amplification protocol. Quantitative PCR analysis was carried out with the LUX (light upon extension) fluorogenic primer method following the protocol in the manufacturer’s manual (Invitrogen), as described by Nazarenko et al. (20).

Mutagenesis Procedure—Site-directed mutagenesis was performed using a PCR-based strategy that employs the pfu Turbo polymerase (Stratagene, La Jolla, CA). A pair of complementary oligonucleotides containing the appropriate point mutations in the sequence of RAMP or deletion mutants were synthesized (Michigan State University Molecular Structure Facility). The PCR for the mutation was as follows: 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 50 °C for 30 s, 68 °C for 8 min; final cycle of 68 °C for 8 min. PCR product was digested for 4 h with DpnI enzyme (Invitrogen) and transformed in to DH5α cells. Mutations were confirmed by automated sequencing (Michigan State University Genomic Technology Support Facility).

Immunofluorescence Microscopy—HEK293 cells were transfected as described above and seeded at 24 h post-transfection onto collagen type I-coated coverslips. Resensitization assays were performed as described and reactions were stopped by fixing cells in 4% paraformaldehyde for 30 min at room temperature. Samples were permeabilized with 0.1% v/v Triton X-100 in PBS and blocked overnight in 0.1% v/v Tween 20 and incubated in 4% paraformaldehyde to allow receptor recycling for indicated times. Note: “1 hr Pretreatment” indicates time just after ADM pretreatment and wash steps, with no recovery time. Cells were fixed and components were visualized using anti-RAMP3 antibody (1:250) with Cy5 anti-rabbit secondary antibody (1:400, in blue) and Cy3 anti-mouse secondary antibody (1:500, in red), respectively; CRLR-GFP is detected with an EGFP tag and shown in green; overlays of staining patterns are shown in the far right panels. Images shown are representative of at least 20 fields imaged per experiment from at least three experiments. Bar scales on all images represent 100 μm.

Fusion Protein Overlays and Western Blotting—10 μg of GST fusion proteins were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose filters. Blots were blocked with 5% w/v fat-free milk powder in Tris-buffered saline with Tween 20 (TTBS: 20 mM Tris, pH 7.4, 500 mM NaCl, 0.1% v/v Tween 20) and incubated overnight at 4 °C in lysates of HEK293 cells with or without overexpression NSF. Blots were then washed three times with TTBS buffer and incubated with anti-NSF monoclonal antibody for 2 h at room temperature. After three washes with TTBS, filters were incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Invitrogen), washed again with TTBS, soaked in SuperSignal West Pico chemiluminescent substrate (Pierce) and exposed to x-ray film. Same protocol, with the exception of the overnight incubation with cell lysate, was followed for immunoblot analysis of RAMP3.

Statistics—Data are presented as mean ± S.E. Single group comparisons exercised a paired Student’s t test method. Statistical significance was set at p < 0.05.

RESULTS AND DISCUSSION

Role of NSF in Resensitization of the CRLR-RAMP Complex—Our laboratory has previously published that RMCs endogenously express the AM1R (CRLR + RAMP2) and the AM2R (CRLR + RAMP3) (8). These data were repeated, and NSF expression was confirmed in the RMCs with reverse transcription-PCR and immunocytochemistry (data not shown). Our laboratory has also reported that pretreatment of RMCs with AM leads to an agonist-stimulated desensitization and internalization of the CRLR-RAMP complex. Phosphatase-dependent resensitization of AM responsiveness was also demonstrated after agonist-stimulated desensitization (8). Measuring cAMP accumulation we repeated these results in this study (Fig. 1). As a preliminary test to determine whether NSF is involved in the resensitization of AM responsiveness, we used a pharmacological inhibitor of NSF, N-ethylmaleimide (NEM). In RMCs treated for 45 s with 50 μM NEM during the resensi-
tization experiment, resensitization was blocked, as measured by cAMP accumulation (Fig. 1). NEM, however, did not affect basal cAMP accumulation or the desensitization response when compared with untreated cells, an important finding given the ability of NEM to interfere with G protein subunits (Fig. 1). These results indicate that NSF plays a role in the sorting of

![Image](https://example.com/image1.png)

