POST-ENDOCYTIC SORTING OF CALCITONIN RECEPTOR-LIKE RECEPTOR AND RECEPTOR ACTIVITY-MODIFYING PROTEIN 1

Graeme S. Cottrell1, Benjamin Padilla1, Stella Pikios1, Dirk Roosterman2, Martin Steinhoff2, Eileen F. Grady1, Nigel W. Bunnett1

From the 1Departments of Surgery and Physiology, University of California, San Francisco, 521 Parnassus Ave, San Francisco CA 94143-0660
2Department of Dermatology, IZKF Münster, and Ludwig Boltzmann Institute for Cell and Immunobiology of the Skin, University of Münster, Von-Esmarch-Strasse 58, 48149 Münster, Germany.

Running title: Trafficking of CLR and RAMP1

Address correspondence to: Nigel W. Bunnett, University of California San Francisco, 521 Parnassus Avenue, San Francisco CA 94143-0660. Tel: (415) 476-0489; Fax: (415) 476-0936; E-mail: nigel.bunnett@ucsf.edu.

Calcitonin receptor-like receptor (CLR) and the receptor activity-modifying protein 1 (RAMP1) comprise a receptor for calcitonin gene related peptide (CGRP). Although CGRP induces endocytosis of CLR/RAMP1, little is known about post-endocytic sorting of these proteins. We observed that the duration of stimulation with CGRP markedly affected post-endocytic sorting of CLR/RAMP1. In HEK and SK-N-MC cells, transient stimulation (10^7 M CGRP, 1 h), induced CLR/RAMP1 recycling with similar kinetics (2-6 h), demonstrated by labeling receptors in living cells with antibodies to extracellular epitopes. Recycling of CLR/RAMP1 correlated with resensitization of CGRP-induced increases in [Ca^{2+}]. Cycloheximide did not affect resensitization, but bafilomycin A1, an inhibitor of vacuolar H^+-ATPases, abolished resensitization. Recycling CLR/RAMP1 were detected in endosomes containing Rab4a and Rab11a, and expression of GTPase-defective Rab4aS22N and Rab11aS25N inhibited resensitization. After sustained stimulation (10^7 M CGRP, >2 h), CLR/RAMP1 trafficked to lysosomes. RAMP1 was degraded ~4-fold more rapidly than CLR (RAMP1, 45% degradation, 5 h; CLR, 54% degradation, 16 h), determined by Western blotting. Inhibitors of lysosomal, but not proteasomal, proteases prevented degradation. Sustained stimulation did not induce detectable mono- or poly-ubiquitination of CLR or RAMP1, determined by immunoprecipitation and Western blotting. Moreover, a RAMP1 mutant lacking the only intracellular lysine (RAMP1K142R) internalized and was degraded normally. Thus, after transient stimulation with CGRP, CLR and RAMP1 traffic from endosomes to the plasma membrane, which mediates resensitization. After sustained stimulation, CLR and RAMP1 traffic from endosomes to lysosomes by ubiquitin-independent mechanisms, where they are degraded at different rates.

Calcitonin gene-related peptide (CGRP) belongs to the calcitonin family of regulatory peptides and is produced by tissue-specific alternate splicing of transcripts from the calcitonin gene (1). CGRP is a potent vasodilator and mediator of neuroinflammatory and pain transmission (2,3). Notably, CGRP has a causative role in migraine headaches, and is thus mediator of human disease (4). In view of the importance of CGRP in health and disease, it is of interest to understand the mechanisms that control cellular responses to this peptide.

Unusually for neuropeptides, the CGRP receptor is a heterodimer comprised of calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1). CLR is a G-protein coupled receptor (GPCR) that shares 55% amino acid sequence identity with the calcitonin receptor (5), while RAMP1, RAMP2 and RAMP3, are single transmembrane proteins with ~30% identity (6). CLR functions as either a CGRP receptor, when co-expressed with RAMP1, or an adenomedullin receptor, when co-expressed with RAMP2 or 3 (6); the extracellular domain of the RAMP imparts this specificity (7,8). When expressed alone, CLR is retained in the endoplasmic reticulum (6) and RAMP1 is retained in the endoplasmic reticulum and the Golgi apparatus (9-11). However, when coexpressed,
CLR and RAMP1 traffic to the plasma membrane. Thus, RAMP1 is also a chaperone that targets CLR to the plasma membrane. However, it remains to be determined if CLR and RAMP1 are invariably associated after receptor activation.

Upon activation with CGRP, CLR, but not RAMP1, is phosphorylated and interacts with β-arrestins (12). β-arrestins are adapters for clathrin and AP2, and the CLR, RAMP1, β-arrestin complex undergoes dynamin-dependent endocytosis in clathrin-coated pits by well-defined mechanisms (11,12). However, little is known about the mechanisms of post-endocytic sorting of GPCRs, such as CLR, or accessory proteins, such as RAMP1, to degradative or recycling pathways. It is important to understand the post-endocytic sorting of CLR and RAMP1 since recycling may permit rapid resensitization of CGRP signaling, whereas degradation would prevent sustained, uncontrolled CGRP signaling during conditions of sustained peptide release.

β-arrestins can influence the rate of recycling of GPCRs. “Class A” receptors (e.g., β2 adrenergic receptor [β2AR], µ-opioid receptor, neurokinin 3 receptor) form low affinity and transient interactions with β-arrestin2, and rapidly recycle (13,14). “Class B” receptors (e.g., neurokinin 1 receptor [NK1R]) form high affinity and sustained interactions with β-arrestin1 and 2, and slowly recycle (13,14). Activated CLR colocalizes with β-arrestin2 and β-arrestin1 for prolonged periods (12) (Bunnett, unpublished observation). Thus, CLR may belong to the “class B” family of slowly recycling GPCRs. However, it is not known whether CLR and RAMP1 can recycle.

Addition of ubiquitin molecules to intracellular lysine residues targets some GPCRs to degradative pathways (e.g., β2AR, chemokine (C-X-C motif) receptor 4, protease-activated receptor 2 [PAR2], NK1R) (15-19). However, other GPCRs traffic to lysosomes by ubiquitin-independent processes (e.g., δ-opioid receptor) (20). The nature of the stimulus also affects ubiquitination and post-endocytic sorting of GPCRs. Thus, after transient stimulation with low concentrations of substance P, the NK1R is not ubiquitinated and rapidly recycles and resensitizes (19,21-23). In contrast, after sustained stimulation with high concentrations of substance P, the NK1R is ubiquitinated and degraded (19). Although CLR and RAMP1 exist in a stoichiometric 1:1 ratio at the plasma membrane (12), and co-internalize and traffic to lysosomes (11,12), their respective rates of degradation have not been examined, and the role of ubiquitination in lysosomal trafficking of CLR and RAMP1 is unknown. It remains to be determined if the duration of stimulation with CGRP affects the post-endocytic sorting of CLR and RAMP1 to recycling or degradative pathways.

We investigated the pathway and mechanism of post-endocytic trafficking of CLR and RAMP1, and determined the importance of this trafficking to the control of CGRP signaling. Our aims were to: (a) determine whether transient stimulation with CGRP induces post-endocytic sorting of CLR and RAMP1 to recycling pathways; (b) define the role of recycling in resensitization of CGRP signaling; (c) determine whether sustained stimulation with CGRP induces post-endocytic sorting of CLR and RAMP1 to degradative pathways; and (d) investigate whether trafficking to degradative pathways involves ubiquitination of CLR and RAMP1.

