UBIQUITIN-DEPENDENT DOWN-REGULATION OF THE NEUROKININ-1 RECEPTOR
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Transient stimulation with substance P (SP) induces endocytosis and recycling of the neurokinin-1 receptor (NK1R). The effects of sustained stimulation by high concentrations of SP on NK1R trafficking and Ca2+ signaling, as may occur during chronic inflammation and pain, are unknown. Chronic exposure to SP (100 nM, 3 h) completely desensitized Ca2+ signaling by NK1R wild type (NK1Rwt). Resensitization occurred after 16 h, and cycloheximide prevented resensitization, implicating new receptor synthesis. Lysine ubiquitination of G-protein coupled receptors is a signal for their trafficking and degradation. Lysine-deficient mutant receptors (NK1RΔ5K/R, C-terminal tail lysines; NK1RΔ10K/R, all intracellular lysines) were expressed at the plasma membrane and were functional because they responded to SP by endocytosis and by mobilization of Ca2+ ions. SP desensitized NK1Rwt, NK1RΔ5K/R and NK1RΔ10K/R. However, NK1RΔ5K/R and NK1RΔ10K/R resensitized 4 to 8-fold faster than NK1Rwt by cycloheximide-independent mechanisms. NK1RΔ325 (naturally occurring truncated variant) showed incomplete desensitization followed by a marked sensitization of signaling. By labeling receptors in living cells using antibodies to extracellular epitopes, we observed that SP induced endocytosis of NK1Rwt, NK1RΔ5K/R and NK1RΔ10K/R. After 4 h in SP-free medium, NK1RΔ5K/R and NK1RΔ10K/R recycled to the plasma membrane, whereas NK1Rwt remained internalized. SP induced ubiquitination of NK1Rwt and NK1RΔ5K/R, determined by immunoprecipitation under non-denaturing and denaturing conditions and detected with antibodies to mono- and poly-ubiquitin. NK1RΔ10K/R was not ubiquitinated. Whereas SP induced degradation of NK1Rwt, NK1RΔ5K/R and NK1RΔ10K/R showed ~50% diminished degradation. Thus, chronic stimulation with SP induces ubiquitination of the NK1R, which mediates its degradation and down-regulation.

Agonist-induced trafficking of G-protein coupled receptors (GPCRs) between the plasma membrane and organelles determines cellular responsiveness. The molecular mechanisms of receptor desensitization have been thoroughly investigated. Many activated GPCRs exemplified by the angiotensin II type 1A receptor (AT1AR), β2 adrenergic receptor (β2AR), neurokinin-1 receptor (NK1R) and protease-activated receptors 1 and 2 (PAR1, PAR2) are phosphorylated by G-protein receptor kinases (1-5). This phosphorylation increases the affinity of receptors for β-arrestins, which translocate to the receptors at the plasma membrane (3,6-10). β-arrestins a) sterically hinder the interaction between GPCRs and heterotrimeric G-proteins to desensitize G-protein signaling (3,11,12); b) are adaptor proteins for clathrin and AP-2 and are thus required for endocytosis of GPCRs (3,7,12,13); and c) serve as molecular scaffolds for the formation of signaling modules that include components of the mitogen-activated protein kinase cascade such as Sre, Raf-1, MEKK and activated extracellular signal regulated kinases 1 and 2 (14-17). However, in comparison to endocytosis, much less is known about the molecular mechanisms of post-endocytic sorting of GPCRs back to the plasma membrane (recycling) or to lysosomes or proteasomes (down-regulation).
The nature of interaction between GPCRs and β-arrestins determines the rate of receptor recycling. GPCRs can be divided into two classes according to their interaction with β-arrestins (18). “Class A” receptors (e.g. β2AR, μ-opioid receptor, α1b-adrenergic receptor, and neurokinin-3 receptor) show a preference for β-arrestin 2 over β-arrestin 1, and interact with low affinity and transiently with β-arrestin 2, to rapidly dissociate and recycle (18-20). “Class B” receptors (e.g. NK1R, AT1AR and neurotensin receptor-1) form high affinity and sustained interactions with both isoforms of β-arrestin, and then slowly recycle to the plasma membrane (18). The existence of Ser and Thr residues within the C-terminal domains of these receptors, which are sites for phosphorylation by G-protein receptor kinases, specifies high affinity interactions with β-arrestins and therefore determines the rates of recycling (21,22). The concentration of agonist can also markedly influence the rate of receptor recycling, possibly by affecting the extent of receptor phosphorylation. For example, trafficking of the NK1R is markedly influenced by the concentration of its agonist, substance P (SP). After stimulation with low concentrations (<1 nM) of SP, the NK1R is minimally phosphorylated, internalizes into endosomes immediately beneath the plasma membrane, and rapidly dissociates from β-arrestins to recycle by Rab4a- and Rab11a-dependent mechanisms (23-25). In contrast, higher concentrations (>10 nM) of SP induce a rapid and extensive phosphorylation of NK1R, internalization into early endosomes in a perinuclear location, and association with β-arrestins for prolonged periods (8). Dephosphorylation of the NK1R and its dissociation from agonist and β-arrestins must occur before the NK1R slowly recycles (25,26).

The ubiquitination of cell-surface receptors and associated proteins, such as β-arrestins, has emerged as an important step in endocytic and post-endocytic sorting of these proteins (27,28). Ubiquitin, a protein of 76 amino acids, is attached to lysine residues of target proteins by a mechanism that is catalyzed by three enzymes. The ubiquitin-activating enzyme, E1, uses ATP to “charge” the ubiquitin molecule to form a ubiquitin C-terminal adenylyl. The activated ubiquitin molecule is then passed to the ubiquitin-conjugating enzymes, E2. Finally, although not in all cases, E3 ubiquitin-ligases bind to the target protein and attach the C-terminus of ubiquitin to lysine residues of the target. Although most species express a single E1, larger numbers of E2 and E3 enzymes provide specificity and scope for regulation. Several GPCRs are ubiquitinated. In yeast, the agonists of the α-mating factor receptors, Ste2p and Ste3p, induce their ubiquitination, a step that is required for internalization and trafficking (29,30). In mammals, agonists induce ubiquitination of β2AR (31), chemokine (C-X-C motif) receptor 4 (CXCR4) (32) and PAR2 (33). However, this ubiquitination is necessary for post-endocytic sorting and degradation rather than for endocytosis. Lysine-deficient mutants of the β2AR (31), δ-opioid (34), CXCR4 (32) and PAR2 (33) all still internalize normally, but the trafficking of these receptors is altered. Nothing is known about the role of ubiquitination in trafficking of the NK1R.