**FIG. 5.** The role of NSF in post-endocytic sorting of CGRP1 (CRLR+R1) and AM1 receptor. A, NSF overexpression does not alter the trafficking of the CGRP receptor after internalization. HEK293 cells were transfected with CRLR and R1, with or without NSF. 48 h post-transfection, cells were pretreated with CGRP (10 nM) for 1 h, washed as described under “Experimental Procedures,” and allowed the indicated recovery times in agonist-free medium, and receptor recycling was measured with whole-cell competition binding using 125I-rCGRP as the ligand and cold rCGRP as the competitor. The number of binding sites/cell was estimated with GraphPad PRISM software. *, p ≤ 0.05; n ≥ 3. B, NSF overexpression does not cause CGRP receptor resensitization after agonist-stimulated endocytosis. HEK293 cells were transfected and pretreated as described in the legend to Fig. 2A, and then membranes were extracted. Adenylate cyclase activity was measured in membranes stimulated with 100 nM CGRP for 15 min. Experiments performed in triplicate and expressed as percent maximal stimulation (% Forskolin). *, p ≤ 0.05; n ≥ 3.

![Image](https://example.com/image2.png)

**FIG. 6.** The effect of RAMP3 PDZ motif deletion on the trafficking of the AM2R (CRLR+RAMP3). A, deletion of RAMP3 PDZ motif blocked the recycling of the CRLR-RAMP3 complex when co-expressed with NSF. HEK293 cells were co-transfected with CRLR, wild-type RAMP3, or RAMP3Δ145-8, and NSF. 48 h post-transfection, cells were pretreated with AM (10 nM) for 1 h, washed as described under “Experimental Procedures,” and allowed 4 h recovery time in agonist-free medium, and receptor recycling was measured with whole-cell binding using 125I-rAM as the ligand and cold rAM as the competitor. The number of binding sites/cell was estimated with GraphPad PRISM software. *, p ≤ 0.05; n ≥ 3. B, deletion of RAMP3 PDZ motif blocked the resensitization of the CRLR-RAMP3 complex after agonist-induced desensitization when co-expressed with NSF. HEK293 cells were transfected and pretreated as described in the legend to Fig. 2A, and then membranes were extracted as described under “Experimental Procedures.” Adenylate cyclase activity was measured in membranes stimulated with 100 nM AM for 15 min. Experiments performed in triplicate and expressed as percent maximal stimulation (% Forskolin). *, p ≤ 0.05; n ≥ 3.
Fig. 7. Localization of CRLR, RAMP3Δ145-8, and NSF in HEK293 cells during a recycling experiment. Untreated and after 1-h ADM (10 nM) pre-treatment, CRLR and RAMP3Δ145-8 internalize similarly to the wild-type CRLR-RAMP3 complex. After a 4-h recovery time in the absence of agonist, CRLR and RAMP3Δ145-8 fail to recycle to the plasma membrane. Experiments were performed as described in the legend to Fig. 4. Fixed cells were stained with anti-RAMP3 antibody (1:200) and anti-NSF antibody (1:500) with Cy3 anti-rabbit secondary antibody (1:400, in green) and Cy5 anti-rabbit secondary antibody (1:250) with Cy3 anti-mouse secondary antibody (1:500, in red), respectively; CRLR-GFP is shown in red; overlays of staining patterns are shown in the far right panels. Images shown are representative of at least 20 fields imaged from at least three experiments. Bar scales on all images represent 100 μm.

the CRLR-RAMP complex following agonist-induced internalization in this endogenous CRLR-RAMP system where the receptor complex is recycled. To fully evaluate the molecular mechanisms of this observation, we used HEK293 cells to examine the interaction of the CRLR-RAMP complex with NSF and the impact of this interaction on receptor trafficking. In contrast to RMCs, HEK293 cells express very low endogenous levels of RAMPs. Kuwasako et al. (2) have demonstrated that in HEK293 cells overexpressing the CRLR-RAMP complex, agonist-induced internalization leads to receptor trafficking to a degradation pathway. In this study the internalized CRLR/RAMP complexes were colocalized with LAMP-1, a lysosomal marker, to show the targeting of the receptor for the degradation pathway. Utilizing adenylate cyclase activity assays, whole-cell ligand binding, and immunofluorescence microscopy we confirmed these findings (Fig. 2, A and B). Pretreatment of HEK293 cells transfected with CRLR and RAMP3 with 10 nM AM for 1 h resulted in desensitization of the adenylate cyclase response from 50% (of forskolin stimulation) in untreated cells to 28% in AM-treated cells (Fig. 2A left axis). Even after the removal of agonist and incubation with buffer alone for indicated times through 4 h, the adenylate cyclase response remained desensitized (Fig. 2A left axis), indicating a lack of resensitization. Consistent findings were obtained with whole-cell binding and immunofluorescence microscopy experiments (Fig. 2A, right axis, and B).

