Recycling and Resensitization of the Neurokinin 1 Receptor

INFLUENCE OF AGONIST CONCENTRATION AND Rab GTPASES*

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Substance P (SP) induces endocytosis and recycling of the neurokinin 1 receptor (NK1R) in endothelial cells and spinal neurons at sites of inflammation and pain, and it is thus important to understand the mechanism and function of receptor trafficking. We investigated how the SP concentration affects NK1R trafficking and determined the role of Rab GTPases in trafficking. NK1R trafficking was markedly influenced by the SP concentration. High SP (10 nM) induced translocation of the NK1R and β-arrestin 1 to perinuclear sorting endosomes containing Rab5a, where NK1R remained for >60 min. Low SP (1 nM) induced translocation of the NK1R to early endosomes located immediately beneath the plasma membrane that also contained Rab5a and β-arrestin 1, followed by rapid recycling of the NK1R. Overexpression of Rab5a promoted NK1R translocation to perinuclear sorting endosomes, whereas the GTP binding-deficient mutant Rab5aS34N caused retention of the NK1R in superficial early endosomes. NK1R translocated from superficial early endosomes to recycling endosomes containing Rab4a and Rab11a, and Rab11aS25N inhibited NK1R recycling. Rapid NK1R recycling coincided with resensitization of SP-induced Ca2+ mobilization and with the return of surface SP binding sites. Resensitization was minimally affected by inhibition of vacuolar H+ ATPase and phosphatases but was markedly suppressed by disruption of Rab4a and Rab11a. Thus, whereas β-arrestins mediate NK1R endocytosis, Rab5a regulates translocation between early and sorting endosomes, and Rab4a and Rab11a regulate trafficking through recycling endosomes. We have thus identified a new function of Rab5a as a control protein for directing concentration-dependent trafficking of the NK1R into different intracellular compartments and obtained evidence that Rab4a and Rab11a contribute to G-protein-coupled receptor recycling from early endosomes.

Agonist binding to G-protein-coupled receptors (GPCRs) induces rapid alterations in the subcellular distribution of receptors and associated proteins that are important for signal transduction. Activation of the β2-adrenergic receptor (β2AR), angiotensin II type 1A receptor (AT1AR), and neurokinin 1 receptor (NK1R) stimulates translocation of G-protein receptor kinases (GRKs) from the cytosol to the plasma membrane, where they phosphorylate receptors (1–4). β-Arrestins directly interact with Rab5a, and this interaction may be required for trafficking (25). Rab4a and Rab11a play an important role in receptor sorting and recycling. Rab4a is partially colocalized with Rab5a in sorting endosomes and mediates rapid recycling of the transferrin receptor (27). Rab11a is present in recycling endosomes and is required for slow recycling of the transferrin receptor from perinuclear endosomes (27). The AT1AR colocalizes with Rab4a and Rab11a during

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‡ The abbreviations used are: GPCR, G-protein-coupled receptor; SP, substance P; NK1R, neurokinin 1 receptor; β2AR, β2-adrenergic receptor; AT1AR, angiotensin II type 1A receptor; GRK, G-protein receptor kinase; GFP, green fluorescent protein; EGFP, enhanced GFP, BSA, bovine serum albumin.
induced translocation of \( \beta \)-arrestin 1-EGFP. KNKR-NKIR cells were either unstimulated (A) or incubated with 10 nM or 1 nM SP for 60 min at 4°C (B). Cells were fixed, and the NKIR was localized by immunofluorescence using anti-FLAG and \( \beta \)-arrestin 1 (\( \beta \)ARR1) by GFP. In unstimulated cells (A), the NKIR was at the cell surface (arrowheads), and \( \beta \)-arrestin 1-EGFP was cytosolic (arrows). Incubation with 10 nM and 1 nM SP (B) induced translocation of \( \beta \)-arrestin 1-EGFP from the cytosol to the cell membrane (arrowheads). Scale bar, 10 \( \mu \)m.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—SP was from Bachem (Torrance, CA). Fura-2-AM and Texas Red succinimidyl ester were from Molecular Probes, Inc. (Eugene, OR). SP was labeled with Texas Red succinimidyl ester as described (35). Iproyl-2,4,3,4-\( ^3 \)HISP (7.4–26 TBq/mmol) was from Amersham Biosciences. Restriction enzymes, T4 ligase, LipofectAMINE, and enzyme-free cell dissociation buffer were from Invitrogen or New England Biolabs, Inc. (Beverly, MA). Enhanced GFP (EGFP) expression vector pEGFP-N1 was from Clontech (Palo Alto, CA). Rabbit anti-Rab11a was from Zymed Laboratories Inc. (South San Francisco, CA). Rabbit anti-Rab4a (D-20), Rabbit anti-Rab5a (S-19), and mouse anti-FLAG and anti-\( \beta \)arrestin 1 (\( \beta \)ARR1) antibodies were from Sigma. All other reagents and antibodies were from Sigma.