The purpose of the present experiments was to investigate the effects of chronic stimulation by agonists on trafficking and signaling of the NK1R, and to determine the contributions of ubiquitination to these processes. The NK1R is a “class B” receptor that is not normally targeted to the degradative pathway and efficiently recycles, although the time for resensitization of signaling depends upon the concentration of agonist used (25). We hypothesized that sustained exposure of the NK1R to high concentrations of SP would down-regulate the receptor due to ubiquitination of intracellular lysine residues. To assess down-regulation, we examined the effects of chronic stimulation with SP on Ca2+ signaling, trafficking, degradation and ubiquitination of the NK1R. To establish the role of intracellular lysine residues and ubiquitin, we engineered mutant receptors lacking either lysine residues in the C-terminal tail or entire intracellular face of the receptor, and generated a chimeric molecule of the NK1R conjugated to ubiquitin.

Experimental Procedures

Reagents. Antibodies were from the following sources: rabbit NK1R 94168 (35); mouse ubiquitin (P4D1) from Santa Cruz Biotechnology (Santa Cruz, CA); mouse IgM polyclubitin (FK1) from Affiniti (Exeter, UK); high affinity rat HA.11 from Roche...
Molecular Biochemicals (Indianapolis, IN); mouse Flag M2, rabbit Flag, mouse β-actin and rabbit γ-tubulin from Sigma Chemical Co. (St. Louis, MO); goat anti-mouse or anti-rat IgG conjugated to FITC or Rhodamine Red-X from Jackson ImmunoResearch Laboratories (West Grove, PA); goat anti-mouse IgM and goat-anti-rabbit IgG conjugated to AlexaFluor680 from Invitrogen (Carlsbad, CA); goat anti-mouse IR Dye™ 800 from Rockland Immunochemicals (Gilbertsville, PA). An AlexaFluor594 labeling kit was from Invitrogen. SP and [Sar²MeO²¹]—SP (SM-SP) were from Bachem (Torrance, CA). SM-SP was labeled with AlexaFluor594 as described (9). Oligonucleotides were from Sigma-Genosys (Woodlands, TX). Restriction enzymes and T4 DNA ligase were from New England Biolabs (Ipswich, MA).

Vector construction. cDNA encoding rat NK₁R with N-terminal Flag was subcloned into pcDNA5/FRT (Invitrogen) (36). NK₁R conjugated to an ubiquitin molecule (NK₁R-Ub) was created by cloning the NK₁R wt cDNA in-frame to ubiquitin (Fig. 1). Briefly, ubiquitin was cloned into pcDNA5/FRT with an upstream Eco RV site, followed by addition of NK₁R wt lacking a stop codon. Lysine-deficient mutants of the NK₁R, in which all predicted C-terminal tail lysines (designated NK₁RΔ5K/R) or all intracellular lysines (NK₁RΔ10K/R) were replaced with arginines, were generated using the QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) (Fig. 1). Point mutations of one, two or three lysine to arginine residues in the C-terminal tail of the NK₁R were similarly generated (Fig. 1). For NK₁RΔ10K/R, the N-terminal Flag was exchanged with HA.11 epitope using PCR. Primer sequences are available on request. All constructs were sequenced.

Cell lines. Sarcoma virus transformed rat kidney epithelial cells (KNRK, American Tissue Type Collection, Rockville, MD) were maintained in DMEM containing 10% HIFBS in 95% air, 5% CO₂ at 37°C (36). KNRK-FLP cells (created with the Flp-In™ system according to the manufacturer’s guidelines (33)) were maintained in medium supplemented with 100 µg/ml zeocin. Stable cell lines were created by co-transfection with pcDNA5/FRT and pOG44, a vector that transiently expresses the Flp recombinase (Invitrogen). Cells were placed in medium containing 300 µg/ml hygromycin B. After 7-10 days, viable cells were pooled and gene expression was assessed by Western blotting, immunofluorescence and Ca²⁺ signaling. Cells were maintained in 150 µg/ml hygromycin B. KNRK cells expressing a truncated mutant of the NK₁R corresponding to a natural variant, KNRK-NK₁RΔ325 were created and maintained as previously described (37,38). In some experiments, KNRK cells were transiently transfected using Lipofectamine™ according to the manufacturer’s guidelines.

Activation of NK₁R and drug treatments. Cells were incubated with vehicle (control), or 100 nM SP or SM-SP (unless otherwise stated) in OptiMEM. To inhibit new protein synthesis, cells were preincubated with cycloheximide (140 µM). The proteolytic activity of the proteasome was inhibited using epoxomicin (10 µM). Intracellular Ca²⁺ release was stimulated by thapsigargin (1 µM). All inhibitors were added for 1 h prior to stimulation with SP or SM-SP.

SDS PAGE and Western blotting. Cells were lysed in 50 mM Tris/HCl pH 7.4, 1% SDS, boiled and centrifuged. Lysates (10 µg) were separated by SDS-PAGE (8 or 10% acrylamide), transferred to PVDF membrane (Immobilon FL, Millipore), and incubated with blocking buffer (LiCOR, Lincoln, NE). Membranes were incubated with primary antibodies in blocking buffer: ubiquitin P4D1, 1:5,000; ubiquitin FK1, 1:1,000; NK₁R 94168, 1:5,000; Flag polyclonal, 1:5,000; β-actin, 1:20,000 and γ-tubulin, 1:10,000; all overnight at 4°C. Membranes were washed and then incubated with secondary antibodies coupled to either AlexaFluor680 or IR Dye™ 800 (1:10-20,000, 1 hr, room temperature). Immunoreactive proteins were viewed using Odyssey Infrared Imaging System (LiCOR). To deglycosylate the NK₁R, a sonicated lysate was treated with the enzymes PNGase F (New England Biolabs), O-glycosidase, sialidase, β-galactosidase and glucosaminidase (QAbio, San Mateo, CA) in 50 mM sodium phosphate, pH 7.5, overnight at 37°C. Membranes were prepared by ultracentrifugation and analyzed by Western blotting.

Immunoprecipitation. For denaturing immunoprecipitation, cells were lysed in 100 µl of
50 mM Tris/HCl pH 7.4, 1% SDS, sonicated and mixed with 4 volumes of RIPA buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 5 mM MgCl2, 10 mM NaF, 0.1 mM NaVO4, 10 mM Na2P2O7, 1 mM EGTA, 0.5% NP40). The lysate was mixed by pipetting, RIPA was added to 1 ml final volume and centrifuged (16,000 g, 20 min, 4°C). For non-denaturing immunoprecipitation, cells were lysed in 1 ml RIPA for 30 min at 4°C, scraped and centrifuged. Supernatants from denaturing and non-denaturing preparations were rotated with antibodies (FLAG M2, 3.5 µg/ml or HA.11, 250 ng/ml) overnight at 4°C. Protein A/G PLUS (Santa Cruz Biotechnology) was added (30 µl) and samples were incubated for 2 h at 4°C. Immunoprecipitates were pelleted, washed three times with 1 ml RIPA, boiled in Laemelli buffer with β-mercaptoethanol and analyzed by Western blotting.