To determine whether NSF overexpression could alter the receptor trafficking in this cell system, NSF was co-transfected with CRLR and RAMP3, and resensitization and recycling assays were performed. Resensitization and recycling were monitored by adenylate cyclase activity assays and whole-cell competition binding, respectively. In addition, visualization of the trafficking of the receptor complex was performed by immunofluorescence microscopy. In the absence of NSF, pretreatment with AM for 1 h resulted in desensitization of the adenylate cyclase response and internalization of the receptor complex. Upon removal of agonist and incubation with buffer alone for 4 h, the adenylate cyclase response remained desensitized and the receptor complex remained internalized, indicating a lack of resensitization (Fig. 3, A and B). In contrast, when NSF was co-transfected in the cells, although the desensitization response (i.e. response after 1-h agonist treatment) was not altered, the cells now underwent time-dependent resensitization (i.e. response after 1-, 2-, or 4-h agonist removal) in response to AM (Fig. 3B). Consistent findings were obtained with whole-cell binding and immunofluorescence microscopy experiments (Figs. 3A and 4). Time course experiments indicated the time course for complete resensitization and recycling of the CRLR-RAMP3 receptor complex to be 4 h in HEK293 cells, as measured by adenylate cyclase, whole-cell binding, and immunofluorescence microscopy experiments (Figs. 3 and 4). All subsequent experiments in HEK293 cells use the 4 h time point to determine receptor complex recycling and resensitization. These results indicate that the presence of NSF alters the intracellular sorting of the CRLR/RAMP3 receptor complex after AM-stimulated endocytosis.

RAMP Isoform-specific Regulation of CRLR-RAMP Receptor Complex Trafficking—To determine whether this effect of NSF was specific for RAMP3, the additional RAMPs (RAMP1 or RAMP2) were tested for their ability to act with NSF to alter the receptor complex life cycle. Interestingly, in contrast to RAMP3, presence of NSF did not alter the resensitization response or recycling pattern of the CRLR/RAMP1 or RAMP2 receptor complexes. Both the activity and receptor number remained at desensitized levels in cells transfected with CRLR+RAMP1 or CRLR+RAMP2 (along with NSF) (Fig. 5,
A–D). Both CRLR/RAMP1 and CRLR/RAMP2 complexes showed no difference in receptor expression levels at the plasma membrane (as measured with whole-cell binding) in untreated cells, as compared with CRLR/RAMP3 complex. These results indicate that RAMP3 must contain a molecular determinant distinct from the other RAMPs that allowed its interaction and activity with NSF.

Role of PDZ Interactions in Trafficking of the CRLR-RAMP3 Complex—We hypothesized that the unique characteristic of RAMP3 that allowed its interaction with NSF to change receptor trafficking was a PDZ motif on its extreme C terminus. To establish if this domain is critical for interaction of the CRLR/RAMP3 complex with NSF, the PDZ motif (-DTLL) on RAMP3 was deleted. Deletion of this domain did not affect basal adenylate cyclase activity or the desensitization response of the CRLR-RAMP3 complex in response to AM, even in the presence of NSF (Fig. 6). In contrast, the deletion of the PDZ motif (-DTLL) significantly affected the resensitization and recycling of the CRLR-RAMP3 receptor complex in the presence of NSF. Both radioligand binding and adenylate cyclase assays showed a loss of recycling and resensitization, respectively, of the receptor complex when RAMP3Δ145-8 was expressed in the presence of NSF, as compared with the wild-type RAMP3 with NSF (Fig. 6, A and B). Mutant RAMP3 (RAMP3Δ145-8) showed the same levels of adenylate cyclase activity and whole-cell binding data, all point mutants within the PDZ motif on CRLR/RAMP3 complex, except R3L147A, blocked the recycling of the CRLR-RAMP3 complex seen when co-expressed with NSF. HEK293 cells were co-transfected with CRLR, wild-type RAMP3 or RAMP3 point mutants, and NSF. 48 h post-transfection, cells were pretreated with AM (10 nM) for 1 h, washed as described under “Experimental Procedures,” and allowed 4 h recovery.