**Constructs of Rab4a and Rab11a**—cDNAs for Rab4a and Rab11a were from the cDNA library of sarcoma virus-transformed rat kidney epithelial cells (26). To generate cell lines stably expressing Rab4a and Rab11a, KNKR-NKIR cells were incubated for 2 h with 5 \( \mu \)g/ml cDNA encoding Rab11a-GFP, Rab11aS25N-GFP, Rab4a-GFP, or Rab4aS24N-GFP and 20 \( \mu \)l/ml LipofectAMINE. Cells were selected for 2 weeks with hygromycin (150 \( \mu \)g/ml) and neomycin (400 \( \mu \)g/ml) and sorted for GFP by flow cytometry (6). To express constructs transiently, KNKR-NKIR cells were incubated for 2 h with 5 \( \mu \)g/ml cDNA and 20 \( \mu \)l/ml LipofectAMINE, and cells were studied after 72 h. For controls, cells were transfected with empty vector without insert. Transiently transfected cells were used for microscopy, and stable cell lines were studied in all other experiments.

**Western Blotting**—Cell lysates (25 \( \mu \)g of protein) were separated by SDS-PAGE (13% acrylamide gels). Proteins were transferred to polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA) and blocked for 1 h at room temperature (1\( \times \) phosphate-buffered saline, 2% BSA, 5% milk powder, 0.1% Tween 20). Blots were incubated with antibodies to Rab4a, Rab5a, and Rab11a at room temperature (1\( \times \)phosphate-buffered saline, 2% BSA, 0.1% Tween 20) and washed, and fluorescence was measured at 340- and 380-nm excitation and 510-nm emission in a F-2000 spectrophotometer (Hitachi Instruments, Irvine, CA). The ratio of the fluorescence at the two excitation wavelengths, which is propor-

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**Fig. 1.** Membrane translocation of \( \beta \)-arrestin 1-GFP. KNKR-NKIR cells were either unstimulated (A) or incubated with 10 nM or 1 nM SP for 60 min at 4°C (B). Cells were fixed, and the NKIR was localized by immunofluorescence using anti-FLAG and \( \beta \)-arrestin 1 (\( \beta \)ARR1) by GFP. In unstimulated cells (A), the NKIR was at the cell surface (arrowheads), and \( \beta \)-arrestin 1-EGFP was cytosolic (arrows). Incubation with 10 nM and 1 nM SP (B) induced translocation of \( \beta \)-arrestin 1-EGFP from the cytosol to the cell membrane (arrowheads). Scale bar, 10 \( \mu \)m.

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**Western Blotting**—Cell lysates (25 \( \mu \)g of protein) were separated by SDS-PAGE (13% acrylamide gels). Proteins were transferred to polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA) and blocked for 1 h at room temperature (1\( \times \)phosphate-buffered saline, 2% BSA, 5% milk powder, 0.1% Tween 20). Blots were incubated with antibodies to Rab4a, Rab5a, and Rab11a at room temperature (1\( \times \)phosphate-buffered saline, 2% BSA, 0.1% Tween 20) and washed, and fluorescence was measured at 340- and 380-nm excitation and 510-nm emission in a F-2000 spectrophotometer (Hitachi Instruments, Irvine, CA). The ratio of the fluorescence at the two excitation wavelengths, which is propor-
tional to [Ca\(^{2+}\)], was calculated (37). To assess desensitization and resensitization, cells were exposed to SP or vehicle, washed, and challenged again with SP.