**Experimental Procedures**

**Reagents.** Rabbit antibodies to the C-terminus of rat CLR (RK11) and human RAMP1 (9891) have been described (24). Sources of other antibodies were: rat high affinity anti-HA (Roche Applied Science, Indianapolis, IN); rabbit anti-Myc and mouse anti-β-actin (A-5441) (Sigma Chemical Co., St. Louis, MO); goat anti-Myc (A-14) and mouse anti-ubiquitin (P4D1) (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-human lysosomal-associated glycomembrane protein 1 (LAMP1) (Developmental Studies Hybridoma Bank, Iowa City, IA); mouse anti-early endosomal antigen 1 (EEA1) (BD Transduction Laboratories, Lexington, KT); goat or donkey anti-mouse, rat or rabbit IgG coupled to horseradish peroxidase, fluorescein isothiocyanate, rhodamine red-X or Cy5 (Jackson ImmunoResearch, West Grove, PA); goat antimouse or rabbit IgG coupled to AlexaFluor®680 (Invitrogen, Carlsbad, CA) and coupled to IRDye®800 (Rockland Immunochemicals, Gilbertsville, PA). Rat α-CGRP was from Bachem (Torrance, CA).
Vector construction. cDNAs encoding rat CLR with an extracellular, N-terminal HA epitope, and rat RAMP1 with an extracellular, N-terminal Myc epitope have been described (24). CLR was subcloned into pcDNA5/FRT to yield pcDNA5/FRT-CLR. To create a vector that expressed CLR and RAMP1, RAMP1 was amplified by PCR together with a CMV promoter and bovine growth hormone polyA tail and subcloned into the BsmI and BstZ17I sites of the pcDNA5/FRT-rCLR. A rat RAMP1 mutant in which the lysine (K142) was mutated to arginine (designated RAMP1K142R) was generated by PCR (forward primer 5'-cgaatggccgagtgacccggtgg-3' and reverse primer 5'-gtgctgcgtttgccctggggtggg-3') using standard techniques and subcloned into pcDNA5/FRT-rCLR. Constructs were sequenced to verify integrity. Other primer sequences are available on request. cDNAs encoding GFP-tagged Rab4a, Rab11a and GTPase defective Rab4aS22N and Rab11aS25N have been described (23).

Transfected cells and cell lines. The generation and maintenance of human embryonic kidney 293 (HEK) FLP cells (Invitrogen) stably expressing rat CLR and rat RAMP1 have been described (24). HEKFLP cells stably expressing CLR and RAMP1 from the same vector (pcDNA5/FRT) or rat NK1R were created with the Flp-In™ system according to the manufacturer's guidelines, and cells were grown in DMEM supplemented with 10% HIFBS and 100 µg/ml of hygromycin B. In some experiments, cells were transiently transfected with CLR, RAMP1, Rab4a, Rab4aS22N, Rab11a or Rab11aS25N using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s guidelines. The human neuroblastoma cell line SK-N-MC was from American Tissue Type Collection (Manassas, VA). SK-N-MC cells were transiently transfected with CLR and RAMP1 and were grown in MEM supplemented with non-essential amino acids and 10% HIFBS. All cells were routinely grown in 95% air, 5% CO2 at 37°C. In control experiments, cells were transfected with vectors without inserts.

Measurement of [Ca2+]. Cells were incubated with 2.5 µM fura-2AM (Invitrogen) for 20 min at 37°C and washed. Fluorescence was measured at 340 and 380 nm excitation and 510 nm emission in a F-2500 spectrophotometer (Hitachi Instruments, Irvine, CA). The ratio of the fluorescence at the two excitation wavelengths, which is proportional to [Ca2+], was calculated, and results are expressed as increase above basal values. To assess desensitization and re-sensitization, cells were challenged with CGRP (10^-7 M, 1 h) or vehicle (control), washed, and [Ca2+] was determined to a second challenge with CGRP (3x10^-8 M).

Fluorescence. Cells were plated at 3x10^5 cells per 35 mm dish onto coverslips coated with poly-D-lysine (100 µg/ml). To localize CLR and RAMP1, cells were fixed in 4% paraformaldehyde in 100 mM PBS, pH 7.4 (20 min at 4°C), and washed for 15 min with 1xPBS containing 0.1% saponin and 1% normal goat serum or 2% normal donkey serum. Proteins were localized using primary antibodies: CLR (RK11, 1:4000), RAMP1 (9891, 1:2000 or goat anti-Myc, 1:100), LAMP1 (1:1000), EEA1 (1:500) (overnight, 4°C). Cells were washed for 15 min with 1xPBS containing 0.1% saponin and 1% normal goat serum or 2% normal donkey serum, and incubated with secondary antibodies conjugated to fluorescein isothiocyanate, rhodamine-red-X or Cy5 (1:200, 2 h room temperature or overnight at 4°C).

 Trafficking of antibody-tagged receptors. To label CLR and RAMP1 at the cell surface, living cells were incubated with rat anti-HA (to detect CLR; 1:100) and rabbit or goat anti-Myc (to detect RAMP1; 1:100) for 30 min at 37°C. Cells were washed with PBS containing Ca2+ and Mg2+ (PBSCM) and stimulated with CGRP. Cells were fixed at specified times, washed and incubated with fluorescent secondary antibodies overnight at 4°C.

Confocal microscopy. Cells were observed by using Zeiss Axiovert, 510Meta and BioRad MRC1000 confocal microscopes with Zeiss Plan Apo x100 (NA 1.3 or 1.4) objective. Images were collected at zoom of 1-2, iris of <3 µm and typically 5-10 optical sections were taken at intervals of 0.5 µm. Images (single optical sections are shown) were colored and processed to adjust contrast and brightness using Adobe Photoshop CS (Adobe Systems, Mountain View, CA, USA).

SDS-PAGE and Western blotting. Cells were lysed in 50 mM Tris/HCl pH 7.4, 1% SDS, boiled and centrifuged. Lysates (5-10 µg protein) were separated by SDS-PAGE (CLR, 8 or 9%; RAMP1, 15% acrylamide gels). Proteins were transferred to
PVDF membranes (Immobilon-P or FL, Millipore, Billerica, MA) and blocked for 1 h at room
temperature (1x PBS, 2% BSA, 5% milk powder,
0.1% Tween\textsuperscript{20} or Odyssey Blocking Buffer). Membranes were incubated with antibodies to rat
CLR (RK11, 1:10000), rat RAMP1 (9891, 1:5000), β-actin (1:20000), or ubiquitin P4D1
(1:1000) overnight at 4°C (1x PBS, 2% BSA, 5% milk powder, 0.1% Tween\textsuperscript{20} or Odyssey Blocking Buffer). Membranes were washed for 30 min (1x PBS, 0.1% Tween\textsuperscript{20}) and incubated with secondary antibody coupled to horseradish
peroxidase (1:10000, 1 h, room temperature). Membranes were washed for 30 min and
immunoreactive proteins were detected by using chemiluminescence (SuperSignal West Pico
Chemiluminescent Substrate, Pierce, Rockford, IL). Alternatively, membranes were incubated with secondary antibodies conjugated to AlexaFluor®680 or IRDye™800 (1:10-20000, 1 h,
room temperature), and blots were analyzed with the Odyssey infrared imaging system (Li-COR
Biosciences, Lincoln, NE).

**Immunoprecipitation.** Cells were plated at 1.5x10\textsuperscript{6}
cells per 100 mm dish and used after 48 h. After
treatments, cells were washed with PBSCM, lysed with 1 ml RIPA buffer (10 mM Tris/HCl, pH 7.4,
150 mM NaCl, 50 mM NaF, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 0.5% w/v sodium deoxycholate, 1% Nonidet P-40) and
centrifuged (10 min, 15000 g, room temperature). Supernatants were transferred to fresh tubes and
immunoprecipitating antibodies added (CLR: rat anti-HA, 500 ng; RAMP1: rabbit anti-Myc, 2 µg).
Samples were rotated for 16 h at 4°C. Protein A/G PLUS (Santa Cruz Biotechnology) was added (30
µl) and samples were rotated for 2 h at 4°C. Immunoprecipitates were pelleted, washed three
times with 1 ml RIPA, boiled in 2x Laemelli buffer, and analyzed by Western blotting. For
denaturing immunoprecipitation (NK,R), cells were lysed in 100 µl of 10 mM Tris/HCl pH 7.4,
1% SDS, sonicated and mixed with 4 volumes of RIPA buffer (10 mM Tris/HCl, pH 7.4, 150 mM
NaCl, 50 mM NaF, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 0.5% w/v sodium deoxycholate, 1% Nonidet P-40). The lysate was mixed by pipetting, RIPA was added to
1 ml final volume and centrifuged (16000 g, 20 minutes, 4°C). Supernatants were rotated with antibody (FLAG M2, 3.5 µg/ml) overnight at 4°C and samples processed as described above.