**Densitometry.** Signals on Western blots were quantified using Odyssey Infrared Imaging System (LI-COR). To quantify NK1R degradation, NK1R signals were compared to β-actin and γ-tubulin signals. To quantify ubiquitination, ubiquitinated NK1R signals were compared to total NK1R signals.

**Fluorescence microscopy.** To localize the NK1R, cells were fixed with 4% paraformaldehyde in 100 mM PBS, pH 7.4 (20 min, 4°C), washed and incubated with primary antibodies in PBS containing 0.1% saponin and 1% goat serum: NK1R 94168 or Flag M2 (1:500, overnight, 4°C). Cells were washed and incubated with secondary antibodies coupled to FITC or Rhodamine Red-X (1:200, 2 h, room temperature). To examine NK1R recycling, live cells were incubated with antibodies to the extracellular epitopes (Flag M2 for NK1Rwt and NK1RΔ5K/R or HA.11 for NK1RΔ10K/R; both 1:100, 2 h, 4°C). Cells were washed and incubated with SP or vehicle for 3 h, washed and incubated in SP-free medium for 4 h. Cells were washed in PBS, fixed in paraformaldehyde, and incubated with secondary antibody as described. To examine binding and internalization of fluorescently tagged SM-SP, cells were incubated with Alexa-SM-SP for 1 h at 4°C, washed, incubated at 37°C for 30 min and fixed. Cells were observed by using a Zeiss Axiovert and BioRad MRC1000 confocal microscopes with Zeiss Plan Apo x100 (NA 1.3) 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 were colored to represent the appropriate fluorophores and processed to adjust contrast and brightness using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA).

**Measurement of [Ca2+]i.** 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.

**Statistics.** Results are presented as the mean and standard error of ≥3 experiments and are compared by ANOVA and Student’s Newman-Kuels (multiple comparisons) or Student’s t-test (2 comparisons), with *p<0.05 considered significant.

**Results**

NK1Rwt, NK1RΔ5K/R, NK1RΔ10K/R and NK1RΔ325 are expressed at the plasma membrane and internalize after binding SP. The ubiquitination of intracellular lysine residues is required for agonist-induced trafficking of certain GPCRs (29-33,39). To examine the importance of ubiquitination for trafficking and regulation of the NK1R, we generated a chimeric receptor, composed of wild-type receptor conjugated to an ubiquitin molecule (NK1R-Ub), and mutant receptors that lacked all lysine residues in the C-terminal tail (NK1RΔ5K/R) or on the entire intracellular face of the NK1R (NK1RΔ10K/R) (Fig. 1). We also studied a truncated form of the NK1R (NK1RΔ325) that lacks 83 residues of the C-terminal tail, including 4 lysines, and which may correspond to a naturally occurring variant of this receptor (40-42).

To verify that the chimeric, mutant and truncated NK1Rs were appropriately localized at the plasma membrane of unstimulated cells, we used immunofluorescence. Using antibodies to the C-terminus of the NK1R (94168) (NK1Rwt and lysine mutants) or the extracellular Flag epitope tag (NK1RΔ325), we detected NK1Rwt, NK1RΔ5K/R, NK1RΔ10K/R and NK1RΔ325 at the plasma
membrane (Fig. 2). Thus, mutation of lysine residues or truncation of the C-terminal tail does not affect the targeting of the receptors to the plasma membrane. In contrast, NK$_1$R-Ub (detected using the 94168 antibody) did not traffic to the plasma membrane but was instead localized in an unidentified subcellular compartment (Fig. 2). This receptor was not studied further.

To determine if agonists induce endocytosis of the wild-type and lysine-deficient receptors, we incubated cells with Alexa-SM-SP. When cells were incubated at 4°C, Alexa-SM-SP was confined to the plasma membrane of cells expressing NK$_1$Rwt, NK$_1$RΔ5K/R or NK$_1$RΔ10K/R (Fig. 3). After 30 min at 37°C, Alexa-SM-SP was completely depleted from the plasma membrane and was detected in endosomes in cells expressing NK$_1$Rwt, NK$_1$RΔ5K/R or NK$_1$RΔ10K/R. An inhibitor of proteasomal proteolysis, MG132, inhibits agonist-stimulated endocytosis of the β$_2$-AR (31). Therefore, we examined the effect of epoxomicin, a selective proteasome inhibitor, on endocytosis of Alexa-SM-SP. Epoxomicin had no effect on endocytosis of Alexa-SM-SP in cells expressing NK$_1$Rwt, NK$_1$RΔ5K/R or NK$_1$RΔ10K/R (Fig. 3). Thus, mutation of the intracellular lysine residues and inhibition of the proteasome does not affect binding or internalization of SP. We have previously reported that although SP stimulates endocytosis of NK$_1$RΔ325, it does so at a reduced rate due to impaired phosphorylation of this receptor and diminished interaction with β-arrestins (15,38,43). Therefore, we did not further examine the trafficking of this variant in the current study.

**Intracellular lysine residues prevent rapid recycling of endocytosed NK$_1$R.** Although ubiquitination of lysines is not required for endocytosis of GPCRs such as CXCR4 or PAR$_3$, it is required for the post-endocytic trafficking of these receptors to lysosomes (32,33,39). To examine the role of lysine residues in the post-endocytic sorting of the NK$_1$R, we incubated living cells expressing NK$_1$Rwt, NK$_1$RΔ5K/R or NK$_1$RΔ10K/R with antibodies to the extracellular epitope tags, and localized the receptors after stimulating them with SP. When cells expressing NK$_1$Rwt, NK$_1$RΔ5K/R or NK$_1$RΔ10K/R were incubated with antibodies at 4°C and immediately fixed, receptors were mostly detected at the plasma membrane (Fig. 4). When cells were incubated with vehicle for 3 h, receptors were also mostly confined to the plasma membrane, although some receptors were clustered at the cell-surface and in endosomes, suggesting either constitutive trafficking of receptors or an effect of antibody binding. When cells were incubated with SP for 3 h, NK$_1$Rwt, NK$_1$RΔ5K/R or NK$_1$RΔ10K/R were depleted from the plasma membrane and present in endosomes in a perinuclear region of the cell. After agonist washout and 4 h of recovery in SP-free medium, NK$_1$Rwt was still present in the perinuclear vesicles, whereas NK$_1$RΔ5K/R and NK$_1$RΔ10K/R were detected at the plasma membrane, which is indicative of receptor recycling (Fig. 4). Thus, lysine residues prevent rapid recycling of the NK$_1$Rwt after prolonged stimulation with SP.