**SP Binding**—To determine the rate of uptake of SP, cells were incubated in Dulbecco’s modified Eagle’s medium/BSA containing 100,000 cpm of [\(\text{propyl}-2,4,3H\)]SP and 1 or 10 nM unlabeled SP for 0–10 min at 37 °C. Cells were placed on ice, washed with ice-cold HBSS, pH 7.4, and washed in acid to separate cell surface (acid-labile) from internalized (acid-resistant) label (38). Cells were washed twice with ice-cold HBSS, pH 5.0, with acetic acid and lysed with 1\(\times\) NaOH, 0.1% SDS, and the lysate was counted. To assess the loss and recovery of binding sites for SP, cells were incubated with 1 or 10 nM SP in Dulbecco’s modified Eagle’s medium/BSA for 10 min at 37 °C, washed, and incubated at 37 °C for 0–20 min. Cells were placed on ice, washed with ice-cold HBSS, pH 5.0, to remove surface SP, and incubated with 100,000 cpm of [\(\text{H}^{3}\)]SP for 90 min at 4 °C. Cells were washed in acid to measure surface binding. The affinity of the NK1R and the number of surface binding sites for SP were determined by Scatchard analysis after stimulation with 1 or 10 nM SP for 10 min at 37 °C. To do so, cells were incubated in HBSS/BSA for 90 min at 4 °C with 0.001–100 nM SP and 100,000 cpm [\(\text{H}^{3}\)]SP. Unbound SP was washed off with HBSS, and surface-bound [\(\text{H}^{3}\)]SP was eluted with acid and counted. In all experiments, nonspecific binding was determined in cells treated with 1 \(\mu\)M SP.

**RESULTS**

**SP-induced Endocytosis and Recycling of the NK1R—\(\beta\)-Arrestins mediate NK1R endocytosis (6, 32), and the NK1R forms stable and high affinity interactions with \(\beta\)-arrestin 1 and 2 at the cell surface and in endosomes (30–33). However, the concentration dependence of SP-induced redistribution of the NK1R and \(\beta\)-arrestins has not been examined. We incubated KNRK-NK1R cells transiently expressing \(\beta\)-arrestin 1-1-GFP with graded concentrations of SP and localized the NK1R by immunofluorescence and \(\beta\)-arrestin 1 using GFP. In unstimulated cells, the NK1R was at the cell surface, and \(\beta\)-arrestin 1-1-GFP was in the cytosol (Fig. 1A). Incubation with 1 or 10 nM SP for 60 min at 4 °C induced membrane translocation of \(\beta\)-arrestin 1 to a similar degree (Fig. 1B). To examine NK1R trafficking, we stimulated the cells for 10 min at 37 °C with 1 or 10 nM SP, washed the cells, and incubated at 37 °C for various times. After 10 nM SP (10 min, 37 °C), both the NK1R and \(\beta\)-arrestin 1 were colocalized in endosomes mostly in a perinuclear location, where they remained colocalized for at least 60 min (Fig. 2A). This sequestration of \(\beta\)-arrestin 1 into endosomes was sufficient to deplete \(\beta\)-arrestin 1 from the cytosol until 60 min. After 1 nM SP (10 min, 37 °C), the NK1R and \(\beta\)-arrestin 1 were colocalized exclusively to endosomes located immediately beneath the plasma membrane (Fig. 2B). After washing, there was some translocation to a perinuclear compartment, but the NK1R and \(\beta\)-arrestin 1 were predominantly colocalized in superficial vesicles. Within 5–30 min, the NK1R recycled to the cell surface, and \(\beta\)-arrestin 1 translocated to the cytosol. Thus, high concentrations of SP induce prolonged sequestration of NK1R and \(\beta\)-arrestin 1 into perinuclear endosomes, thereby depleting \(\beta\)-arrestins from the cytosol. In contrast, low concentrations of SP result in a brief association of NK1R and \(\beta\)-arrestin 1 in superficial endosomes, followed by rapid recycling of the NK1R to the cell surface and redistribution of \(\beta\)-arrestin 1 to the cytosol. We similarly examined trafficking of the NK1R in cells that were not transfected with \(\beta\)-arrestin 1-1-GFP and obtained the same results (not shown), suggesting that overexpression of \(\beta\)-arrestin 1 does not influence NK1R trafficking.