**Activation of CLR and RAMP1 and drug
treatments.** Two days after plating cells or after
transfection, cells were washed three times with
PBSCM and placed in DMEM containing 0.1%
BSA. Cells were stimulated with 10\textsuperscript{-7} M rat α-
CGRP, unless otherwise stated. To inhibit new
protein synthesis, cells were treated with
cycloheximide (140 µM, Sigma). To inhibit
vacuolar-type H\textsuperscript{+}-ATPases, cells were treated with
bafilomycin A\textsubscript{1} (1 µM) (A. G. Scientific, San
Diego, CA). These inhibitors were pre-incubated
with cells 1 h prior to stimulation with CGRP, and
were present throughout stimulation and recovery
phases of the experiments. Lysosomal proteases
were inhibited using ZPAD, E64d (Bachem; 200
µM and 20 µM, respectively) and pepstatin A
(Roche; 10 µM). The proteolytic activity of the
proteasome was inhibited using epoxomicin
(BioMol, Plymouth Meeting, PA; 10 µM). Controls included appropriate vehicle.

**Densitometry.** Signals on Western blots were
quantified using the Odyssey Infrared Imaging
System (Li-COR Biosciences) or blots were
digitized using an Epson Perfection 3200 PHOTO
scanner, and analyzed by densitometry with NIH
Image 1.63. To quantify CLR and RAMP1
degradation, signals were compared to β-actin
signals.

**Statistics.** Data are presented as the mean and
standard error of ≥3 experiments. Results are
compared by Student’s t-test or ANOVA and
Student-Newman-Keuls’s test, with *p<0.05
considered significant.

**Results**

**CLR and RAMP1 traffic to early endosomes and
lysosomes.** To examine the effects of CGRP on
trafficking of CLR and RAMP1, we incubated
HEK cells stably expressing CLR and RAMP1
(HEK-CLR-RAMP1 cells) with CGRP (10\textsuperscript{-7} M)
for 0-120 min; we localized CLR, RAMP1 (using
antibodies RK11 and 9891, to the C-terminus of
CLR and RAMP1, respectively), EEA1 and
LAMP1 by indirect immunofluorescence. In
unstimulated cells, CLR and RAMP1 were at the
plasma membrane (Fig. 1A, B, arrow heads). After
10 min with CGRP, CLR and RAMP1 were
depleted from the plasma membrane and were
colocalized with EEA1 in early endosomes (Fig.
1A, B, white arrows). CLR and RAMP1 were
prominently localized to early endosomes at 30 and 60 min (not shown). After 120 min with CGRP, CLR and RAMP1 were sometimes colocalized with EEA1 (Fig. 1A, B, white arrows), but were also detected in vesicles that did not contain EEA1 (Fig. 1A, B, yellow arrows). These vesicles were lysosomes, since at 120 min CLR and RAMP1 were prominently colocalized with LAMP1 (Fig. 1C, D, white arrows). However, CLR and RAMP1 were also detected in some vesicles that did not contain LAMP1 and are probably early endosomes (Fig. 1C, D, yellow arrows). At earlier times (e.g. 60 min), CLR and LAMP1 were rarely detected in lysosomes (not shown).

These results show that activated CLR and RAMP1 traffic from the plasma membrane to early endosomes and lysosomes, which is consistent with other reports of CLR and RAMP1 trafficking (11). At early times (10-60 min), the receptors are most prominently in early endosomes. At later times (120 min), the receptors are more prominently sorted to lysosomes. However, even after sustained activation, lysosomal sorting is incomplete and some receptors are retained in early endosomes. We therefore examined whether CLR and RAMP1 can recycle from early endosomes after transient stimulation with CGRP.

**CLR and RAMP1 recycle after transient stimulation with CGRP.** Although continuous stimulation of cells with high concentrations of CGRP induces trafficking of CLR and RAMP1 to lysosomes (11), many GPCRs efficiently recycle, especially after brief stimulation with agonist (22,23). We examined CLR and RAMP1 recycling in cycloheximide-treated HEK-CLR-RAMP1 cells, to avoid synthesis of new receptors, which would also traffic to the plasma membrane. Cells were incubated with CGRP for 1 h to induce trafficking of both proteins to early endosomes. Cells were washed and incubated in CGRP-free medium for 0-6 h, to allow recycling. CLR and RAMP1 were simultaneously detected by indirect immunofluorescence using a rabbit antibody the C-terminus of CLR (RK11), and a goat antibody to an N-terminal Myc epitope of RAMP1. In unstimulated cells, CLR and RAMP1 colocalized at the plasma membrane (Fig. 2A, arrows). After 1 h with CGRP, CLR and RAMP1 were depleted from the plasma membrane and were colocalized in vesicles (Fig. 2A, arrows). These vesicles colocalized with EEA1 and are thus early endosomes (not shown). At 2 h after washing and recovery in agonist-free medium, CLR and RAMP1 were present in some endosomes but were also detected at the plasma membrane, and both proteins were almost exclusively colocalized at the plasma membrane after 6 h recovery (Fig. 2A, arrow heads).

The reappearance of CLR and RAMP1 at the plasma membrane of cycloheximide-treated cells suggests that these proteins can recycle. However, mobilization of CLR and RAMP1 from intracellular stores could also account for the reappearance of CLR and RAMP1 at the plasma membrane. To exclude this possibility, we examined trafficking of CLR and RAMP1 that were tagged at the cell surface using antibodies to extracellular epitopes. To label surface receptors, living HEK-CLR-RAMP1 cells were simultaneously incubated with antibodies to the extracellular epitope tags of CLR (HA) and RAMP1 (Myc). Cells were washed to remove unbound antibodies, incubated with CGRP for 1 h, washed, and recovered in CGRP-free medium for 0-6 h. At specified times, cells were fixed, permeabilized and incubated with fluorescent secondary antibodies to detect CLR and RAMP1. In unstimulated cells, CLR and RAMP1 were detected at the cell surface (Fig. 2B, arrow heads). When cells were incubated with vehicle for 1 h, CLR and RAMP1 remained at the cell surface (not shown). After 1 h with CGRP, CLR and RAMP1 were colocalized in endosomes (Fig. 2B, arrows) with EEA1 (not shown). Within 2 h of washing and incubation in CGRP-free medium, CLR and RAMP1 were detected at the plasma membrane, and after 6 h recovery both proteins were detected exclusively at the plasma membrane (Fig. 2B, arrow heads).

The reappearance of antibody-tagged CLR and RAMP1 at the plasma membrane indicates that CLR and RAMP1 can recycle after transient stimulation with CGRP. Importantly, antibody-tagged CLR and RAMP1 (Fig. 2B) traffic to similar locations at similar times as non-tagged receptors (Fig. 2A), suggesting that the antibodies do not influence trafficking.

**Recycling of CLR and RAMP1 mediates resensitization of CGRP signaling.** Since CLR and RAMP1 recycle after transient incubation with
CGRP, we determined if recycling mediates resensitization of CGRP-induced increases in [Ca\(^{2+}\)].

We first established a time course of resensitization. HEK-CLR-RAMP1 cells were exposed to CGRP (10\(^{-7}\) M) or vehicle (control) for 1 h, washed to remove agonist and then re-challenged with CGRP (3\times10^{-8} \text{ M}) at 0-8 h. The change in [Ca\(^{2+}\)] to the rechallenge was determined. Immediately after removal of agonist, cells were completely desensitized to CGRP (Fig. 3A). Resensitization was not complete until 6-8 h after removal of CGRP (at 8 h, 95\pm5\% resensitization compared to vehicle control, p>0.05). Thus, resensitization coincides with recycling of CLR and RAMP1.