To further test the hypothesis that the absence of the PDZ motif on the RAMP2 accounts for the lack of interaction of the CRLR-RAMP2 complex with NSF, and hence the inability of the CRLR-RAMP2 complex to follow a recycling pathway, the PDZ motif of RAMP3, the amino acids -DTLL, were substituted on the C terminus of RAMP2, in exchange for its original four C-terminal amino acids (-EAQA). The RAMP2ΔDTLL mutant showed similar levels of adenylate cyclase activity and whole-cell radioligand binding without pretreatment and after desensitization, as compared with wild-type RAMP2 in control experiments (Fig. 8). The RAMP2ΔDTLL mutant also showed...
similar receptor expression levels at the plasma membrane (as measured with whole-cell binding) in untreated cells, as with the wild-type CRLR/RAMP2 complex. Resensitization assays (measured with adenylate cyclase activity and recycling assays (measured by whole-cell binding) were performed in HEK293 cells transfected with CRLR, RAMP2ΔDTLL, and NSF, as described previously. Similar to the CRLR-RAMP3 complex, the CRLR-RAMP2ΔDTLL complex underwent resensitization and recycling in the presence of NSF, as assessed by adenylate cyclase and whole-cell binding (Fig. 8, A and B). These findings provide additional evidence that the PDZ motif on the RAMP3 is the site of interaction of the receptor complex with NSF, causing a change in receptor trafficking from a degradative to a recycling pathway.

To further identify the critical amino acids in the PDZ binding domain that regulate the RAMP3/NSF interaction, point mutations of the amino acids of the RAMP3 PDZ motif to alanine were performed. The functional effects of the point mutations were analyzed with resensitization assays, measured by adenylate cyclase activity, and recycling assays, measured by whole-cell binding, as described before. Our results indicate that mutation of Asp145, Thr146, or Leu148 to alanine were performed. The functional effects of the point mutations of the amino acids of the RAMP3 PDZ motif to alanine disrupted the RAMP3 interaction with NSF and inhibited the resensitization and recycling of the CRLR/mutant RAMP3 complex after AM-induced endocytosis in HEK293 cells (Fig. 9, A and B). Mutation of Leu147 to alanine had no effect on the resensitization or recycling of the receptor complex in the presence of NSF (Fig. 9). Control experiments with the point mutations co-transfected with CRLR showed similar levels of adenylate cyclase activity and whole-cell binding as compared with wild-type RAMP3 without pretreatment and after desensitization. RAMP3 point mutants also showed no difference in receptor expression levels at the plasma membrane (as measured with whole-cell binding) in untreated cells, as compared with the wild-type CRLR/RAMP3 complex.

To examine whether the PDZ motif on RAMP3 is interacting with NSF, overlay assays were performed. This was accomplished using GST-RAMP3 fusion proteins in an overlay assay with cell lysates of HEK293 cells overexpressing NSF. Control experiments run with GST protein showed no detectable bands when incubated with NSF lysates and probed with an NSF antibody (Fig. 10a). Importantly, wild-type RAMP3 fusion proteins showed interaction with NSF in the cell lysates of HEK293 cells overexpressing NSF in the overlay assay (Fig. 10a). In addition, RAMP3Δ145-8 fusion proteins, lacking the PDZ motif on RAMP3, showed no detectable bands when incubated with NSF lysates and probed with an NSF antibody (Fig. 10a). Lysates of HEK293 cells not overexpressing NSF showed no detectable bands when run with GST-RAMP3 in the overlay assay and probed for NSF (Fig. 10b). When blots used in the overlay assay were stripped and probed for RAMP3, bands were detected in the GST-RAMP3 and GST-RAMP3Δ145-8 lanes in the exact location as in the overlay assay when probed for NSF (Fig. 10c). These data demonstrate an interaction between RAMP3 and NSF via the PDZ motif on RAMP3, an interaction that is capable of regulating CRLR-RAMP3 (AM2R) complex trafficking.