**Expression of Wild-type and Mutant Rab Proteins**—To evaluate the contributions of Rab GTPases to SP-induced trafficking and signaling of the NK1R, we overexpressed wild-type Rab4a, -5a, and -11a or the GTP binding-deficient mutants Rab4aS24N, Rab5aS34N, and Rab11aS25N in KNRK-NK1R cells. Rab proteins were tagged with GFP for detection. We first examined the relative levels of expression of the Rab-GFP constructs by Western blotting using an antibody to GFP. Rab-GFP proteins of the anticipated apparent molecular mass (~55 kDa; Rab (~25 kDa) + GFP (~30 kDa)) were detected in all cell lines (Fig. 3A, lanes 2–7). All constructs were expressed at comparable levels with the exception of Rab11aS25N-GFP, which was consistently expressed at lower levels. Specificity of the GFP antiserum was confirmed by analysis of KNRK-NK1R cells, where no GFP immunoreactivity was detected (Fig. 3A, lane 1). We then compared the relative levels of expression of endogenous Rabs with the transfected GFP-tagged Rabs by Western blotting using antibodies to Rab4a, Rab5a, and Rab11a. In KNRK-NK1R cells, antibodies to Rab5a (Fig. 3C, lane 1) and Rab11a (Fig. 3D, lane 1) detected single proteins of the anticipated apparent weight, although the antibody to Rab4a detected several additional unidentified proteins (Fig. 3B, lane 1). In the Rab-transfected cell lines, wild-type and...
mutant Rab4a-GFP and Rab5a-GFP constructs were expressed at levels that were markedly higher than those of endogenous Rab4a and -5a (Fig. 3, B and C). The relative levels of expression were compared by densitometry. Rab4a-GFP was expressed 8.5-fold higher, Rab4aS24N 6.4-fold higher, Rab5a 2.7-fold higher, and Rab5aS34N 3.5-fold higher than the endogenous Rabs. Although wild-type Rab11a-GFP was expressed at levels comparable with that of the endogenous Rab11a (0.7-fold), Rab11aS25N-GFP was expressed at consistently lower levels than endogenous Rab11a (0.3-fold) (Fig. 3D). However, these comparisons of the levels of expression of endogenous Rabs in rat-derived KNRK cells and the overexpressed canine Rabs may be complicated by differences in the interaction of the Rab antibodies with Rabs from different species. Together, these results confirm expression of wild-type and mutated Rabs in the transfected cell lines.

**Rab5a-mediated Trafficking of NK1R from Superficial Vesicles to Endosomes in a Perinuclear Location**

We have shown that Alexa594-labeled SP (10–100 nM) colocalizes with Rab5a in early endosomes and that expression of the GTP binding-deficient mutant Rab5aS34N partially inhibits NK1R endocytosis and induces its accumulation in superficial vesicles (26). We now sought to determine the role of Rab5a in translocation of the receptor from superficial vesicles to perinuclear endosomes. In KNRK cells coexpressing NK1R and Rab5a-GFP, incubation with 1 nM SP for 10 min at 37 °C induced translo-
cation of NK1R to large endosomes in a perinuclear region that also contained Rab5a (Fig. 4A). In contrast, in cells that were not overexpressing Rab5a, the NK1R was confined to endosomes located immediately beneath the plasma membrane after exposure to 1 nM SP (Fig. 2B). Thus, overexpression of Rab5a alone altered NK1R trafficking, inducing translocation to a perinuclear region and preventing retention in superficial endosomes. This result suggests that Rab5a mediates transport of the NK1R to endosomes in a perinuclear location. Expression of Rab5aS34N-GFP caused the accumulation of the NK1R in vesicles located beneath the plasma membrane and prevented translocation to perinuclear regions, even in cells exposed to high concentrations (10 nM) of SP (Fig. 4B) that otherwise promoted translocation of the NK1R to endosomes in a perinuclear region (Fig. 2A). These results indicate that GDP/GTP exchange of Rab5a is important for NK1R transport from superficial vesicles to perinuclear regions of the cell.

To determine whether this sorting function of Rab5a is a general phenomenon, we compared the effects of overexpression of Rab5aS34N on trafficking of two different ligands, Texas Red-labeled SP and transferrin. KNKR-NK1R cells expressing Rab5aS34N-GFP were incubated with 100 nM Texas Red SP or 50 μg/ml Texas Red transferrin for 10 min at 37 °C. In cells expressing Rab5aS34N, SP was confined to the cell surface and to superficial vesicles (Fig. 5A), whereas it proceeded to perinuclear vesicles in untransfected cells (not shown). Expression of Rab5aS34N partially inhibited but did not abolish the translocation of transferrin from the plasma membrane to perinuclear endosomes (Fig. 5B). Thus, Rab5a plays a more prominent role in translocation of the NK1R to endosomes in a perinuclear region than it does for the transferrin receptor.

Rab4a- and Rab11a-mediated Recycling of the NK1R—
Rab4a and Rab11a participate in translocation of proteins from perinuclear compartments and superficial vesicles to the plasma membrane, and Rab11a also mediates the late step of induced vesicle transport from Golgi apparatus to the plasma membrane (36, 39). To determine their role in recycling of the NK1R, we examined KNKR-NK1R cells coexpressing Rab4a-GFP, Rab11a-GFP, or the GTP binding-deficient mutants Rab4aS24N-GFP or Rab11aS25N-GFP. Cells were incubated with 1 nM SP for 10 min at 37 °C, washed, and incubated for a 10-min recovery in SP-free medium. NK1R was detected using FLAG antibody and Rabs with GFP.