To determine whether new protein synthesis is required for resensitization, we treated HEK-CLR-RAMP1 cells with cycloheximide. Even in the presence of cycloheximide, CGRP signaling returned to control levels after 8 h (101\pm4\%, Fig. 3B). Thus, resensitization of CGRP signaling is not dependent on new receptor synthesis following a 1 h exposure to CGRP.

Endosomal acidification promotes dissociation of ligand from GPCRs, and inhibitors of this acidification can prevent receptor recycling and resensitization (22). Therefore, we examined the effects of bafilomycin A\(_1\), an inhibitor of vacuolar-type H\(^{+}\)-ATPases, on resensitization of CGRP signaling. After 2 h recovery, CGRP-induced Ca\(^{2+}\) mobilization was resensitized to 59\pm3\% in vehicle-treated cells, but bafilomycin A\(_1\) abolished resensitization (Fig. 3C). Thus, activity of vacuolar-type H\(^{+}\)-ATPases is required for resensitization.

**Rab4a and Rab11a partially mediate resensitization.** Rab-GTPases mediate the vesicular transport required for the resensitization of some GPCRs [reviewed in (25,26)]. We investigated the role of Rab4a and Rab11a in resensitization of CLR and RAMP1, since these GTPases also mediate NK\(_{i}\)R recycling and resensitization (23). HEK-CLR-RAMP1 cells were transiently transfected with GFP-tagged Rab4a or Rab11a or with GTPase defective Rab4aS22N or Rab11aS25N, which act as dominant negative mutants. Cells were exposed to CGRP (10\(^{-7}\) M) or vehicle (control) for 1 h, washed to remove agonist and then re-challenged with CGRP (3\times10^{-8} \text{ M}) at 8 h. In cells transfected with empty vector or Rab4a, resensitization was complete after 8 h (vector control, 92\pm4\%; Rab4a, 93\pm3\%) (Fig. 3D). However, in cells transfected with Rab4aS22N, resensitization was significantly reduced (Rab4aS22N, 77\pm4\%, p<0.05). In cells transfected with empty vector or Rab11a, resensitization was complete after 8 h (vector control, 92\pm4\%; Rab11a, 95\pm5\%) (Fig. 3E). However, in cells transfected with Rab11aS25N, resensitization was significantly reduced (Rab11aS25N, 69\pm3\%, p<0.05).

These results show that disruption of Rab4a or Rab11a diminishes resensitization of responses to CGRP by >20\% and >30\%, respectively. However, unlike inhibition of vacuolar-type H\(^{+}\)-ATPases, dominant negative Rab4a and Rab11a did not abolish resensitization. Disruption of these Rabs also reduces, but does not abolish, resensitization of the NK\(_{i}\)R (23). Thus, activity of Rab4a and Rab11a is only partially required for resensitization of CLR and RAMP1.

Inhibition of endocytosis, for example by expression of dominant negative mutants of dynamin and Rab5a, can impede resensitization of GPCRs, such as the NK\(_{i}\)R, presumably by disrupting normal intracellular processing of receptors (27). Therefore, we examined whether overexpression of Rab4aS22N or Rab11aS25N affected CGRP-induced endocytosis of CLR. HEK-CLR-RAMP1 cells expressing Rab4aS22N-GFP or Rab11aS25N-GFP were incubated with CGRP (10\(^{-7}\) M) or vehicle for 30 min. CLR and EEA1 were localized by indirect immunofluorescence. Rab4aS22N-GFP and Rab11aS25N-GFP were uniformly distributed in the cytosol and also detected in enlarged vesicles (Fig. 4A). In vehicle-treated cells, CLR was present at the plasma membrane (not shown). After incubation with CGRP for 30 min, CLR colocalized with EEA1 in early endosomes (Fig. 4A). Thus, Rab4a and Rab11a are not required for CLR endocytosis. These Rabs are also not required for NK\(_{i}\)R endocytosis (23).

We determined whether CLR and RAMP1 colocalize in endosomes with Rab4a and Rab11a during recycling. To detect recycling receptors (and avoid detection of receptors in intracellular stores), CLR and RAMP1 at the cell surface were tagged with antibodies to extracellular epitopes.
(HA for CLR and Myc for RAMP1). Cells were exposed to CGRP (10^{-7} M) for 1 h, washed to remove agonist, and incubated in CGRP-free medium for 4 h. Cells were fixed and permeabilized; CLR and RAMP1 were detected by using fluorescent secondary antibodies, and Rab4a and Rab11a were detected using GFP. In unstimulated cells, CLR and RAMP1 were colocalized at the plasma membrane, and Rab4a and Rab11a were detected in vesicles (Fig. 4B,C). After stimulation with CGRP and washing, CLR and RAMP1 were present in vesicles some of which contained Rab4a and Rab11a (Fig. 4B,C, arrows). Thus, during recycling CLR and RAMP1 are present in endosomes containing Rab4a and Rab11a, and disruption of these Rabs inhibits resensitization. These results are consistent with a role for Rab4a and Rab11a in CLR and RAMP1 recycling.

Together, these data suggest that after transient stimulation, CGRP signaling slowly resensitizes by mechanisms that depend on recycling of CLR and RAMP1. Resensitization requires activity of vacuolar H\(^{+}\)ATPase, and is partially dependent on activity of Rab4a and Rab11a, but does not require the synthesis of new receptors.

**CLR and RAMP1 traffic to lysosomes after continuous stimulation with CGRP to be degraded with different kinetics.** After continuous incubation of cells with CGRP for 2 h, CLR and RAMP1 were colocalized with LAMP1, indicative of trafficking to lysosomes and degradation by lysosomal proteases (Fig. 1C, D). To examine the kinetics of degradation of CLR and RAMP1, we treated HEK-CLR-RAMP1 cells with CGRP (10^{-7} M) for various times, and assessed protein levels by Western blotting, using antibodies to the C-terminus of CLR (RK11) and RAMP1 (9891). Cells were incubated with cycloheximide to prevent new protein synthesis. After continuous incubation with CGRP for 1, 3 or 5 h, there was no detectable degradation of CLR (Fig. 5A). Degradation of CLR was detected only after prolonged incubation with CGRP for 8 or 16 h (54±3% degradation compared to vehicle-treated cells at 16 h, p<0.05, Fig. 5B). In contrast, continuous incubation with CGRP for 5 h resulted in marked degradation of RAMP1 (45±13% degradation compared to vehicle-treated cells, p<0.05, Fig. 5C). Thus, RAMP1 is more susceptible to degradation than CLR.

To confirm that CLR and RAMP1 are degraded in lysosomes after sustained stimulation with CGRP, we treated HEK-CLR-RAMP1 cells with lysosomal protease inhibitors (ZPAD, E64d, pepstatin A) and examined the levels of both proteins. These lysosomal inhibitors completely prevented degradation of CLR after 16 h and RAMP1 after 5 h incubation with CGRP (Fig. 6A,B). In contrast, degradation of CLR and RAMP1 was unaffected by epoxomicin, an inhibitor of proteasomal proteases, or by DMSO, the vehicle for the inhibitors (Fig. 6A,B). Thus, after continuous exposure to CGRP, CLR and RAMP1 are degraded by lysosomal proteases.

To confirm the lysosomal trafficking and degradation of CLR and RAMP1, we simultaneously localized CLR (using RK11 antibody), RAMP1 (using Myc antibody) and LAMP1. HEK-CLR-RAMP1 cells were incubated with CGRP (10^{-7} M) or vehicle for 8 or 16 h, in the presence of cycloheximide. CLR, RAMP1 and LAMP1 were simultaneously detected by indirect immunofluorescence. In unstimulated cells, CLR and RAMP1 were at the plasma membrane (Fig. 7, arrow heads). After 8 and 16 h, RAMP1 was barely detectable, and levels of CLR were minimally reduced, even though CLR was present in lysosomes (Fig. 7, arrows). The lysosomal protease inhibitors prevented the loss of immunoreactive RAMP1 at 16 h.