**Intracellular lysine residues promote down-regulation of SP signaling.** To establish the role of the intracellular lysine residues or the C-terminal tail in the desensitization and resensitization of SP signaling, we incubated cells expressing either NK$_1$Rwt, NK$_1$RΔ5K/R, NK$_1$RΔ10K/R or NK$_1$RΔ325 with SP (100 nM) or vehicle (control) for 3 h, washed the cells, and then measured the effect of SP (10 nM) on [Ca$^{2+}$]i at 0-16 h after washing. Vehicle-treated control cells expressing NK$_1$Rwt, NK$_1$RΔ5K/R or NK$_1$RΔ10K/R responded similarly to SP, indicating that mutation of intracellular lysine residues does not affect receptor coupling to Ca$^{2+}$ signaling (Fig. 5A). The response to SP of vehicle-treated cells expressing NK$_1$RΔ325 was lower than in other cell lines, which probably reflects the reduced level of expression in this line as reflected by diminished cell-surface detection of immunoreactive receptor (Fig. 2). Immediately after the 3 h stimulation with SP, cells expressing NK$_1$Rwt, NK$_1$RΔ5K/R or NK$_1$RΔ10K/R were unresponsive to SP, indicating complete desensitization of these receptors (Fig. 5A). Thus, intracellular lysine residues do not affect desensitization of SP-induced increases in [Ca$^{2+}$]. However, when cells expressing NK$_1$RΔ325 were incubated with SP for 3 h and then immediately challenged, SP responses were 43±6% of control values (100%), indicating only partial
desensitization of the truncated receptor. This result is consistent with other reports of diminished desensitization of NK\textsubscript{1}RΔ325 (44) that relates to its impaired interaction with β-arrestins (15). To determine if the desensitization of Ca\textsuperscript{2+} signaling of NK\textsubscript{1}R was attributable to depletion of intracellular stores of Ca\textsuperscript{2+} ions, we challenged cells with thapsigargin. Thapsigargin (1 μM) stimulated a similar increase in [Ca\textsuperscript{2+}], in cells exposed to SP or vehicle for 3 h, suggesting that intracellular Ca\textsuperscript{2+} stores are intact (results not shown).

In cells expressing NK\textsubscript{1}Rwt, incubation with SP for 3 h resulted in a sustained desensitization of SP-induced increases in [Ca\textsuperscript{2+}], that did not fully resensitize to control levels until 16 h after washing (Fig. 5B). Resensitization was biphasic, and may thus be mediated by several processes. In marked contrast to the slow resensitization of NK\textsubscript{1}Rwt, in cells expressing NK\textsubscript{1}RΔ325 (Δ10K/R), responses were fully resensitized after 4 h, and in cells expressing NK\textsubscript{1}RΔ10K/R, responses were fully resensitized after 2 h (Fig. 5B). Thus, lysine residues are required for the sustained down-regulation of SP-induced Ca\textsuperscript{2+} signaling by the NK\textsubscript{1}Rwt after prolonged stimulation with SP. In cells expressing NK\textsubscript{1}RΔ325 (which did not completely desensitize), responses to SP resensitized after 30 min to 174±11% and after 1 h to 238±15% of the control values (100%) (Fig. 5B). Thus, lack of the C-terminal tail of the NK\textsubscript{1}R not only diminishes desensitization, but also sensitizes responses to subsequent challenge with SP.

To assess whether the recovery of SP signaling was due to receptor recycling or receptor synthesis, we incubated cells with cycloheximide to prevent new protein synthesis. In cells expressing NK\textsubscript{1}Rwt, cycloheximide strongly inhibited resensitization of responses to SP measured at 16 h (13±2% of control values, Fig. 5C). In cells expressing NK\textsubscript{1}RΔ5K/R or NK\textsubscript{1}RΔ10K/R, cycloheximide had minimal effect on resensitization of responses to SP measured at 4 h (NK\textsubscript{1}RΔ5K/R, 76±3%; NK\textsubscript{1}RΔ10K/R, 71±13% of control values (100%), Fig. 5C). Thus, resensitization of SP signaling in cells expressing NK\textsubscript{1}Rwt requires new protein synthesis, whereas resensitization in cells expressing NK\textsubscript{1}RΔ5K/R and NK\textsubscript{1}RΔ10K/R does not.

Intracellular lysine residues promote SP-induced degradation of the NK\textsubscript{1}Rwt. The observations that sustained incubation with SP resulted in long-lasting down-regulation of the NK\textsubscript{1}Rwt and that cycloheximide prevented this resensitization suggest that the NK\textsubscript{1}R is degraded after chronic stimulation. To examine the effects of SP on the levels of NK\textsubscript{1}R, we exposed cells expressing NK\textsubscript{1}Rwt, NK\textsubscript{1}RΔ5K/R and NK\textsubscript{1}RΔ10K/R to vehicle or SP for 16 h in the presence and absence of cycloheximide and analyzed total receptor levels using Western blotting. In vehicle-treated cells, NK\textsubscript{1}Rwt, NK\textsubscript{1}RΔ5K/R and NK\textsubscript{1}RΔ10K/R were detected as broad bands of immunoreactivity of apparent molecular masses of ~75 to 110 kDa (Fig. 6A, B, C). There were no detectable signals from analysis of lysates from untransfected KNRK cells, indicating specificity (Fig. 6A, B, C). Treatment of cell lysates with PNGase F, O-glycosidase, sialidase, β-galactosidase and glucosaminidase reduced the apparent molecular mass to ~46 kDa (Fig. 6D). Thus, the broad immunoreactive band represents glycosylated NK\textsubscript{1}R. In the absence of cycloheximide, incubation with SP for 16 h resulted in a ~3-4-fold increase in levels of NK\textsubscript{1}Rwt, NK\textsubscript{1}RΔ5K/R and NK\textsubscript{1}RΔ10K/R relative to levels of γ-tubulin or β-actin when compared by densitometry (Fig. 6A, B, C, E). However, when cycloheximide-treated cells were incubated with SP for 16 h, levels of NK\textsubscript{1}Rwt were reduced by 40-50%, whereas levels of NK\textsubscript{1}RΔ5K/R and NK\textsubscript{1}RΔ10K/R were only reduced by 30-35% and 20-25% respectively (Fig. 6A, B, C, F). Thus, lysine residues promote SP-induced degradation of the NK\textsubscript{1}R.