In unstimulated cells, Rab4a was detected in vesicles and NK1R was at the cell surface (Fig. 6A). After 10 min with 1 nM SP, NK1R was partially colocalized with Rab4a in some endosomes in a superficial location (Fig. 6, A and C). After 10 min of recovery, NK1R was recycled and Rab4a resumed its steady state distribution in the cytosol and in scattered vesicles. Expression of Rab4aS24N did not prevent SP-induced endocytosis of the NK1R (arrows). Scale bar, 10 μm.

Fig. 6. Rab4a-mediated intracellular trafficking of NK1R. Cells expressing Rab4a-GFP (A) or Rab4aS24N-GFP (B) were unstimulated (upper rows) or were incubated with 1 nM SP for 10 min at 37 °C, washed, and incubated for 0 or 10 min at 37 °C. C, expanded view of a cell from A after a 10-min stimulation with SP. NK1R was localized by immunofluorescence using anti-FLAG and Rab4a by GFP. After incubation with SP, NK1R was partially localized with Rab4a in endosomes (arrows). Expression of Rab4aS24N did not prevent SP-induced endocytosis of the NK1R (arrows). Scale bar, 10 μm.

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In unstimulated cells, Rab4a was detected in vesicles and NK1R was at the cell surface (Fig. 6A). After 10 min with 1 nM SP, NK1R was partially colocalized with Rab4a in some endosomes in both superficial and perinuclear locations (Fig. 6, A and C). After 10 min of recovery, NK1R was present at the cell surface. This pattern of trafficking was not markedly altered by expression of Rab4aS24N; the NK1R was present in endosomes in a superficial location after 10 min with 1 nM SP and recycled 10 min after washing (Fig. 6B). However, the colocalization of NK1R and Rab4a in some endosomes suggests a role of Rab4a in NK1R trafficking (Fig. 6C).

In unstimulated cells, Rab11a was present in the cytosol and in vesicles scattered throughout the cell, and NK1R was present at the cell surface (Fig. 7A). After 10 min with 1 nM SP, there was a marked redistribution of Rab11a and NK1R to prominent endosomes that were located immediately beneath the plasma membrane (Fig. 7, A and C). Within 10 min of washing, the NK1R was recycled and Rab11a resumed its steady state distribution in the cytosol and in scattered vesicles. Expression of Rab11aS25N did not affect endocytosis of...
the NK1R, since after 10 min with 1 nM SP, the NK1R was detected in superficial and perinuclear vesicles (Fig. 7B). However, after a 10-min recovery, the NK1R was retained in these vesicles, and there was diminished recycling to the cell surface. The strong colocalization of the NK1R and Rab11a and the diminished recycling in cells expressing Rab11aS25N suggest that Rab11a plays an important role in recycling of the NK1R.

**Desensitization of and Resensitization of the NK1R**—We assessed desensitization and resensitization of the NK1R by measuring SP-induced mobilization of intracellular Ca\(^{2+}\). To verify that exposure of cells to low concentrations of SP caused desensitization, we challenged cells repeatedly with 1 nM SP at 10-min intervals. Challenge with 1 nM SP caused a prompt increase in [Ca\(^{2+}\)]\(_i\), that rapidly returned to basal levels. A second challenge with 1 nM SP had minimal affect on [Ca\(^{2+}\)]\(_i\), indicating desensitization of the NK1R (Fig. 8A). However, if cells were washed between challenges, there was complete resensitization (Fig. 8B). Thus, exposure of cells to 1 nM SP for 10 min completely desensitizes the NK1R, and at this time the NK1R is present in endosomes in a superficial location and at the cell surface (Fig. 2B). To examine resensitization, cells were incubated with 1 or 10 nM SP for 10 min at 37 °C, washed, and challenged again with the same concentration of SP at 5–10 min after washing. In cells exposed to 1 nM SP, there was >100% resensitization to a second challenge with 1 nM SP at 5 or 10 min later (Fig. 8C). Thus, the NK1R is resensitized at a time when it is present at the cell surface and not fully sequestered with β-arr251. In contrast, in cells exposed to 10 nM SP, there was no detectable resensitization at 5 or 10 min, times when the NK1R was sequestered with β-arr251 in endosomes in a perinuclear location (see Fig. 2A).

After exposure of cells to high concentrations of SP (10 nM), which induces translocation of the NK1R to endosomes in a perinuclear region, resensitization requires endosomal acidification, which is necessary for dissociation of SP and dephosphorylation of the NK1R. Thus, under these conditions, resensitization is inhibited by ammonium chloride, the vacuolar H\(^+\)-GTPase inhibitor bafilomycin A1 and the protein phosphatase 2A inhibitor okadaic acid (15, 16). We therefore evaluated the effects of these treatments on rapid resensitization of the NK1R. Cells were preincubated (30 min) with ammonium chlo-
ride (20 mM), bafilomycin A (0.1 μM), or okadaic acid (0.1 μM), incubated with 1 nM SP for 10 min at 37 °C, washed, and challenged at 5 or 10 min with 1 nM SP. There was complete resensitization at both time points in cells treated with ammonium chloride (Fig. 8 C). Bafilomycin A and okadaic acid slowed resensitization after 5 min, but at 10 min resensitization was complete. Thus, resensitization at 5 min is partially dependent, and resensitization at 10 min is independent of endosomal acidification and protein phosphatase 2A.

Rab4a- and Rab11a-mediated Resensitization of the NK1R—During rapid recycling of the NK1R that follows challenge with low concentrations of SP, the NK1R was detected in endosomes in a superficial location with Rab4a and Rab11a, and expression of Rab11aS25N impeded recycling of the NK1R. We therefore hypothesized that Rab4a- and Rab11a-dependent recycling of the NK1R was required for rapid resensitization. Cells expressing NK1R alone or NK1R with Rab4a, Rab4aS24N, Rab11a, or Rab11aS25N were incubated with 1 nM SP for 10 min, washed, and challenged with 1 nM SP 5, 10, or 20 min after washing. Exposure to 1 nM SP for 10 min induced similar desensitization in all cell lines (not shown). In cells expressing the NK1R alone, there was >100% resensitization of SP-induced Ca²⁺ mobilization at all times (Fig. 9). When the interval between SP changes was 5 min, overexpression of wild-type and GTP binding-defective Rab4a and Rab11a strongly inhibited resensitization. Resensitization at 10 min was also suppressed by Rab4aS24N and Rab11aS25N, whereas resensitization at 20 min was unaffected by expression of Rab4a, Rab11a or the dominant negative mutants. Thus, Rab4a- and Rab11a-dependent recycling of the NK1R is required for rapid resensitization.

Loss and Recovery of SP Binding Sites at the Cell Surface—To determine how the concentration of SP affects the rate of endocytosis of the NK1R, we incubated KNRK-NK1R cells with [³H]SP and 1 or 10 nM unlabeled SP for 2.5–10 min at 37 °C. Cells were washed with acid to separate cell surface (acid-labile) from internalized (acid-resistant) label. In cells incubated with 1 or 10 nM unlabeled SP, there was a rapid endocytosis of [³H]SP, such that >50% of specifically bound SP was internalized within 10 min at 37 °C (Fig. 10A).

To quantify the loss and recovery of SP binding sites at the cell surface, we exposed KNRK-NK1R cells to 1 or 10 nM SP for...
Cells exposed to 10 nM SP, there was a rapid loss of surface SP wash. Compared with unstimulated cells, incubation with 1 nM rab11a, or Rab11aS25N were preincubated with 1 nM SP for 10 min at 37 °C, washed, and challenged again with 1 nM SP at 5, 10, or 20 min after the washing. Results are expressed as a percentage of responses in cells not preincubated with SP. Note that expression of wild-type and mutant Rab4a and 11a markedly impeded resensitization. *p < 0.05 compared with NK1R cells (analysis of variance and Student-Newman-Kuels test) (n = 3 experiments).

To determine whether the loss of surface binding sites for SP is due to a reduction in the number of receptors at the cell surface or a reduction in the affinity of surface receptors for SP, we analyzed SP binding by Scatchard analysis. KNRK-NK1R cells were incubated with 1 or 10 nM SP for 10 min at 37 °C. Cells were washed, and surface binding sites were quantified by incubating the cells with [3H]SP and graded concentrations of unlabeled SP (0.001–100 nM) for 90 min at 4 °C. Cells were washed, and surface-bound ligand was collected by an acid wash. Compared with unstimulated cells, incubation with 1 nM SP had no effect on the affinity of SP binding to surface NK1R (Fig. 8A). This observation indicates that the NK1R can bind SP but not signal at this time.

The change in SP binding observed after NK1R stimulation is mediated by an alteration in the number of binding sites and not a change in the affinity of NK1R.

**DISCUSSION**

**Concentration-dependent Trafficking on the NK1R**—The first aim of this investigation was to determine how the SP concentration affects intracellular trafficking of the NK1R. Our results show that the concentration of SP has a dramatic effect on the pathway of NK1R trafficking. A high concentration of SP (10 nM) induced sequestration of NK1R, β-arrestin 1, and Rab5a into sorting endosomes in a perinuclear location. The NK1R remained associated with β-arrestin 1 in these endosomes for prolonged periods (>60 min), and SP binding sites did not recover at the cell surface until >30 min after removal of the ligand. These observations are in agreement with previous studies of NK1R trafficking using similar concentrations of SP (15, 26, 40, 41). The slow recycling of the NK1R by the long pathway after exposure to high concentrations of SP is prevented by inhibitors of endosomal acidification and by a phosphatase inhibitor (15, 16). These results are consistent with the hypothesis that recycling requires receptor endocytosis, dissociation of SP from the NK1R in acidified endosomes, dephosphorylation of the NK1R by endosomal phosphatases, and dissociation of β-arrestins.

Exposure to a low concentration of SP (1 nM) triggers a different pathway of NK1R recycling. Here the NK1R and β-arrestin 1 colocalized in endosomes located immediately beneath the plasma membrane. The NK1R rapidly returned to the plasma membrane, and β-arrestin 1 redistributed to the cytosol. During stimulation with 1 nM SP, binding sites rapidly returned to the cell surface. Although bafilomycin and okadaic acid diminished receptor resensitization at early time points, they had no effect on resensitization after 10 min. These results suggest that endosomal acidification and endosomal phosphatases play a minor role in rapid recycling and resensitization of the NK1R.

We hypothesize that the extent of agonist-induced phosphorylation of the NK1R determines association with β-arrestins and regulates the pathway and kinetics of NK1R trafficking. The capacity of GPCRs, including the NK1R, to form stable and high affinity interactions with β-arrestin 1 and 2 depends on phosphorylation of clusters of serine and threonine residues in the carboxyl-terminal domains of receptors (30–33). The NK1R contains numerous serine and threonine residues in the carboxyl terminus, and a truncated NK1R, which lacks many of these residues, fails to interact with β-arrestins (12). Although the precise sites of SP-induced phosphorylation of the NK1R are unknown, GRK2, GRK3, and GRK5 can phosphorylate the NK1R in reconstituted systems and membrane preparations (42–44). In intact cells, SP induces a concentration-dependent phosphorylation of the human and rat NK1R that is probably mediated by GRKs, since it is unaffected by inhibition of protein kinase C, which can also phosphorylate the receptor (34, 45). Of direct relevance to the present work, 1 nM SP induces minimal phosphorylation of the rat NK1R expressed in KNRK cells, whereas 10 nM SP causes extensive phosphorylation (34).

Thus, although we did not examine SP-induced phosphorylation of the NK1R in the present study, these results are consistent with the hypothesis that the extent of phosphorylation affects interaction with β-arrestin 1 and 2 and thereby controls the pathway of receptor trafficking. After stimulation with high concentrations of SP that are known to cause extensive phosphorylation of the NK1R, the NK1R and β-arrestins remain colocalized for prolonged periods in sorting endosomes, and recycling and resensitization are slow and heavily dependent on endosomal phosphatases. In contrast, after stimulation with low concentrations of SP that induce minimal phosphorylation of the NK1R, the NK1R colocalizes with β-arrestins only transiently, and recycling and resensitization are rapid and minimally dependent on phosphatase activity. Confirmation of this hypothesis will require identification of the NK1R residues that are phosphorylated in response to different concentrations of SP and evaluation of the importance of this phosphorylation for association of the NK1R with β-arrestins and other trafficking proteins.

**Rab-mediated Trafficking of the NK1R**—Our second aim was to determine the role of Rab GTPases in intracellular trafficking of the NK1R. Rab GTPases participate in multiple steps of vesicular transport. By expressing GFP-tagged Rabs, we investigated the translocation of the NK1R through different endosomal compartments. Rab5a participates in endocytosis and intracellular trafficking of receptors as well as in the formation of early endosomes (19). Activation of Rab5a is required for endocytosis of certain receptors. For example, expression of
Rab5aS34N causes retention of the transferrin receptor, β2AR, and muscarinic 4A receptor at the plasma membrane, indicating that GTP-bound Rab5a is crucial for vesicle budding from the plasma membrane (20, 22–24, 46). In contrast, we observed that Rab5aS34N did not substantively inhibit endocytosis of the NK1R but instead induced its retention in vesicles located beneath the plasma membrane. Overexpression of wild-type Rab5a altered the trafficking of the NK1R from endosomes in a superficial to a perinuclear location. Together, our results suggest that GDP/GTP exchange on Rab5a is a critical control mechanism in NK1R trafficking. GTP-bound Rab5a may interact with the NK1R in superficial vesicles and mediate translocation of the receptor to sorting endosomes in a perinuclear region. In support of our results, Rab5aS34N does not prevent endocytosis of the AT1AR but impedes its translocation to perinuclear sorting endosomes (25).