Thus, sustained incubation with CGRP induces trafficking of CLR and RAMP1 to lysosomes. RAMP1 is rapidly degraded in lysosomes, as assessed by Western blotting, using an antibody to the C-terminus, and immunofluorescence, using an antibody to the N-terminus. CLR is less susceptible to lysosomal proteolysis, determined by Western blotting. Since immunoreactive CLR was detectable in lysosomes after prolonged stimulation with CGRP (16 h), the C-terminus, to which the antibody was raised, may be intact.

**CLR and RAMP1 traffic to lysosomes by ubiquitin–independent mechanisms.** The covalent addition of ubiquitin molecules to intracellular lysine residues is required for the post-endocytic sorting of some (15-19), but not all (20) GPCRs to lysosomes for degradation. To determine whether sustained stimulation with CGRP caused CLR or
CLR and RAMP1 can recycle and traffic to lysosomes in SK-N-MC cells. To determine whether CGRP can induce recycling and lysosomal trafficking of CLR and RAMP1 in cells naturally expressing these proteins, we studied human SK-N-MC cells (28). The CLR antibody RK11 is raised to the C-terminal 18 residues of rat CLR, which differs from human CLR by 4 amino acid substitutions and thus does not fully cross-react with the human receptor (Bunnett, unpublished observation). Therefore, we transiently expressed epitope-tagged CLR and RAMP1 in SK-N-MC cells. This approach allowed simultaneous detection of both proteins using antibodies to epitope tags and permitted the study of recycling of antibody tagged receptors.

To examine recycling, CLR and RAMP1 at the cell surface were tagged with antibodies by incubating living SK-N-MC cells with antibodies to the extracellular epitopes of CLR (HA) and RAMP1 (Myc). Cells were transiently stimulated with CGRP (10⁷ M) or vehicle (control) for 1 h, washed and recovered in CGRP-free medium for 0-6 h. Cells were fixed, permeabilized and incubated with fluorescent antibodies to detect CLR and RAMP1. In unstimulated cells (0 h) or after incubation with vehicle for 1 h (not shown), CLR and RAMP1 were primarily detected at the plasma membrane (Fig. 10, arrow heads). When cells were incubated with CGRP for 1 h, CLR and RAMP1 colocalized in intracellular vesicles (Fig. 10, arrows). After agonist washout and 2 h incubation in CGRP-free medium, CLR and RAMP1 were in intracellular vesicles with weak colocalization at the plasma membrane, and after 6 h both proteins were mostly present at the plasma membrane (Fig. 10, arrow heads). Thus, after transient incubation with CGRP, CLR and RAMP1 internalize and recycle in SK-N-MC cells.

To examine whether sustained stimulation with CGRP induces trafficking of CLR and RAMP1 to lysosomes, SK-N-MC cells transfected with CLR and RAMP1 were incubated with CGRP (10⁻⁷ M) for 8 h. We localized CLR (using RK11), RAMP1 (using Myc antibody) and LAMP1 by indirect immunofluorescence. In unstimulated cells, CLR and RAMP1 were at the cell surface (Fig. 11, arrow heads). After 8 h with CGRP, RAMP1 was barely detectable, and CLR was present in LAMP1-containing lysosomes (Fig.

CLR and RAMP1 can recycle and traffic to lysosomes in SK-N-MC cells. To determine whether CGRP can induce recycling and lysosomal trafficking of CLR and RAMP1 in cells naturally expressing these proteins, we studied human SK-N-MC cells (28). The CLR antibody RK11 is raised to the C-terminal 18 residues of rat CLR, which differs from human CLR by 4 amino acid substitutions and thus does not fully cross-react with the human receptor (Bunnett, unpublished observation). Therefore, we transiently expressed epitope-tagged CLR and RAMP1 in SK-N-MC cells. This approach allowed simultaneous detection of both proteins using antibodies to epitope tags and permitted the study of recycling of antibody tagged receptors.

To examine recycling, CLR and RAMP1 at the cell surface were tagged with antibodies by incubating living SK-N-MC cells with antibodies to the extracellular epitopes of CLR (HA) and RAMP1 (Myc). Cells were transiently stimulated with CGRP (10⁷ M) or vehicle (control) for 1 h, washed and recovered in CGRP-free medium for 0-6 h. Cells were fixed, permeabilized and incubated with fluorescent antibodies to detect CLR and RAMP1. In unstimulated cells (0 h) or after incubation with vehicle for 1 h (not shown), CLR and RAMP1 were primarily detected at the plasma membrane (Fig. 10, arrow heads). When cells were incubated with CGRP for 1 h, CLR and RAMP1 colocalized in intracellular vesicles (Fig. 10, arrows). After agonist washout and 2 h incubation in CGRP-free medium, CLR and RAMP1 were in intracellular vesicles with weak colocalization at the plasma membrane, and after 6 h both proteins were mostly present at the plasma membrane (Fig. 10, arrow heads). Thus, after transient incubation with CGRP, CLR and RAMP1 internalize and recycle in SK-N-MC cells.

To examine whether sustained stimulation with CGRP induces trafficking of CLR and RAMP1 to lysosomes, SK-N-MC cells transfected with CLR and RAMP1 were incubated with CGRP (10⁻⁷ M) for 8 h. We localized CLR (using RK11), RAMP1 (using Myc antibody) and LAMP1 by indirect immunofluorescence. In unstimulated cells, CLR and RAMP1 were at the cell surface (Fig. 11, arrow heads). After 8 h with CGRP, RAMP1 was barely detectable, and CLR was present in LAMP1-containing lysosomes (Fig.
Inhibitors of lysosomal proteases prevented loss of immunoreactive RAMP1, and CLR was more readily detected (Fig. 11). Thus, after sustained stimulation with CGRP, CLR and RAMP1 traffic to lysosomes in SK-N-MC cells, where RAMP1 is rapidly degraded, and CLR is degraded more slowly.

**Discussion**

Our results show that the duration of stimulation markedly influences the trafficking and fate of CLR and RAMP1. After transient stimulation with CGRP, CLR and RAMP1 recycle from early endosomes, and activity of vacuolar H⁺-ATPase, Rab4a and Rab11a is required for resensitization of CGRP signaling. Thus, recycling of CLR and RAMP1 mediate resensitization after transient stimulation. After sustained stimulation with CGRP, CLR and RAMP1 traffic from early endosomes to lysosomes, where RAMP1 is rapidly degraded and CLR is slowly degraded. Neither CLR nor RAMP1 undergo detectable ubiquitination after sustained stimulation and the single intracellular lysine residue of RAMP1 is not necessary for CGRP-induced activation, endocytosis or degradation of RAMP1. This differential regulation of CLR and RAMP1 may markedly influence the responsiveness of cells, depending upon whether there is transient or sustained release of CGRP.

**Transiently activated CLR and RAMP1 recycle.** By labeling surface CLR and RAMP1 in living cells using antibodies to extracellular epitopes, we observed that transient stimulation with CGRP (1 h) induced recycling of CLR and RAMP1 from early endosomes to the plasma membrane with similar kinetics. This recycling was observed in HEK cells stably expressing CLR and RAMP1, and in SK-N-MC cells transiently expressing these proteins. Thus, recycling occurs in a model cell line (HEK) as well as in cells that naturally express CGRP receptors.

Our finding that CLR and RAMP1 efficiently recycle after transient stimulation and agonist removal contradicts other reports that CLR and RAMP1 are degraded in HEK cells (11,12). We do not know the reason for these differences. One possibility is that our method of labeling surface CLR and RAMP1 with antibodies to extracellular epitopes allows for more sensitive detection of recycling receptors. Although we cannot exclude the possibility that antibodies could affect trafficking of internalized receptors, we have shown that such antibody-conjugated receptors can traffic to lysosomes after sustained activation with CGRP (Bunnett, unpublished). Moreover, CGRP induced identical trafficking of both non-tagged and antibody-tagged CLR and RAMP1 (Fig. 2), suggesting that the antibodies did not influence agonist-stimulated receptor trafficking. Differences in receptor trafficking could also be related to the existence of adapter proteins, which may bind the internalized receptor complex to induce trafficking of CLR and RAMP1 through different pathways. For example, overexpression of N-ethylmaleimide-sensitive factor (NSF), which binds to murine RAMP3 by a PDZ domain, switches CLR/RAMP3 from a degradative to a recycling pathway (29). Human RAMP3 also interacts through a PDZ domain with Na⁺/H⁺ exchanger regulatory factor-1 (NHERF-1), which prevents the internalization of the CLR/RAMP3 complex, while leaving plasma membrane targeting and desensitization intact (30). NHERF-1 has been implicated in altering the trafficking (from degradative to recycling) of other receptors such as the β₂AR (31) and the κ-opioid receptor (32). RAMP1 does not contain PDZ domains, but this does not exclude the possibility of interactions with other trafficking proteins.