**RAMP3 and NSF Regulation of Receptor Trafficking in Unaltered Cell Lines**—It was important to establish whether our observations in the HEK293 cells were transferable to unaltered cell lines. Reexamining the rat mesangial cells used in the first set of experiments, we employed RNA interference technology to knockdown RAMP3 expression. Having demonstrated the requirement of NSF in the cells for efficient receptor resensitization (Fig. 1), the RAMP3 RNA interference experiment would determine whether RAMP3 was also required for effective receptor resensitization. In both mRNA and protein expression studies, RAMP3 expression dramatically decreased, while control experiments using lacZ knockdown showed no significant alteration in RAMP3 expression when compared with wild-type cells (Fig. 11A and B; data not shown). Resensitization assays were performed and cAMP accumulation was measured to determine the effect of RAMP3 RNA interference on receptor resensitization in rat mesangial cells. RNA interference for RAMP3 in RMCs showed similar levels of cAMP accumulation as compared with wild-type RMCs without pretreatment and after desensitization (Fig. 11c). Strikingly, when allowed sufficient recovery time in the absence of agonist following desensitization, RMCs with RAMP3 RNA interference showed an inability to resensitize, unlike the wild-type RMCs (Fig. 11c). This finding demonstrates that NSF and RAMP3 are both critical for receptor targeting for resensitization in rat mesangial cells, an unaltered cell line absent of the issues of overexpression.

As a further test of our proposed model, we employed a cell line that does not express RAMP3 but does express the AM1R...
lated using a standard curve ranging from 10 to 10^4 fmol of cAMP. cAMP levels were measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer’s instructions. Determination of cAMP level was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences) according to the manufacturer’s instructions. cAMP levels in Rat2 fibroblast cells were calculated using a standard curve ranging from 10 to 10^4 fmol of cAMP. Each experiment was done in duplicate and repeated at least three times. Data are expressed as percent maximal response (% Forskolin). *, p ≤ 0.05; n ≥ 3 experiments.

(CRLR and RAMP2). We hypothesized that, with the absence of RAMP3, this cell line would fail to resensitize following agonist pretreatment and that expression of RAMP3 in these cells would allow a switch in receptor targeting for resensitization. Rat2 fibroblast cells have been shown by Choksi et al. (21) to lack RAMP3 expression but do express the AM1R (CRLR and RAMP2). We repeated this finding and confirmed the expression of NSF in Rat2 fibroblast cells with reverse transcription-PCR and immunocytochemistry (data not shown). We performed resensitization assays with the Rat2 fibroblast cells and measured resensitization by cAMP accumulation. The Rat2 fibroblast cells exhibited a decrease in cAMP accumulation after agonist pretreatment and failed to resensitize, as predicted, when allowed to recover in the absence of agonist for 4 h. Interestingly, consistent with our model, RAMP3 expression in the Rat2 cells showed no alteration in cAMP accumulation without pretreatment and after desensitization when compared with wild-type Rat2 cells, but now showed resensitization when allowed recovery time in the absence of agonist (Fig. 12). Furthermore, expression of the RAMP3 PDZ motif mutant in the Rat2 cells was unable to cause receptor resensitization, while showing similar levels of cAMP accumulation as compared with wild-type Rat2 cells without pretreatment and following desensitization (Fig. 12). RAMP3 and RAMP3a145-8 showed similar levels of transfection efficiency in the Rat2 cells, as measured by immunocytochemistry (data not shown). This data further confirms the crucial role of RAMP3 in the targeting of the AM2R for resensitization/recycling after agonist-stimulated desensitization. Additionally, it suggests that differential RAMP expression in cells may determine the sorting of the AM-R from the endosomes after internalization.

While many GPCRs utilize similar mechanisms for endocytosis, the functional consequences of endocytosis vary from receptor to receptor. Internalized receptors that are trafficked through a rapid recycling pathway are restored to the plasma membrane in a functional state to achieve resensitization. On the other hand, receptors that are internalized and targeted to late endosomes and lysosomes experience proteolytic degradation, thus promoting attenuation of receptor signaling and down-regulation of the receptor. The CRLR-RAMP system...
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shows sorting of the receptor complex by both trafficking pathways in different cell lines. That is, in RMCs the CRLR-RAMP complex follows a post-endocytic recycling pathway, whereas in HEK293 cells and Rat2 fibroblast cells the receptor complex undergoes degradation (2, 8). Findings in this report demonstrate some of the cellular and molecular mechanisms responsible for the trafficking of the CRLR-RAMP receptor complex. We have shown that the PDZ type I motif at the C terminus of RAMP3 interacts with NSF to target the CRLR/RAMP3 complex for recycling after internalization. NSF is commonly known to interact with αSNAP (soluble NSF attachment protein) and SNARE proteins to form the 20 S particle, a complex that plays a critical role in intracellular membrane fusion and exocytosis (14–16). While other laboratories have demonstrated involvement of NSF with the trafficking of receptors, including the β2-AR and AMPA receptors, this study is the first demonstration of NSF acting in concert with a heterodimeric partner of a receptor to alter the trafficking of the GPCR (11, 12).

Targeting of the receptor complex after agonist-stimulated internalization demonstrates a novel role for RAMP3. This new function for RAMP3 may explain the altered RAMP expression patterns in certain animal models of disease. RAMP3 expression has been shown to be increased during the transition from left ventricular hypertrophy to heart failure in Dahl salt-sensitive and deoxycorticosterone acetate (DOCA)-salt spontaneously hypertensive rats and in the myocardium of rats with chronic heart failure, where recycling of the receptor complex would be advantageous for continued ligand responsiveness (22–24). In fact, in various cardiosrenal disease states where AM is protective, circulating plasma levels of AM have been shown to be increased. For example, in chronic glomerulonephritis, type I diabetes, and type II diabetes plasma AM levels are elevated (25–27). In addition, AM delivery through adenoviral infection has been shown to decrease cardiac hypertrophy and renal damage in rat models of hypertension and improves diabetic rats (28–32). If indeed, AM is exerting protective effects against these diseases, then overcoming desensitization of the receptor complex would be important. It is of interest to determine whether NSF expression or localization is altered in these disease states as well. Furthermore, studies identifying additional molecular mechanisms of CRLR-RAMP trafficking may be valuable for therapeutic targeting.

While not only discovering a novel function for the RAMPs in post-endocytic trafficking, these findings also demonstrate a functional difference between the AM1R (CRLR+RAMP2) and AM2R (CRLR+RAMP3) receptor complexes, despite very similar second messenger systems and the physiological responses thus far identified. This study demonstrates the first difference between the RAMP2 and RAMP3 isoforms in the trafficking of the AM1R and AM2R. We suggest that this novel difference in AM1 and AM2 receptors may lead to either or both of the following two consequences: 1) there could be yet discovered roles of AM that differentially act through AM1 and AM2R because of this novel role of RAMP3; or 2) in disease conditions, expression of RAMP3 may be preferentially stimulated to effect recycling of CRLR-RAMP3 complex to affect physiological consequences of AM. This could result in a beneficial or harmful effect depending on the system in question (cardiovascular diseases versus cancer, respectively).

It is important to address the possibility of a species-specific effect, as the prototypic class I PDZ motif is present only in the human RAMP3 and not other species thus far sequenced. The human RAMP3 contains the classical PDZ class I motif (DTLL), while the two rodent species sequenced (rat and mouse) have a C-terminal amino acid sequence of DRLL. The resensitization experiments in the rat mesangial cells demonstrate the importance of the rat RAMP3 for efficient receptor targeting for resensitization. Furthermore, the resensitization experiments in the Rat2 fibroblast cells illustrate the ability of human RAMP3 to alter receptor targeting in a rat cell line to promote receptor resensitization/recycling after agonist pretreatment. Taken together, these data suggest that the lack of complete conservation between species of the C-terminal PDZ motif on RAMP3 does not alter our proposed model.

Finally, recent data suggest that RAMP3 can interact with receptors other than CRLR. It is important to determine whether this novel role of RAMP3 in receptor trafficking is specific for CRLR or also for the other receptors RAMPs interact with, namely VPAC, PTH1- and -2-R, and glucagon receptors (6). In addition to AM, Roh et al. (33) have reported that intermedin, a newly discovered peptide from the calcitonin gene peptide superfamily, can also bind the CRLR-RAMP3 complex. If this reported function for RAMP3 is specific for AM, or if another peptide like intermedin could indeed be this phenomenon, remains to be tested.

This study has shown that the heterodimeric partner of CRLR, RAMP3, is capable of altering the trafficking of the receptor complex after endocytosis by interacting with NSF via its PDZ domain. This demonstrates a novel function for the RAMP accessory proteins and the first difference between the AM1R and AM2R. With recent reports of RAMPs complexing and regulating additional GPCRs, these findings may reveal a more widespread form of regulation of the GPCR life cycle.

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