SP induces a gradual increase in the ubiquitination of the NK\textsubscript{1}R. Addition of ubiquitin molecules to intracellular lysine residues of GPCRs can mediate agonist-induced trafficking and degradation of receptors (29-33,39). To determine whether prolonged incubation with SP caused ubiquitination of the NK\textsubscript{1}R, cells expressing NK\textsubscript{1}Rwt, NK\textsubscript{1}RΔ5K/R or NK\textsubscript{1}RΔ10K/R were incubated with SP for 0-5 h. The receptor was immunoprecipitated under non-denaturing or denaturing conditions, and Western blots were probed for ubiquitin and the NK\textsubscript{1}R.

When analyzed under non-denaturing conditions, in cells expressing NK\textsubscript{1}Rwt that were not exposed to SP, there were minimal levels of
ubiquitin detected with an antibody (P4D1) that interacts with mono- and poly-ubiquitinated proteins (Fig. 7A, B). Incubation with SP for 1, 3 or 5 h increased the levels of ubiquitinated proteins within the precipitated NK₁R-protein complex by ~6-fold over basal at 5 h (Fig. 7A, B). In contrast, no ubiquitinated proteins were observed in the immunoprecipitated NK₁RΔ10K/R-protein complex (Fig. 7C). There was also no detectable ubiquitation of NK₁Rwt after incubation of cells with lower concentrations of SP (1 or 10 nM, 15 - 60 min) (data not shown).

Since many proteins such as β-arrestins associate with activated and internalized GPCRs, we sought to determine if the ubiquitin observed was directly associated with the NK₁R by immunoprecipitation under denaturing conditions. Incubation of cells expressing NK₁Rwt with SP for 5 h resulted in a ~3 increase in the levels of ubiquitinated NK₁R detected using P4D1 (Fig. 8A, B). In cells expressing NK₁RΔ5K/R, SP induced a low level of ubiquitination of the NK₁R (Fig. 8C).

We found that SP stimulated ubiquitination of NK₁Rwt, but that NK₁RΔ10K/R, lacking all intracellular lysines, was not ubiquitinated. SP induced a low level of ubiquitination of NK₁RΔ5K/R, which lacks only those lysine residues in the C-terminal tail (Fig. 1). Together, these results suggest that SP induces ubiquitination of lysine residues both within the C-terminus and intracellular loops of the NK₁R. In an attempt to identify the precise sites of ubiquitination of the NK₁R, we studied mutant receptors lacking single or multiple lysine residues in the C-terminal tail (Fig. 1). All these mutant were expressed at the plasma membrane and mobilized [Ca²⁺], in response to SP, but were still ubiquitinated after chronic exposure to agonist (data not shown). These mutant receptors were not studied further.

Proteins can undergo two different types of ubiquitination, mono-ubiquitination (where only a single ubiquitin molecule is added) and poly-ubiquitination (where the ubiquitin is added in chains). Using antibody FK1, which recognizes only poly-ubiquitin chains, we also observed that SP exposure resulted in poly-ubiquitination of NK₁Rwt (Fig. 8C). In non-transfected KNRK cells, there was minimal immunoreactivity to P4D1, FK1 or NK₁R antibodies (Fig. 7A, C, Fig. 8A, C), indicating specificity. P4D1 and FK1 antibodies recognized multiple ubiquitinated proteins in whole cell lysates. Thus, lysine residues on the intracellular face of the NK₁Rwt are targets for ubiquitination after prolonged stimulation with SP. Furthermore, NK₁Rwt is poly-ubiquitinated but we cannot rule out the possibility of mono-ubiquitination, which requires further investigation.

**Discussion**

Our results show that the concentration of agonist and the duration of stimulation markedly influence trafficking and signaling of the NK₁R. Whereas brief stimulation with low concentrations of SP stimulates endocytosis and recycling of the NK₁R, after chronic stimulation with high concentrations of SP, the NK₁R internalizes and is destined for degradation rather than recycling. Recovery of cellular responses after chronic stimulation is slow and requires protein synthesis, whereas receptor recycling mediates resensitization after transient stimulation. This concentration-dependent trafficking of the NK₁R depends on ubiquitination of lysine residues in the intracellular C-terminal tail and loop domains of the receptor. Chronic stimulation induces gradual ubiquitination of the NK₁R, which is not observed in mutant receptors lacking intracellular lysine residues. Moreover, these mutant receptors efficiently and rapidly recycle even after chronic stimulation with SP. Thus, chronic stimulation by SP induces ubiquitination of intracellular lysine residues of the NK₁R, which is required for down-regulation of SP signaling. This regulation may be particularly relevant during chronic inflammation and pain, where there may be sustained release of tachykinins, and may thus serve to prevent uncontrolled inflammatory and nociceptive signaling.

**Role of intracellular lysine residues in NK₁R trafficking**

Our data suggest that after prolonged exposure to high concentrations of agonist, the intracellular facing lysine residues of the NK₁R play a role in the post-endocytic sorting and degradation of this receptor. Mutation of lysine residues in the C-terminal tail (NK₁RΔ5K/R) and the whole intracellular face (NK₁RΔ10K/R) or truncation of
the C-terminal tail (NK₁RΔ325) did not affect targeting of receptors to the plasma membrane as all receptors were localized to the plasma membrane similar to NK₁Rwt. Similarly, mutation of intracellular lysine residues does not affect targeting of β₂AR, CXCR4, δ-opiod and PAR₂ to the plasma membrane (31-34). Both NK₁RΔ5K/R and NK₁RΔ10K/R were able to bind Alexa-SM-SP in a similar manner to NK₁Rwt, and SP mobilized Ca²⁺ ions in cells expressing NK₁RΔ5K/R and NK₁RΔ10K/R, which together indicate that all receptors were functional. In cells expressing NK₁RΔ5K/R and NK₁RΔ10K/R, Alexa-SM-SP internalized into early endosomes in a similar fashion to NK₁Rwt, suggesting that intracellular lysines are not required for receptor internalization. Our results are consistent with other reports that lysine mutation does not affect agonist binding, signaling and endocytosis of other GPCRs, including β₂AR, CXCR4, δ-opiod and PAR₂ (31-34). In contrast to the β₂AR (31), blocking the proteolytic activity of the proteasome did not affect the agonist-induced internalization of NK₁R. The incubation of cells with proteasomal inhibitors can lead to a reduction in the pool of “free” ubiquitin and thus the ubiquitination of β-arrestin may be impaired, leading to diminished internalization of the receptor. However, the exact mechanism by which the proteasome affects the internalization of the β₂AR and perhaps other GPCRs remains to be determined.

We found that lysine mutation had a dramatic effect on the post-endocytic sorting of the NK₁R after chronic stimulation with high concentrations of SP. The concentration of SP has a marked effect on post-endocytic sorting of the NK₁R. After stimulation with low concentrations of SP (1 nM), the NK₁R enters endosomes located immediately beneath the plasma membrane, from which it rapidly recycles by Rab4a- and Rab11a-dependent mechanisms (25). After stimulation with higher concentrations of SP (10 nM), the NK₁R traffics to endosomes in a perinuclear location, from which it slowly recycles (25). Using antibodies to extracellular epitopes to label receptors in living cells, we now report that chronic stimulation with still higher concentrations of SP (100 nM) stimulates trafficking of NK₁Rwt, NK₁RΔ5K/R and NK₁RΔ10K/R to endosomes in a perinuclear location. However, after agonist removal and incubation in SP-free medium for 4 h, both NK₁RΔ5K/R and NK₁RΔ10K/R recycle, whereas NK₁Rwt is localized to perinuclear vesicles. Thus, lysine residues prevent rapid recycling of the NK₁R. Similar observations were made for PAR₂, when mutation of lysine residues allowed the mutant to escape from its normal lysosomal targeting and return to the cell surface (33).

Role of intracellular lysine residues in down-regulation of NK₁R signaling

Our results show that chronic stimulation of cells with a high concentration of SP strongly desensitizes SP-induced activation of NK₁Rwt, and that responses to SP recover slowly, reaching control levels after 16 h. SP similarly desensitized NK₁RΔ5K/R and NK₁RΔ10K/R, which indicates that the intracellular lysines are not necessary for desensitization. Further, this desensitization was not due to depletion of Ca²⁺ stores as thapsigargin was able to release similar levels Ca²⁺ in treated and non-treated cells. However, NK₁RΔ10K/R and NK₁RΔ5K/R resensitized 4-8-fold more rapidly than NK₁Rwt, respectively. Moreover, whereas cycloheximide inhibited the slow resensitization of NK₁Rwt, suggesting a requirement for new protein synthesis, cycloheximide did not affect resensitization of NK₁RΔ5K/R and NK₁RΔ10K/R, which is thus independent of new protein synthesis and most probably depends on recycling of these receptors. These results are consistent with assessment of the effects of SP on receptor levels, determined by Western blotting. In the absence of cycloheximide, SP strongly stimulated expression of NK₁Rwt, NK₁RΔ5K/R and NK₁RΔ10K/R, which could also promote resensitization. This apparent upregulation of the NK₁R may be either due to SP-induced expression of the receptor, or could reflect a balance between the slow rate at which the receptor is degraded and its constitutive expression by the cytomegalovirus promoter. However, when cells were preincubated with cycloheximide, SP caused marked degradation of NK₁Rwt, whereas NK₁RΔ5K/R and NK₁RΔ10K/R were degraded 30 and 50% less than NK₁Rwt respectively. These findings implicate lysine residues as important targets in the pathway that leads to degradation of NK₁Rwt. Our observations are supported by reports that intracellular lysine residues in CXCR4 and
PAR2 are necessary for lysosomal targeting and degradation (32,33). However, since some NKβRΔ10K/R was still targeted to the degradative pathway, other mechanisms that do not require intracellular lysines also target the NKβR for destruction. In support of this suggestion, a mutant of the δ-opioid receptor lacking all intracellular lysine residues is still efficiently targeted to and degraded in the lysosome (34). Indeed, sorting of the δ-opioid receptor through the endosomal pathway to the lysosome is facilitated by vacuolar protein-sorting proteins such as Vps4 and Hrs (45). However, Hrs has also been implicated in mediating resensitization of the β2AR (46). Thus, lysine residues may not be the only signal to target the NKβR for degradation.

Notably, a naturally occurring variant, NKβRΔ325, which lacks most of the C-terminal tail including four of the five lysine residues in this domain did not completely desensitize after chronic exposure to SP, and after washing showed a profound sensitization of SP signaling to ~2.5-fold higher than control values. Our observations of diminished desensitization are consistent with other reports that NKβRΔ325 shows diminished desensitization and endocytosis after brief exposure to low concentrations of SP (38,43,44). The explanation of these effects is probably that the truncated receptor lacks many Ser and Thr residues in the C-terminal tail, and thus shows diminished phosphorylation by G-protein receptor kinases and defective interaction with β-arrestins (15). The truncated NKβR could permit the maintenance of robust SP signaling, even during chronic stimulation where full length NKβR is down-regulated.  

**SP-induced ubiquitination of the NKβR**

Ubiquitinated lysine residues serve as signals or adaptor molecules for pre- and post-endocytic sorting of cell-surface proteins, including GPCRs. Using non-denaturing immunoprecipitation, we observed that prolonged incubation with SP resulted in the appearance of ubiquitinated proteins within the NKβRwt complex, whilst within the NKβRΔ10K/R immunoprecipitated complex no such proteins could be detected. To ensure that the ubiquitinated protein was indeed NKβR and not associated proteins such as β-arrestins, which are ubiquitinated after activation of some GPCRs (31), we also denatured cellular lysates prior to immunoprecipitation. Using this method, we observed ubiquitinated forms of the NKβRwt. Proteins can be modified by ubiquitin in two general ways, either by addition of single ubiquitin molecules to one or more lysines (mono- and multimono-ubiquitination), or by the sequential addition of ubiquitin to form branched chains (poly-ubiquitination). The type of ubiquitination affects that final destination of the fated protein, with poly-ubiquitinated substrates usually degraded by proteasomal proteases and mono-ubiquitinated proteins destined for the lysosome. Under denaturing conditions and using antibodies that selectively recognize either mono- or poly-ubiquitin (P4D1) or poly-ubiquitin alone (FK1), we determined that the NKβRwt was associated with poly-ubiquitin chains. However, this does not exclude that possibility that the NKβR is also mono-ubiquitinated at some sites. Our results suggest that the NKβR is targeted for degradation by the proteasome, although further experiments are required to identify the sites of NKβR degradation. It is possible that the antibodies that we used do not fully discriminate between mono- and poly-ubiquitin. However, we have previously used these reagents to demonstrate that PAR2, which is irreversibly activated by proteolytic cleavage, is mono-ubiquitinated and targeted to lysosomes for degradation (33). In contrast, the inositol 1,4,5-trisphosphate receptor is poly-ubiquitinated and degraded by the proteasome (47). We observed that NKβRΔ5K/R, which lacks all lysines in the C-terminal tail, is still ubiquitinated, albeit to a lesser extent, after prolonged exposure to SP. Moreover, mutation of individual lysines did not affect ubiquitination. Thus, several lysine residues within the C-tail and intracellular loops of the NKβR are likely targets for E3 ubiquitin ligases. Further experimentation is required to identify critical lysine residues, and to determine whether the NKβR is mono- or poly-ubiquitinated at these residues. Although we observed that mutation of lysine residues had a marked effect on NKβR recycling and resensitization, these effects may not necessarily be related to alterations in receptor ubiquitination, since lysine residues could affect trafficking independently from their role in receptor ubiquitination. Additional studies are required to investigate this possibility.
We detected ubiquitinated NK$_1$R only after prolonged stimulation with SP (100 nM, > 1 h), which indicates that ubiquitination occurs long after internalization of the receptor and its trafficking to an undefined intracellular location. In contrast, other GPCRs such as PAR$_2$ (ubiquitinated by the E3-ligase c-Cbl (33)) and CXCR4 (ubiquitinated by the E3-ligase AIP4 (39)) are rapidly ubiquitinated, either at the plasma membrane or in early endosomes. Indeed, agonists of PAR$_2$ stimulate trafficking of c-Cbl to the plasma membrane and endosomes. Instead, SP-induced ubiquitination of the NK$_1$R may require prolonged retention of the receptor in an intracellular compartment, which may facilitate the assembly of ubiquitinating machinery that, under conditions of low agonist concentration, may not form. Alternatively, chronic stimulation may induce repeated rounds of receptor endocytosis and recycling, which could alter the receptor in such a way as to promote ubiquitination. These possibilities and the E3-ligase that mediates the conjugation of ubiquitin to the NK$_1$R remain to be investigated.

Even in the absence of new protein synthesis, some NK$_1$Rwt recycled back to the plasma membrane, as indicated by the 15% recovery of SP signaling in the presence of cycloheximide at 16 h. Moreover, after prolonged exposure to SP, resensitization of Ca$^{2+}$ signaling was biphasic, suggesting the existence of several mechanisms of resensitization. Recycling of non-ubiquitinated or perhaps de-ubiquitinated receptors may mediate rapid resensitization. Recycled receptors may escape ubiquitination and degradation. Alternatively, addition of ubiquitin may serve to retain the NK$_1$R within the cell until a deubiquitination step, which then allows the receptor to traffic back to the plasma membrane to signal normally. Indeed, we found that NK$_1$R when directly conjugated to a ubiquitin molecule fails to reach the plasma membrane. Activated tumor necrosis factor receptor associated factor-6 recycles back to the plasma membrane after its deubiquitination by an unknown ubiquitin-specific protease (48). Furthermore, ubiquitinated adenosine A$_2A$ receptors are deubiquitinated by the ubiquitin-specific protease Usp4, which allows trafficking to the plasma membrane (49). Further experimentation is required to determine whether the NK$_1$R also undergoes deubiquitination.

In summary, our findings suggest a role for the lysine residues and ubiquitination in the recycling and degradation of the NK$_1$R after chronic stimulation with SP. This mechanism is likely to play an important role in preventing sustained stimulation by SP, which could otherwise cause uncontrolled inflammatory and nociceptive signaling.

**References**


Footnotes

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1 The abbreviations used are: angiotensin II type 1A receptor, AT1AR; β2 adrenergic receptor, β2AR; chemokine (C-X-C motif) receptor 4, CXCR4; G-protein coupled receptor, GPCR; neurokinin-1 receptor, NK1R; protease-activated receptor 1, PAR1; protease-activated receptor 2, PAR2; substance P, SP; [Sar9MetO211]-substance P, SM-SP.

Figure Legends

Figure 1. Epitope tagged NK1R, NK1R lysine and truncated mutants, and NK1R-ubiquitin chimera. The structure of NK1R is illustrated with N-terminal Flag (NK1Rwt, NK1RΔ5K/R, NK1RΔ325, NK1R-Ub) or HA.11 (NK1RΔ10K/R). The lysine residues mutated to arginine and the site of truncation are indicated. # indicates the mutated residues of the point mutations within the C-terminal tail.

Figure 2. Expression of NK1Rwt, NK1R-Ub and lysine and truncated mutants. NK1Rwt, NK1RΔ5K/R, NK1RΔ10K/R and NK1RΔ325 expressed in KNRK cells were detected at the plasma membrane by immunofluorescence and confocal microscopy. NK1R-Ub was detected in intracellular compartments. Scale bar = 10 µm.

Figure 3. Endocytosis of Alexa-SM-SP. Cells expressing NK1Rwt, NK1RΔ5K/R or NK1RΔ10K/R were incubated with 100 nM Alexa-SM-SP for 1 h at 4°C, washed and incubated in SP-free medium at 37°C for 0 or 30 min. In all cell types, Alexa-SM-SP was detected at the plasma membrane at 0 min (arrow heads), and at 30 min, Alexa-SM-SP was depleted from the plasma membrane and detected in endosomes in a perinuclear location (arrows). Endocytosis was unaffected by the proteasomal inhibitor epoxomicin. Scale bar = 10 µm.

Figure 4. Internalization and post-endocytic trafficking of NK1Rwt and mutants. Cells expressing NK1Rwt, NK1RΔ5K/R or NK1RΔ10K/R were incubated with Flag M2 (NK1Rwt, NK1RΔ5K/R) or HA.11 (NK1RΔ10K/R) antibodies for 2 h at 4°C to label surface receptors. Unstimulated cells were incubated in SP-free medium for 0 or 3 h. SP-stimulated cells were incubated with 100 nM SP for 3 h, washed and
allowed to recover in SP-free medium for 0 or 4 h. Cells were fixed and the NK\(_R\) was detected by immunofluorescence and confocal microscopy. In unstimulated cells, the NK\(_R\) was mostly detected at the plasma membrane at both 0 and 3 h (arrow heads). There was some internalized NK\(_R\), suggesting constitutive internalization in the absence of agonist. Incubation with SP for 3 h resulted in loss of NK\(_R\)Rwt, NK\(_i\)RA5K/R and NK\(_i\)RA10K/R from the plasma membrane and redistribution of receptors to vesicles in a perinuclear location (arrows). After 4 h recovery, NK\(_i\)Rwt was still retained in vesicles (arrows), whereas NK\(_i\)RA5K/R and NK\(_i\)RA10K/R were present at the plasma membrane (arrow heads). Scale bar = 10 \(\mu\)m.

**Figure 5. Desensitization and resensitization of SP-induced increases in [Ca\(^{2+}\)].** Cells expressing NK\(_i\)Rwt, NK\(_i\)RA5K/R, NK\(_i\)RA10K/R or NK\(_i\)RA325 were pre-incubated with 100 nM SP or vehicle (veh. control) for 3 h, washed, incubated in SP-free medium for 0-16 h, and then challenged with 10 nM SP. A, Responses to 10 nM SP immediately after pre-incubating with SP or vehicle for 3 h and washing. Vehicle-treated cells responded similarly to SP (although the response was smaller in NK\(_i\)RA325 cells). Pre-incubation with SP abolished responses in cells expressing NK\(_i\)Rwt, NK\(_i\)RA5K/R or NK\(_i\)RA10K/R. Responses in cells expressing NK\(_i\)RA325 were diminished but not abolished. B, Time course of resensitization, with responses expressed as a percentage of control levels in cells pre-incubated with vehicle. In cells expressing NK\(_i\)Rwt resensitization was complete after 16 h, whereas resensitization was more rapid in cells expressing NK\(_i\)RA5K/R (4 h) and NK\(_i\)RA10K/R (2 h). In cells expressing NK\(_i\)RA325, there was a markedly enhanced resensitization to levels that were 2.5-fold higher than in vehicle-treated cells. C, Effects of cycloheximide on resensitization, expressed as a percentage of values in vehicle-treated cells also incubated with cycloheximide. Cycloheximide abolished resensitization of NK\(_i\)Rwt (measured at 16 h) but had no effect on resensitization of NK\(_i\)RA5K/R or NK\(_i\)RA10K/R (measured at 4 h). * P<0.05 compared to control. n=3.

**Figure 6. Effects of SP on levels of NK\(_i\)Rwt, NK\(_i\)RA5K/R or NK\(_i\)RA10K/R.** Cells expressing NK\(_i\)Rwt, NK\(_i\)RA5K/R or NK\(_i\)RA10K/R were incubated with vehicle (Veh) or SP (100 nM SP) for 16 h in the absence or presence of cycloheximide (CHX), A, B and C, Western blots (10 \(\mu\)g protein per lane) were probed for the NK\(_i\)R using the polyclonal 94168 antibody to the carboxyl-terminus of the receptor, or for \(\gamma\)-tubulin or \(\beta\)-actin as loading controls. D, Western blot of NK\(_i\)Rwt before and after deglycosylation (Deglyc.). E and F, Densitometric analyses of Western blots, in which levels of NK\(_i\)R were compared to \(\gamma\)-tubulin or \(\beta\)-actin, and normalized to vehicle treatment. In the absence of cycloheximide, SP caused a ~3-fold increase in levels of detectable NK\(_i\)Rwt (A, E), NK\(_i\)RA5K/R (B, E) and NK\(_i\)RA10K/R (C, E). In the presence of cycloheximide, SP caused a 40-50% reduction in levels of NK\(_i\)Rwt (A, F), but only a 30-35% reduction in levels of NK\(_i\)RA5K/R (B, F), and a 20-25% reduction in levels of NK\(_i\)RA10K/R (C, F). There were no detectable signals for NK\(_i\)R in untransfected KNRK cells, indicating specificity. Deglycosylation reduced the molecular weight of the NK\(_i\)R to the predicted size. * P<0.05 compared to control. n=3.

**Figure 7. Ubiquitination of the NK\(_i\)R detected under non-denaturing conditions.** Cells expressing NK\(_i\)Rwt or NK\(_i\)RA10K/R were incubated with 100 nM SP for 0, 1, 3 or 5 h. The NK\(_i\)R was immunoprecipitated (IP) under non-denaturing conditions using monoclonal antibodies to the aminoterminal Flag or HA.11 epitopes. Western blots (WB) were probed for ubiquitin using the monoclonal P4D1 antibody, which interacts with mono- and poly-ubiquitinated proteins, or for the NK\(_i\)R using the polyclonal 94168 antibody, which interacts with the carboxyl-terminus of the receptors. A, Incubation with SP for 1-5 h resulted in appearance of high molecular weight forms of ubiquitinated NK\(_i\)Rwt. B, Densitometric analysis of Western blots, in which ubiquitinated NK\(_i\)Rwt signals were compared to total NK\(_i\)R signals, and normalized to 0 min. C, There was no detectable ubiquitination of NK\(_i\)RA10K/R after incubation with SP. There were no detectable signals for NK\(_i\)R or ubiquitin in untransfected KNRK cells,
indicating specificity. Multiple ubiquitinated proteins were detected in Western blots of lysates of KNRK cells. n≥3.

**Figure 8. Ubiquitination of the NK₁R detected under denaturing conditions.** Cells expressing NK₁Rwt or NK₁RΔ5K/R were incubated with 100 nM SP for 0 or 5 h. The NK₁R was immunoprecipitated (IP) under denaturing conditions using monoclonal antibody to the amino-terminal Flag epitope. Western blots (WB) were probed for ubiquitin using the monoclonal P4D1 antibody, which interacts with mono- and poly-ubiquitinated proteins (A, C), or the monoclonal FK1 antibody, which interacts with poly-ubiquitinated proteins (D). Blots were reprobed for the NK₁R using the polyclonal Flag antibody. A, Incubation with SP for 5 h resulted in appearance of high molecular weight forms of mono- or poly-ubiquitinated NK₁Rwt. B, Densitometric analysis of Western blots, in which ubiquitinated NK₁Rwt signals were compared to total NK₁R signals, and normalized to 0 min. C, Incubation with SP for 5 h resulted in a low level of high molecular weight forms of mono- or poly-ubiquitinated NK₁RΔ5K/R. D, Incubation with SP for 5 h resulted in appearance of high molecular weight forms of poly-ubiquitinated NK₁Rwt. There were no detectable signals for NK₁R or ubiquitin in untransfected KNRK cells, indicating specificity. Multiple ubiquitinated proteins were detected in Western blots of lysates of KNRK cells. n≥3.
Rat NK₁R

Flag
or HA,11

N

C

393

374

337

350

Lysine

Truncation NK₁RΔ325

148

248

245

243

317

337


NK₁R-Ub: K³⁹³-TMTESSFYSNL³⁴⁰⁷-I-ubiquitin
NK₁RΔ5K/R: K317-393R
NK₁RΔ10K/R: K61-393R

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Fig 1
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Fig 6
Ubiquitin-dependent down-regulation of the neurokinin-1 receptor
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