Our observation that the nature of agonist exposure (transient or sustained) alters trafficking of CLR and RAMP1 supports observations of other GPCRs. For example, brief stimulation of the NK₂R with low concentrations of substance P induces rapid recycling and resensitization (23), whereas sustained stimulation with high concentrations of substance P causes ubiquitination and degradation of this receptor (19).

Recycling mediates resensitization of transiently activated CLR and RAMP1. Various mechanisms mediate resensitization of cellular responses to agonists of GPCRs, including receptor recycling (22,23), mobilization of receptors from intracellular stores (33), and synthesis of new receptors (18). Our results indicate that CLR and RAMP1 recycling mediate resensitization after transient stimulation with CGRP. Resensitization of CGRP-induced Ca²⁺ mobilization coincided with recycling of CLR and RAMP1. The slow
rates of resensitization and recycling are consistent with that of a “class B” GPCR that forms sustained, high affinity interactions with β-arrestin 1 and 2. Cycloheximide did not affect resensitization, which is thus independent of synthesis of new receptor proteins. However, bafilomycin A1 completely prevented resensitization, suggesting a requirement for activity of vacuolar-type H+-ATPases. Endosomal acidification promotes recycling and resensitization of other GPCRs, most probably by inducing dissociation of receptors from their ligands (21,22). GTPase-defective mutants of Rab4a and Rab11a also inhibited resensitization by ~20-30%, and during recycling CLR and RAMP1 were detected in endosomes containing wild-type Rab4a and Rab11a. Thus, Rab4a and Rab11a contribute to recycling and resensitization of CLR and RAMP1. In support of these results, Rab11a mediates recycling and resensitization of the β2AR (34), the angiotensin II type 1A receptor (35) and the NK1R (23).

After sustained activation, CLR and RAMP1 traffic to lysosomes to be degraded with different kinetics. In the continued presence of CGRP, CLR and RAMP1 trafficked to LAMP1-containing lysosomes in HEK cells and SK-N-MC cells. These results support another report of CGRP-induced trafficking of CLR to lysosomes (11).

After sustained activation, CLR and RAMP1 were degraded, determined by Western blotting. Inhibitors of lysosomal but not proteasomal proteases inhibited degradation of CLR and RAMP1, confirming lysosomal degradation. However, CLR and RAMP1 were degraded in lysosomes with very different kinetics. Analysis of Western blots indicated that CLR was degraded after >8 h with CGRP, whereas RAMP1 was degraded within 5 h. Structural differences may account for the different rates of degradation. Thus, degradation of high molecular weight, heptahelical CLR may proceed more slowly than smaller, single-membrane-spanning RAMP1. Indeed, the NK1R is also degraded slowly after prolonged stimulation with substance P (19). Alternatively, differences in the rate of CLR and RAMP1 trafficking to lysosomes may account for different rates of degradation. In support of this possibility, another GPCR, protease-activated receptor 2 (PAR2), is rapidly ubiquitinated and degraded in lysosomes after activation (18). Thus, certain heptahelical proteins are rapidly degraded in lysosomes, whereas others are more slowly degraded. Further experimentation is required to determine whether susceptibility to degradation or differential rates of lysosomal trafficking mediate the differences in the kinetics of CLR and RAMP1 degradation. However, the increased susceptibility of RAMP1 to degradation could allow CLR to couple to newly synthesized or stored RAMP1, or even to RAMP2 or RAMP3, prior to recycling.

Notably, although degradation of CLR was detected by Western blotting after 8-16 h of stimulation with CGRP, immunoreactive CLR was still detected in lysosomes by immunofluorescence at these times. This difference may be attributable to difficulties in quantifying fluorescent signals by microscopy. Alternatively, the C-terminus of the receptor, against which our CLR antibody was raised, may remain intact for prolonged periods. CLR and RAMP1 are targeted to lysosomes without modification by ubiquitin. The covalent attachment of ubiquitin to intracellular-facing lysine residues promotes the post-endocytic trafficking of certain GPCRs to lysosomes. For example, agonists promote ubiquitination of β2AR, chemokine (C-X-C motif) receptor 4, PAR2 and the NK1R, and in the case of chemokine receptor 4 and PAR2, ubiquitination-defective mutant receptors are retained in early endosomes and avoid lysosomal trafficking and degradation (15-19). This ubiquitination acts as a signal for the recruitment of adapter proteins that traffic the ubiquitinated receptor for degradation by proteasomal or lysosomal proteases. However, we were unable to detect ubiquitination of either CLR or RAMP1 after sustained stimulation with CGRP, although we have previously used similar methods to examine ubiquitination of NK1R (19) and PAR2 (18). Furthermore, a RAMP1 mutant, in which the only intracellular-facing lysine residue was mutated to arginine, was still degraded in a similar manner to wild-type RAMP1, indicating that modification of this lysine residue is not involved in targeting RAMP1 to lysosomes. This finding is in agreement with a previous report that deletion of the C-terminal tail of RAMP1 (to remove a conserved SK motif found in all RAMPs) had little effect on its surface expression, function or intracellular trafficking (36). Additional experiments are required to determine the
mechanisms of post-endocytic sorting of CLR and RAMP1 to lysosomes. Other receptors, such as the δ-opioid receptor, are still efficiently targeted to and degraded in the lysosome in the absence of ubiquitination (20). Vacuolar sorting proteins such as Vps4 and Hrs facilitate trafficking of the δ-opioid receptor through the endosomal pathway to lysosomes (37). The possible contributions of these proteins to CLR and RAMP1 trafficking are unknown.

Physiological consequences of differential trafficking of CLR and RAMP1. Members of the CGRP family of peptides are expressed throughout the central and peripheral nervous systems (38-40), where they control vasodilatation, nociception, motor function, secretion, audition, olfaction and feeding (2,3). An important role for CGRP in human disease is suggested by the report that a CGRP receptor antagonist is an effective treatment for migraine (4). Since CLR and RAMP1 mediate these diverse biological actions of CGRP, the mechanisms that we describe to regulate trafficking and signaling of CLR and RAMP1 are likely to affect multiple biological processes.

Under physiological conditions, where CGRP is probably briefly released, transient activation of CLR and RAMP1 would be expected to promote endocytosis and recycling of both proteins. These mechanisms would permit cells to respond again to CGRP without the need to synthesize new receptors. In contrast, under conditions where there is continuous release of CGRP, as may occur during disease, sustained stimulation would induce lysosomal trafficking of CLR and RAMP1, which may serve to protect against uncontrolled stimulation. Whether this differential trafficking of CLR and RAMP1 also affects signaling of internalized receptors, perhaps by altering interactions with scaffolding proteins and recruited signaling molecules, remains to be determined. However, the availability of agonist may not only determine the fate of the internalized receptor but also its signaling properties and biological actions.

Footnotes

This work was supported by grants from the National Institutes of Health: DK39957 (NWB), DK43207 (NWB), DK57840 (NWB) and DK52388 (EFG).

1The abbreviations used are: CLR, calcitonin receptor-like receptor; RAMP1, receptor activity-modifying protein; CGRP, calcitonin gene-related peptide; GPCR, G-protein coupled receptor; NK1R, neurokinin 1 receptor; β2AR, β2 adrenergic receptor; PAR2, protease-activated receptor 2; LAMP1, lysosomal-associated glycomembrane protein 1; EEA1, early endosomal antigen 1.