Less is known about the role of Rab4a and Rab11a in GPCR trafficking. After endocytosis, the transferrin receptor traffics to endosomes containing Rab5a, Rab4a, and then Rab11a (27). Thus, Rab4a may regulate trafficking of receptors between Rab5a-positive early endosomes and Rab11a-positive recycling endosomes. Our results show that the NK1R rapidly enters Rab4a-positive sorting endosomes. Expression of GDP/GTP-binding deficient Rab4aS24N or wild-type Rab4a did not markedly influence endocytosis or recycling of the NK1R, as detected by immunofluorescence. In contrast to Rab5a, Rab4a appears not to be involved in directing the intracellular transport of NK1R into perinuclear compartments. However, overexpression of Rab4aS24N or wild-type Rab4a delayed resensitization of NK1R. We cannot explain why overexpression of Rab4aS24N or Rab4a did not affect recycling of immunoreactive NK1R but did inhibit resensitization of response to SP. Since the Rab4a-GFP constructs were expressed at markedly higher levels than the endogenous Rab4a, it is unlikely that these results are due to the inability of the constructs to interfere with Rab4a-mediated trafficking. However, Rab4a may be required for normal processing of the NK1R that is necessary for resensitization but not recycling.

SP induced a marked redistribution of Rab11a to recycling endosomes, where there was prominent colocalization with the NK1R. Rab11aS25N did not affect endocytosis of the NK1R but caused its retention in endosomes and thus inhibited NK1R recycling. Overexpression of Rab11aS25N or wild-type Rab11a also impeded resensitization of the NK1R. These results suggest that Rab11a plays a major role in trafficking of the NK1R from early or recycling endosomes to the plasma membrane and thereby contributes to resensitization. In support of our results, Rab11aS25N reduces recycling of the β2AR. Overexpression of Rab11a promotes recycling of the AT1AR (29).

Mechanisms of Intracellular Trafficking of the NK1R—The carboxyl terminus of the AT1AR contains several domains that interact with Rab5a, including a Rab5a binding domain and a guanine-nucleotide exchange element, which determine the interaction of the receptor with Rab5a and thereby control its intracellular trafficking (25). Deletion of the guanine-nucleotide exchange element (AT1RΔ349) does not prevent receptor endocytosis, but the truncated receptor fails to translocate to large perinuclear endosomes and instead is retained in small vesicles that do not completely colocalize with Rab5a (25). We hypothesize that the NK1R has similar regulatory domains in the carboxyl terminus. We have previously reported that a point mutation within a putative Rab5a binding domain of the NK1R (Y305F) at the beginning of the carboxyl tail and truncation of the NK1R to delete the putative guanine nucleotide exchange factor (NK1RΔ354) prevent the perinuclear translocation of the NK1R and instead result in its accumulation in superficially located endosomes after stimulation with SP (40). These mutations have a similar effect on trafficking of the NK1R as that observed after expression of Rab5aS34N, further supporting the existence of Rab5a-interacting domains in the carboxyl terminus of the NK1R.

The interaction of the NK1R with Rab5a is probably required for translocation of the receptor to perinuclear sorting endosomes, which specifies the long pathway of receptor recycling. Since the extent of NK1R phosphorylation appears to correlate with the pathway of recycling, we hypothesize that the degree of phosphorylation determines the interaction between the NK1R and Rab5a and thus defines the pathway and kinetics of NK1R recycling. Maximal phosphorylation of the carboxyl ter-
minus of the NK1R may favor a loop conformation that permits the guanine-nucleotide exchange element to stabilize the NK1R-Rab5a(GTP) interaction. Association of Rab5a(GTP) with NK1R would thus direct the NK1R to the long pathway of recycling. Conversely, minimally phosphorylated NK1R may adopt a conformation in which the guanine-nucleotide exchange element remains separated from the NK1R-associated Rab5a. As a result, the NK1R-Rab5a interaction is not stabilized, which prevents the perinuclear translocation of NK1R. Experiments to investigate these hypotheses are under way.

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