References

1. Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S., and Evans, R. M. (1982) *Nature* **298**(5871), 240-244
2. van Rossum, D., Hanisch, U. K., and Quirion, R. (1997) *Neurosci Biobehav Rev* **21**(5), 649-678
3. Brain, S. D., and Grant, A. D. (2004) *Physiol Rev* **84**(3), 903-934
4. Olesen, J., Diener, H. C., Husstedt, I. W., Goadsby, P. J., Hall, D., Meier, U., Pollentier, S., and Lesko, L. M. (2004) *N Engl J Med* **350**(11), 1104-1110
5. Njuki, F., Nicholl, C. G., Howard, A., Mak, J. C., Barnes, P. J., Girgis, S. I., and Legon, S. (1993) *Clin Sci (Lond)* **85**(4), 385-388
6. McLatchie, L. M., Fraser, N. J., Main, M. J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M. G., and Foord, S. M. (1998) *Nature* **393**(6683), 333-339
7. Kuwasako, K., Kitamura, K., Onitsuka, H., Uemura, T., Nagoshi, Y., Kato, J., and Eto, T. (2002) *FEBS Lett* **519**(1-3), 113-116
8. Kuwasako, K., Kitamura, K., Nagoshi, Y., Cao, Y. N., and Eto, T. (2003) *J Biol Chem* **278**(25), 22623-22630
9. Fraser, N. J., Wise, A., Brown, J., McLatchie, L. M., Main, M. J., and Foord, S. M. (1999) *Mol Pharmacol* **55**(6), 1054-1059
10. Christopoulos, G., Perry, K. J., Morfis, M., Tilakaratne, N., Gao, Y., Fraser, N. J., Main, M. J., Foord, S. M., and Sexton, P. M. (1999) *Mol Pharmacol* **56**(1), 235-242
11. Kuwasako, K., Shimakake, Y., Masuda, M., Nakahara, K., Yoshida, T., Kitaura, M., Kitamura, K., Eto, T., and Sakata, T. (2000) *J Biol Chem* **275**(38), 29602-29609
12. Hilairet, S., Belanger, C., Bertrand, J., Laperriere, A., Foord, S. M., and Bouvier, M. (2001) *J Biol Chem* **276**(45), 42182-42190
13. Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G., and Barak, L. S. (2000) *J Biol Chem* **275**(22), 17201-17210
14. Schmidlin, F., Roosterman, D., and Bunnett, N. W. (2003) *Am J Physiol Cell Physiol* **285**(4), C945-958
15. Shenoy, S. K., McDonald, P. H., Kohout, T. A., and Lefkowitz, R. J. (2001) *Science* **294**(5545), 1307-1313
16. Marchese, A., and Benovic, J. L. (2001) *J Biol Chem* **276**(49), 45509-45512
17. Marchese, A., Raiborg, C., Santini, F., Keen, J. H., Stenmark, H., and Benovic, J. L. (2003) *Dev Cell* **5**(5), 709-722
Figure 1. CGRP-induced trafficking of CLR (A, C) and RAMP1 (B, D) to early endosomes and lysosomes in HEK-CLR-RAMP1 cells. Cells were exposed to vehicle (no CGRP) or CGRP (10^{-7} M) for 10 or 120 min. Proteins were detected by indirect immunofluorescence using antibodies to the C-terminus of CLR (RK11) and RAMP1 (9891), and to EEA1 or LAMP1. A, B. In unstimulated cells, CLR and RAMP1 were at the plasma membrane (arrow heads). After 10 min with CGRP, CLR and RAMP1 colocalized with EEA1 in early endosomes (arrows). After 120 min, CLR and RAMP1 colocalized with EEA1 in some endosomes (white arrows), but were also detected in vesicles that did not contain EEA1 (yellow arrows). C, D. After 120 min with CGRP, CLR and RAMP1 colocalized with LAMP1 in some lysosomes (white arrows), but were also detected in vesicles that did not contain LAMP1 (yellow arrows). Scale bar = 10 µm.

Figure 2. Endocytosis and recycling of CLR and RAMP1 in HEK-CLR-RAMP1 cells. A. Cycloheximide-treated cells were exposed to vehicle (no CGRP) or CGRP (10^{-7} M) for 1 h, washed and recovered in CGRP-free medium for 2 or 6 h. Receptors were detected by indirect immunofluorescence using antibodies to the C-terminus of CLR (RK11) and the N-terminus of RAMP1 (Myc). In unstimulated cells, CLR and RAMP1 were at the plasma membrane (arrow heads). After 1 h with CGRP, CLR and RAMP1 colocalized in endosomes (arrows). After 2-6 h recovery, CLR and RAMP1 were detected at the plasma membrane (arrow heads). B. Recycling of antibody-tagged receptors. Living cells were labeled with antibodies to extracellular N-terminal epitopes of CLR (HA) and RAMP1 (Myc). Cells were exposed to vehicle (no CGRP) or CGRP (10^{-7} M) for 1 h, washed and incubated in CGRP-free medium for 2 or 6 h. Cells were fixed, permeabilized and incubated with fluorescent secondary antibodies to detect CLR and RAMP1. In unstimulated cells, CLR and RAMP1 were at the cell surface (arrow heads). CGRP induced endocytosis of CLR and RAMP1 into the same endosomes (arrows). After 2 or 6 h, CLR and RAMP1 had recycled to the cell-surface (arrow heads). Scale bar = 10 µm.

Figure 3. Desensitization and resensitization of CGRP-induced mobilization of Ca^{2+} ions. HEK-CLR-RAMP1 cells were incubated with CGRP (10^{-7} M) or vehicle (control, Con.) for 1 h, washed, and incubated in CGRP-free medium for 0-8 h. At the indicated times, cells were challenged with CGRP (3x10^{-8} M) and [Ca^{2+}], was measured. A. Time course of resensitization. CGRP signaling returned to control levels after 6-8 h. B. Effects of cycloheximide (CHX), which did not prevent resensitization at 8 h. C. Effects of bafilomycin A1, which abolished resensitization at 2 h. *p<0.05 compared to vehicle control. D, E. Effects of overexpression in HEK-CLR-RAMP1 cells of vector control (empty vector), wild-type Rab4a, Rab4aS22N, wild-type Rab11a or Rab11aS25N. Overexpression of wild-type Rab4a or Rab11a did not affect resensitization at 8 h, whereas overexpression of Rab4aS22N or Rab11aS25N inhibited resensitization by >20% and >30%, respectively. *p<0.05 compared to CGRP-treated cells expressing wild-type Rabs or vector control.

Figure 4. Role of Rab-GTPases in CGRP-induced trafficking of CLR and RAMP1 in HEK-CLR-RAMP1 cells. A. Cells transiently transfected with dominant negative Rab4aS22N-GFP or Rab11aS25N-GFP were incubated with CGRP (10^{-7} M) for 30 min, fixed and CLR was detected by indirect immunofluorescence using antibody to the C-terminus (RK11). CGRP induced endocytosis of CLR into
EEA1 containing early endosomes in cells expressing mutants Rabs (arrows). B, C. Trafficking of antibody-tagged receptors. Living cells were labeled with antibodies to extracellular N-terminal epitopes of CLR (HA) and RAMP1 (Myc). Cells expressing wild type Rab4a-GFP (B) or Rab11a-GFP (C) were exposed to vehicle (no CGRP) or CGRP (10^{-7} M) for 1 h, washed and incubated in CGRP-free medium for 4 h. Cells were fixed, permeabilized and incubated with fluorescent secondary antibodies to detect CLR and RAMP1. In unstimulated cells, CLR and RAMP1 were present at the cell surface (arrow heads) and Rab4a and Rab11a were present in intracellular vesicles (arrows). After recovery for 4 h, CLR and RAMP1 were detected in some Rab 4a- and Rab11a-positive vesicles (arrows). Scale bar = 10 μm.

**Figure 5.** Effects of CGRP on levels of CLR and RAMP1 in HEK-CLR RAMP1 cells. Cells were incubated with vehicle (Veh) or CGRP (10^{-7} M) for the indicated times, and blots were probed for CLR (using C-terminal antibody RK11), RAMP1 (using C-terminal antibody 9891) and β-actin. Upper panels are representative blots of n=3 experiments and lower panels are densitometric analyses of CLR:β-actin or RAMP1:β-actin (means of n≥3 experiments). All cells were treated with cycloheximide for the duration of the experiment. A. CLR was not degraded in the continued presence of CGRP from 0-5 h. B. After incubation with CGRP for 8 h, there was minimal degradation of CLR, but after 16 h there was >50% degradation of CLR. C. RAMP1 was degraded in the continued presence of CGRP for 5 h. *p<0.05 compared to vehicle.

**Figure 6.** Effects of protease inhibitors on degradation of CLR and RAMP1 in HEK-CLR RAMP1 cells. Cells were incubated with vehicle (Veh) or CGRP (10^{-7} M) for the indicated times, and blots were probed for CLR (using C-terminal antibody RK11), RAMP1 (using C-terminal antibody 9891) and β-actin. Upper panels are representative blots of n≥3 experiments, and lower panels are densitometric analyses of the CLR:β-actin or RAMP1:β-actin (means of n≥3 experiments). All cells were treated with cycloheximide for the duration of the experiment. A. Lysosomal inhibitors (LI: ZPAD+E64d+pepsstatin A) inhibited degradation of CLR after 16 h with CGRP, but the proteasomal inhibitor epoxomicin (EP) and vehicle (DMSO) had no effect. B. Lysosomal inhibitors inhibited degradation of RAMP1 after 5 h with CGRP, but the proteasomal inhibitor epoxomicin and vehicle had no effect. *p<0.05 compared to vehicle.

**Figure 7.** Endocytosis, lysosomal targeting and degradation of CLR and RAMP1 in HEK-CLR-RAMP1 cells. Cycloheximide-treated cells were pre-incubated with lysosomal inhibitors (Lys. inhib., ZPAD+E64d+pepsstatin A) or vehicle (DMSO), and were unstimulated (no CGRP) or incubated with CGRP (10^{-7} M) for 8 or 16 h. Proteins were detected by indirect immunofluorescence using antibodies to the C-terminus of CLR (RK11), an N-terminal epitope of RAMP1 (Myc), and LAMP1. In unstimulated cells, CLR and RAMP1 were at the cell surface (arrow heads). CGRP induced endocytosis of CLR and RAMP1. In the absence of protease inhibitors, immunoreactive RAMP1 was diminished at 8 and 16 h, suggesting degradation. Lysosomal inhibitors preserved RAMP1. In contrast, levels of immunoreactive CLR were minimally reduced after incubation with CGRP for 8 or 16 h, even in the absence of protease inhibitors. Both CLR and RAMP1 were detected in LAMP1-positive lysosomes (arrows). Scale bar = 10 μm.

**Figure 8.** Effects of agonists on ubiquitination of CLR, RAMP1 and NK1R. HEK-CLR-RAMP1 cells (A, B), HEK-NK1R cells (C) or HEK cells expressing vector without insert (vector control, vc) were incubated with CGRP (10^{-7} M) or substance P (SP) for the indicated times. A. CLR was immunoprecipitated (IP) using an antibody to the HA epitope and Western blots (WB) were probed for ubiquitin (Ub) using antibody P4D1 or CLR using antibody RK11. B. RAMP1 was immunoprecipitated using an antibody to the Myc epitope and Western blots were probed for ubiquitin using antibody P4D1 or RAMP1 using antibody 9891. Although the ubiquitin antibody detected ubiquitinated proteins in a whole cell lysate, immunoprecipitated CLR and RAMP1 were not detectably ubiquitinated. There were no signals for CLR and RAMP1 in vector control cells, confirming specificity of detection. C. NK1R was immunoprecipitated using an antibody to the Flag epitope and Western blots probed for ubiquitin using antibody P4D1 or NK1R using antibody 94168. Substance P induced NK1R ubiquitination after 3-5 h, as we have previously shown (19). Representative blots of n≥3 experiments are shown.
**Figure 9.** CGRP-induced trafficking and degradation of RAMP1 and RAMP1K142R. **A.** HEK cells transiently expressing CLR and RAMP1 or CLR and RAMP1K142R were exposed to vehicle (no CGRP) or CGRP ($10^{-7}$ M) for 30 min. Receptors were detected by indirect immunofluorescence using antibodies to an N-terminal epitope of RAMP1 (Myc) and the C-terminus of CLR (RK11). In unstimulated cells, RAMP1 and RAMP1K142R were at the cell surface with CLR (arrow heads). After 30 min with CGRP, both RAMP1 and RAMP1K142R were in endosomes with CLR (arrows). **B.** HEK cells expressing CLR and RAMP1 or CLR and RAMP1K142R were incubated with vehicle (Veh) or CGRP ($10^{-7}$ M) for 5 h, and blots were probed for RAMP1 and β-actin. Upper panels are representative blots of n≥3 experiments and the lower panel is a densitometric analysis of the RAMP1:β-actin (means of n≥3 experiments). All cells were treated with cycloheximide. Both RAMP1 and RAMP1K142R were degraded in the continued presence of CGRP for 5 h. *p<0.05 compared to vehicle. Scale bar = 10 µm.

**Figure 10.** Endocytosis and recycling of antibody-tagged CLR and RAMP1 in SK-N-MC cells. Living cells were labeled with antibodies to extracellular N-terminal epitopes of CLR (HA) and RAMP1 (Myc). Cells were exposed to vehicle (no CGRP) or CGRP ($10^{-7}$ M) for 1 h, washed and incubated in CGRP-free medium for 2 or 6 h. Cells were fixed, permeabilized and incubated with fluorescent secondary antibodies to detect CLR and RAMP1. In unstimulated cells, CLR and RAMP1 were at the cell surface (arrow heads). CGRP induced endocytosis of CLR and RAMP1 into the same endosomes (arrows). After 2 or 6 h, CLR and RAMP1 had recycled to the cell surface (arrow heads). Scale bar = 10 µm.

**Figure 11.** Endocytosis, lysosomal targeting and degradation of CLR and RAMP1 in SK-N-MC cells. Cycloheximide-treated cells were pre-incubated with lysosomal inhibitors (Lys. inhib., ZPAD+E64d+pepsatin A) or vehicle (DMSO), and were unstimulated (no CGRP) or incubated with CGRP ($10^{-7}$ M) for 8 h. Proteins were detected by indirect immunofluorescence using antibodies to the C-terminus of CLR (RK11), an N-terminal epitope of RAMP1 (Myc), and LAMP1. In unstimulated cells, CLR and RAMP1 were at the cell surface (arrow heads). CGRP induced endocytosis of CLR and RAMP1. In the absence of protease inhibitors, immunoreactive RAMP1 was markedly reduced and levels of immunoreactive CLR were minimally diminished after 8 h with CGRP. Lysosomal inhibitors preserved RAMP1. Both CLR and RAMP1 were detected in LAMP1-positive lysosomes (arrows). Scale bar = 10 µm.
Figure 1
Figure 2

A

CLR (RK11) RAMP1 (Myc) Merge

No CGRP

CGRP 10^{-7} M, 1 h

Wash

Recovery 2 h

Recovery 6 h

B

Antibody-tagged receptors

CLR (HA11) RAMP1 (Myc) Merge

No CGRP

CGRP 10^{-7} M, 1 h

Wash

Recovery 2 h

Recovery 6 h
Figure 3
Figure 4
Figure 5

Time of incubation with CGRP, 10^{-7} M or vehicle (h)

A

B

C

WB: CLR (RK11)

CLR

β-actin

WB: RAMP1 (9891)

RAMP1

Time of incubation with CGRP, 10^{-7} M or vehicle (h)
Figure 6
Figure 7
Figure 8
Figure 10
Figure 11
Post-endocytic sorting of calcitonin receptor-like receptor and receptor activity-modifying protein 1
Graeme S. Cottrell, Benjamin Padilla, Stella Pikios, Dirk Roosterman, Martin Steinhoff, Eileen F. Grady and Nigel W. Bunnett

*J. Biol. Chem.* published online February 19, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M606338200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts