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Delineation of the Endocytic Pathway of Substance P and Its Seven-Transmembrane Domain NK1 Receptor

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Many of the actions of the neuropeptide substance P (SP) that are mediated by the neurokinin 1 receptor (NK1-R) desensitize and resensitize, which may be associated with NK1-R endocytosis and recycling. We delineated this endocytic pathway in transfected cells by confocal microscopy using cyanine 3-SP and NK1-R antibodies. SP and the NK1-R were internalized into the same clathrin immunoreactive vesicles, and then sorted into different compartments. The NK1-R was colocalized with a marker of early endosomes, but not with markers of late endosomes or lysosomes. We quantified the NK1-R at the cell surface by incubating cells with an antibody to an extracellular epitope. After exposure to SP, there was a loss and subsequent recovery of surface NK1-R. The loss was prevented by hypertonic sucrose and potassium depletion, inhibitors of clathrin-mediated endocytosis. Recovery was independent of new protein synthesis because it was unaffected by cycloheximide. Recovery required endosomal acidification because it was prevented by an H^+-ATPase inhibitor. The fate of internalized ^{125}I-SP was examined by chromatography. SP was intact at the cell surface and in early endosomes, but slowly degraded in perinuclear vesicles. We conclude that SP induces clathrin-dependent internalization of the NK1-R. The SP/NK1-R complex dissociates in acidified endosomes. SP is degraded, whereas the NK1-R recycles to the cell surface.

INTRODUCTION

Endocytosis of cell surface receptors and their ligands and subsequent intracellular sorting are fundamental processes with diverse functions (Goldstein *et al.*, 1985; Trowbridge *et al.*, 1993). The pathway of endocytosis and sorting has been delineated for many of the single transmembrane domain receptors. Some receptors, exemplified by the transferrin receptor (Tf-R)[1] and the low density lipoprotein receptor, are constitutively internalized via clathrin-coated pits into early endosomes (Pearse, 1982; Larkin *et al.*, 1983; Heuser and Anderson, 1989). Other receptors, such as the epidermal growth factor receptor, are internalized after ligand binding (Dunn *et al.*, 1986). Once internalized, receptors and their ligands are sorted in acidified endosomes into distinct pathways. Iron dissociates from transferrin in early endosomes (also known as the sorting endosomes), and transferrin and its receptor return to the cell surface in recycling endosomes (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983). Low density lipoprotein and epidermal growth factor also dissociate from their receptors and then progress to late endosomes and lysosomes, where these ligands are degraded (Carpenter and Cohen, 1976; Goldstein *et al.*, 1985). The low density lipoprotein receptor generally returns in recycling endosomes to the cell surface, although it may also recycle through the Golgi apparatus (Green and Kelly, 1992). In contrast, the epidermal growth factor receptor is degraded with its ligand

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[1] Abbreviations used: BSA, bovine serum albumin; CI MP-R, cation-independent mannose 6-phosphate receptor; cy3-SP, cyanine 3.18-labeled substance P; DMEM, Dulbecco's modified Eagle's medium; HPLC, high pressure liquid chromatography; KNRK cells, Kirsten murine sarcoma virus-transformed rat kidney cells; NK1-R, neurokinin 1 receptor; PBS, phosphate-buffered saline; SP, substance P; Tf-R, transferrin receptor.

in lysosomes (Stoscheck and Carpenter, 1984). Endocytosis and recycling of the Tf-R and low density lipoprotein receptor allow for the continued uptake of low density lipoproteins and iron by a wide variety of cells, whereas endosomes containing the epidermal growth factor receptor participate in signal transduction (Kay *et al.*, 1986).

Far less is known about endocytosis of receptors with seven-transmembrane domains, a large and functionally diverse family that includes receptors for many neurotransmitters and hormones. Some of these receptors, such as the β_2-adrenergic and thrombin receptors, are internalized into early endosomes within minutes of ligand binding, and subsequently sorted into recycling or degradative pathways (von Zastrow and Kobilka, 1992; Hoxie *et al.*, 1993; Brass *et al.*, 1994). However, for many receptors with seven-transmembrane domains the pathway of endocytosis and sorting into recycling or degradative pathways has not been delineated, and the intracellular fate of the internalized ligand is unknown. The internalization and recycling of these receptors is of considerable functional importance because it contributes to desensitization, resensitization, and down-regulation of cellular responses to ligands.

The seven-transmembrane domain neurokinin 1 receptor (NK1-R) mediates many of the actions of the neuropeptide substance P (SP) (Otsuka and Yoshioka, 1993). SP induces internalization of the NK1-R both in transfected epithelial cells and in endothelial cells of post-capillary venules in the rat (Bowden *et al.*, 1994; Garland *et al.*, 1994). However, the pathway of endocytosis of NK1-R and SP has not been delineated.

We examined endocytosis of the NK1-R and SP using NK1-R antibodies, fluorescent SP, and ^{125}I-SP to achieve the following: 1) simultaneously observe endocytosis of ligand and receptor; 2) delineate the intracellular pathway of SP and the NK1-R using markers of early endosomes (Tf-R), late endosomes (cation-independent mannose 6-phosphate receptor, [CI MP-R]), and lysosomes; 3) determine the role of clathrin in endocytosis; 4) examine NK1-R recycling using inhibitors of protein synthesis and endosomal acidification; and 5) determine the location and mechanism of SP degradation. The results show that SP and the NK1-R are rapidly internalized via clathrin-coated pits into the same endosomes. SP dissociates from the NK1-R in an acidified perinuclear compartment. SP is degraded intracellularly whereas the NK1-R recycles to the plasma membrane.

MATERIALS AND METHODS

Reagents and Antibodies

SP was purchased from Bachem Bioscience (King of Prussia, PA). Bolton-Hunter ^{125}I-SP (2000 Ci/mmol) was obtained from Amersham (Chicago, IL). Bis-functional cyanine 3.18 was a gift from Dr. Lauren Ernst, Biological Detection Systems (Pittsburgh, PA). Bafilomycin A_1 was a gift from Dr. Jonathan R. Green, Ciba-Geigy (Basel, Switzerland). The NK1-R antagonists CP 96,345 and RP 67589 were obtained from Pfizer (Groton, CT) and Rhone-Poulenc (Vitry-sur-Seine, France), respectively.

A rabbit polyclonal antiserum (#11884–5) to the intracellular C-terminus of the rat NK1-R, and a mouse monoclonal antibody (M2) to the Flag peptide from International Biotechnologies (New Haven, CT), have been fully characterized (Vigna *et al.*, 1994). A mouse monoclonal antibody to the Tf-R was a gift from Dr. Ian Trowbridge (The Salk Institute, San Diego, CA). A rabbit polyclonal antibody to the CI MP-R was a gift from Dr. Rae Lyn Burke (Chiron, Emeryville, CA). A mouse monoclonal antibody (GM10) to membranes from insulin secretory granules, which recognizes lysosomes (Grimaldi *et al.*, 1987), was a gift from Dr. John Hutton (Cambridge, UK). A mouse monoclonal antibody (X22) to the heavy chain of clathrin was a gift from Dr. Francis Brodsky (University of California, San Francisco, CA). FITC-conjugated swine anti-rabbit IgG was obtained from DAKO (Carpinteria, CA). Affinity-purified FITC-conjugated goat anti-mouse IgG and rhodamine-conjugated and Texas Red-conjugated goat anti-rabbit IgG were obtained from Cappel Research Products (Durham, NC). ^{125}I-sheep anti-mouse Ig (species-specific F (ab')$_2$) were obtained from Amersham Corporation.

Generation and Characterization of Fluorescently Labeled SP

We labeled SP with the fluorophore cyanine 3.18 (Bunnett *et al.*, 1995) to allow observation of SP endocytosis. SP (0.66 mM) was incubated in 0.1 M NaH_2CO_3 (pH 9.3) with 0.3 mM cyanine 3.18 for 30 min at room temperature, 1/10 vol of 0.1 M glycine (pH 9.3) was added, and the products were separated by reverse-phase high pressure liquid chromatography (HPLC) using a C-18 column (Garland *et al.*, 1994; Bunnett *et al.*, 1995). Unlabeled SP eluted from the column at 18.1 min, and cy3-SP eluted at 19.4 min. Mass spectrometry revealed that the molecular weight of the cy3-SP was 2028.6, suggesting that SP was labeled with a single cyanine 3.18 molecule on the Lys^3 residue. Cy3-SP induced a prompt increase in $[Ca^{2+}]_i$ in KNRK Flag NK1-R cells (below) with an EC_{50} of 0.45 nM, measured using Fura-2/AM, similar to that of unlabeled SP (Okamoto *et al.*, 1994; Vigna *et al.*, 1994). The specificity of cy3-SP binding to KNKR Flag-NK1R cells and the homogeneity of the cell population were examined by cell sorting and microscopy (Bunnett *et al.*, 1995). When KNRK Flag-NK1R cells were incubated with cy3-SP, there was at least one log-fold greater intensity in the cyanine 3 channel than when KNRK CMV control cells were incubated with cy3-SP. The uniformity of the peak indicated that most cells expressed similar levels of NK1-R. When 100 nM cy3-SP was incubated with KNRK Flag NK1-R cells for 60 min at 4°C, a strong, crisp signal was localized to the plasma membrane. Pre-incubation with 1 μM unlabeled SP, 10 μM CP 96,345 or 1 μM RP 67589 eliminated surface binding. There was no surface labeling when cy3-SP was incubated with KNRK CMV cells. There was no detectable cy3-SP in endosomes when KNRK CMV cells were incubated with 100 nM cy3-SP for 60 min at 4°C, washed, and incubated in SP-free medium at 37°C for 10 min. Thus, SP is not taken up by receptor-independent mechanisms in these cells.

Cell Culture

Kirsten murine sarcoma virus-transformed rat kidney cells (KNRK, American Type Culture Collection, Rockville, MD) were transfected with cDNA encoding the rat NK1-R and an N-terminal Flag peptide in pcDNA I Neo, or with pRC/CMV as a control (Okamoto *et al.*, 1994; Vigna *et al.*, 1994). KNRK Flag NK1-R and KNRK CMV cells were maintained as described (Vigna *et al.*, 1994). The Flag-NK1-R has been characterized by SP binding (K_d 5.63 ± 1.54 nM, ~80,000 binding sites per cell) and SP-induced calcium mobilization (EC_{50}

0.66 nM) (Vigna *et al.*, 1994). Cells were plated 24–48 h before use on poly-L-lysine–coated glass coverslips or plastic wells. During experiments, the cells were incubated in DMEM containing 0.1% bovine serum albumin (DMEM-BSA).

Microscopical Examination of Endocytosis of the NK1-R and Cy3-SP

We used NK1-R and Flag antibodies and cy3-SP to localize internalized receptor and its ligand. To identify the cellular compartments containing the NK1-R and cy3-SP, we used antibodies to the Tf-R (a marker for early endosomes), the CI MP-R (late endosomes), lysosomes (GM10 antibody), and clathrin (coated pits).

Cells were incubated with 10 nM unlabeled SP or 100 nM cy3-SP in DMEM-BSA for 60 min at 4°C for equilibrium binding, washed, and medium at 37°C was added (for optimal visualization it was necessary to use cy3-SP at a 10-fold higher concentration). To allow endocytosis, cells were incubated at 37°C for 0–8 h, and then fixed with 4% paraformaldehyde in 100 mM PBS (pH 7.4) for 20 min at 4°C. Cells exposed to unlabeled SP were incubated in PBS with 1% normal goat serum and 0.1% saponin for three periods of 5 min, and primary antibodies (anti-NK1-R 1:1000–1:8000; anti-Flag 10 μg/ml; anti-clathrin, 1–5 μg/ml; anti-Tf-R, 1:4000; anti-CI MP-R 1:2000; and GM10 1:6000) were added for 2–4 h at 37°C. For cells exposed to cy3-SP, the concentration of saponin was reduced to 0.0025–0.05% and primary antibodies were added for 1–2 h at 4°C, to optimally maintain the cyanine 3.18 signal. Cells were washed and incubated with secondary antibodies (1:50 or 1:200) for 60 min at room temperature. The cells were washed, fixed as described, and mounted. Specificity of the NK1-R and Flag antibodies was confirmed by pre-absorption with the appropriate peptide antigen and by staining KNRK CMV cells (Vigna *et al.*, 1994). For double labeling, cells were incubated simultaneously with the primary antibodies (polyclonal NK1-R antibody plus monoclonal clathrin, Tf-R, or GM10 antibodies; monoclonal Flag antibody plus polyclonal CI MP-R antibody), washed, and then incubated with a mixture of affinity-purified fluorescent antibodies raised in goats. The specificity of the staining in double-labeling experiments was verified by omitting one of the primary antibodies. Endocytosis of cy3-SP was also directly observed in living cells. The cells were incubated with 100 nM cy3-SP for 60 min at 4°C, washed, and transferred to the stage of an inverted microscope equipped with a microincubator (PDMI-2, Medical Systems, Greenvale, NY), which maintained the temperature at 18°C or 37°C.

Cells were examined with Zeiss Axioplan or Axiovert microscopes, and images were captured using a cooled CCD color video camera system (ZVS-47EC, Optronics Engineering, Goleta, CA) directly to a Macintosh Centris 650 computer using a ColorSnap 32+ video capture board (Computer Friends, Portland, OR) (image size 640 × 480 pixels). Cells were also examined using a Zeiss Laser Scan Inverted 410 microscope with an argon-krypton laser, and images of 512 × 512 pixels were obtained. Images were processed using Adobe Photoshop 2.5 (Adobe Systems, Mountain View, CA).

Quantification of Cell-Surface Flag Immunoreactivity

We quantified Flag immunoreactivity at the cell surface at various times after incubation with SP by reacting nonpermeabilized cells with an antibody to the extracellular Flag epitope, followed by a ^{125}I-labeled secondary antibody. Cells were incubated with 10 pM-1 μM SP in 250 μl DMEM-BSA for 60 min at 4°C, washed, and incubated at 37°C for 0–4 h. Flag antibody (0.1 μg/ml) was added for 60 min at 4°C. Cells were washed, and incubated with ^{125}I-sheep anti-mouse Ig (0.1 μCi/well) for 60 min at 4°C. Cells were washed in PBS at 4°C, and lysed with 0.5 M NaOH overnight at room temperature. Radioactivity and protein content of the lysate were measured. Binding was normalized for protein content or cell number (expressed as cpm/mg protein or cpm/10^6 cells). Specific binding was determined by subtracting nonspecific binding to KNRK CMV cells from total binding. Nonspecific binding to KNRK CMV cells was 2.17 ± 0.34% of that measured in KNRK Flag NK1-R cells (mean ± SE, triplicate observations, n = 6 experiments). Specific binding of the Flag antibody to KNRK Flag NK1-R cells was unaffected by incubation for 0–240 min at 37°C and by previous exposure to SP at 4°C. Therefore, surface Flag immunoreactivity remained constant without exposure to agonist and the ability of the Flag antibody to bind the receptor was unaffected by SP binding. When the effects of drugs were examined, each treatment group had its own control of cells that were not exposed to SP but otherwise treated identically. Treatment with drugs alone did not consistently affect surface Flag immunoreactivity. To calculate the extent of SP-induced internalization, we compared the specific binding of KNRK Flag NK1-R cells treated with SP to that of cells incubated with medium alone.

Degradation of Internalized SP

We examined the metabolic fate of internalized ^{125}I-SP by HPLC. Cells were incubated with 0.5 nM ^{125}I-SP plus 9.5 nM unlabeled SP in DMEM-BSA for 60 min at 4°C, washed, and incubated at 37°C for 0–60 min. Cells were treated with 0.2 M acetic acid and 50 mM NaCl (pH 2.5) on ice for 5 min to separate acid-sensitive (cell surface) label from acid-resistant (internalized) label (Garland *et al.*, 1994). Cell surface and internalized fractions were analyzed by reverse-phase HPLC as previously described (Garland *et al.*, 1994).

Drug Treatments

The effects of inhibitors of endocytosis on internalization of the NK1-R were quantified in binding experiments with the Flag antibody. Hyperosmolar sucrose and potassium depletion block formation of clathrin-coated pits (Larkin *et al.*, 1983; Heuser and Anderson, 1989; Hansen *et al.*, 1993a), and phenylarsine oxide cross links proteins that have sulfur groups (Frost and Lane, 1985). Cells were pre-incubated with 0.45 M sucrose for 30 min at 37°C and 0.45 M sucrose was added to all subsequent solutions (Garland *et al.*, 1994). Cells were pre-incubated with 80 μM phenylarsine oxide for 5 min at 37°C, washed, and allowed to recover for 30–60 min at 37°C (Garland *et al.*, 1994). Cells were potassium depleted exactly as described (Larkin *et al.*, 1983; Hansen *et al.*, 1993a).

The effects of cycloheximide, brefeldin A, and acidotropic agents on endocytosis of the NK1-R and intracellular degradation of ^{125}I-SP were examined. Brefeldin A causes disassembly of the Golgi apparatus and mixing with the endoplasmic reticulum, and induces alterations in the morphological appearance of endosomes and lysosomes (Klausner *et al.*, 1992). Acidotropic agents included bafilomycin A_1, an inhibitor of vacuolar-type H^+-ATPase (Yoshimori *et al.*, 1991), and monensin, NH_4Cl, and chloroquine, which prevent intracellular degradation of ^{125}I-SP (Garland *et al.*, 1994). Cells were preincubated with 70 μM cycloheximide, 1 μM bafilomycin A_1, 50 μM monensin, 10 mM NH_4Cl, 500 μM chloroquine, or 10 μg/ml brefeldin A for 30–60 min at 37°C before addition of SP, and the drugs were included in all solutions during the experiment. In all experiments control cells were incubated with appropriate carrier solutions. We confirmed that 70 μM cycloheximide prevented protein synthesis by metabolic labeling of cells with [^{35}S]methionine (Wikström and Lodish, 1991). Thus, incorporation of [^{35}S]methionine into cells was reduced by 95% by treatment with cycloheximide. We used acridine orange, as described, to confirm that 1 μM bafilomycin A_1 abolished the orange fluorescence of cytoplasmic vesicles and thus prevented acidification (Yoshimori *et al.*, 1991).

Data Analysis

Results are expressed as mean ± SE. All microscopy experiments were repeated at least three times with two coverslips per observa-

tion. Slides were reviewed independently by three investigators to avoid bias, and representative cells are presented as photomicrographs. Binding experiments with the Flag antibody were repeated at least three times, with triplicate observations per experiment. Degradation experiments with ^{125}I-SP were repeated four times. Differences between multiple groups were examined by an analysis of variance and a Bonferroni *t*-test. Differences between two groups were examined by Student's *t*-test. A $p < 0.05$ was considered statistically significant.

RESULTS

The NK1-R and Cy3-SP Were Internalized into the Same Endosomes and then Sorted into Different Compartments

Endocytosis of SP and the NK1-R was examined simultaneously in KNRK Flag NK1-R cells. Cells were incubated with cy3-SP at 4°C, washed, warmed to 37°C, and processed for immunofluorescence using the NK1-R antibody and a FITC-labeled secondary antibody. Without warming, both cy3-SP and the NK1-R were confined to the cell surface (Figure 1, A and E). After 10 min at 37°C, they were still co-localized but now were present in numerous small vesicles under the plasma membrane (Figure 1, B and F). These are early endosomes because cy3-SP and FITC-labeled transferrin are internalized into the same vesicles after 10 min of incubation at 37°C (Garland *et al.*, 1994). At this time, all of the detectable cy3-SP was removed from the cell surface and was present in endosomes. In contrast, some NK1-R immunoreactivity was still detected on the cell surface (Figure 1B, arrowheads), although the staining intensity was reduced compared with that observed before warming. After 30 min of incubation at 37°C, cy3-SP and the NK1-R were present in perinuclear vesicles (Figure 1, C and G). Some colocalization was observed. After 60 and 120 min, the cy3-SP remained in the center of the cell, where it was present in large vesicles that did not contain NK1-R immunoreactivity (Figure 1D, arrowheads). In sharp contrast, the NK1-R was detected in numerous very small vesicles located beneath the plasma membrane, at the plasma membrane itself, and in larger perinuclear vesicles (Figure 1H). At later times, the cy3-SP signal was weak and diffuse and could not be detected in distinct vesicles or at the cell surface. After 4–8 h, the intensity of the NK1-R immunoreactivity at the plasma membrane was greater, and intracellular staining declined.

To verify the precise timing of endocytosis, we directly observed internalization of cy3-SP in living KNRK Flag-NK1R cells. At 4°C, cy3-SP was confined to the plasma membrane. Within 2 min of incubation at 37°C, cy3-SP was found in numerous small vesicles underlying the plasma membrane, and there was diminished surface staining. After 20 min, cy3-SP was evident in larger vesicles located in a perinuclear region of the cell. In parallel experiments, after equilibrium binding of cy3-SP, the temperature was raised to 18°C, which is permissive for endocytosis but does not allow delivery to lysosomes (Griffiths *et al.*, 1988). Within 2 min of incubation at 18°C, cy3-SP was internalized into small vesicles beneath the plasma membrane. Cy3-SP remained in small, peripherally located vesicles when the cells were maintained at 18°C for up to 40 min. However, when these same cells were warmed to 37°C, cy3-SP entered larger, more centrally located vesicles.

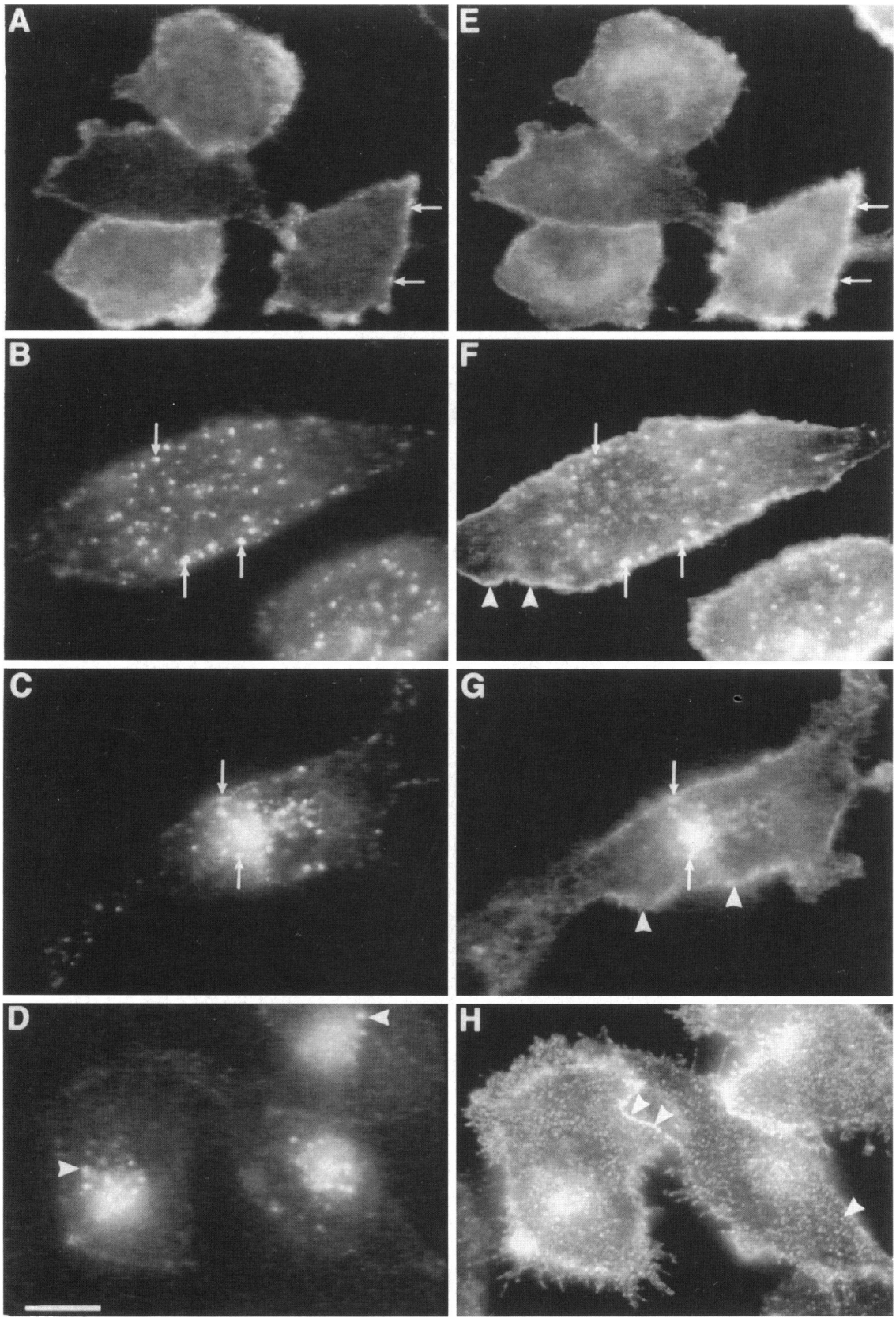
The NK1-R Was Usually Colocalized with the Tf-R but not with the CI MP-R or a Lysosomal Marker

The organelles containing the internalized NK1-R were identified by reacting cells with NK1-R or Flag antibodies and antibodies to the Tf-R, the CI MP-R, and lysosomes. Both the NK1-R and Flag antibodies give a similar pattern of labeling in KNRK Flag NK1-R cells (Garland *et al.*, 1994). KNRK Flag NK1-R cells were incubated with unlabeled SP at 4°C, washed, warmed to 37°C, and processed for immunofluorescence.

At 4°C, NK1-R immunoreactivity was confined to the cell surface and Tf-R immunoreactivity was predominantly localized in vesicles located beneath the plasma membrane and in a perinuclear region. Internalized NK1-R was mostly co-localized with the Tf-R at all time points studied (10–120 min at 37°C). At 30 min, optical sections just below the plasma membrane or through the center of cells showed multiple small vesicles throughout the cytoplasm that contained NK1-R and Tf-R (Figure 2, A and B). Colocalization in the same vesicles was confirmed by superimposition of the images, which gave a yellow signal (Figure 2C). Vesicles containing NK1-R immunoreactivity invariably contained Tf-R immunoreactivity, although some vesicles contained Tf-R but not NK1-R. Thus, internalized NK1-R is usually colocalized in endosomes with a recycling receptor.

After 30–60 min at 37°C, the CI MP-R was present in vesicles in the same perinuclear region as those vesicles containing the NK1-R (localized with the

Figure 1. Simultaneous detection of cy3-SP (left panels) and NK1-R immunoreactivity (right panels) in KNRK Flag NK1-R cells at various times after warming to 37°C. Cells were incubated with 100 nM cy3-SP for 60 min at 4°C, washed, and incubated in SP-free medium at 37°C for 0 min (A and E), 10 min (B and F), 30 min (C and G), or 120 min (D and H). Cells were fixed and incubated with the NK1-R antibody and a FITC-labeled secondary antibody. Note the colocalization (arrows) of cy3-SP and NK1R-immunoreactivity at 0, 10, and 30 min after warming. By 120 min, cy3-SP and NK1-R immunoreactivity are in different cellular compartments (arrowheads). Bar, 10 μm.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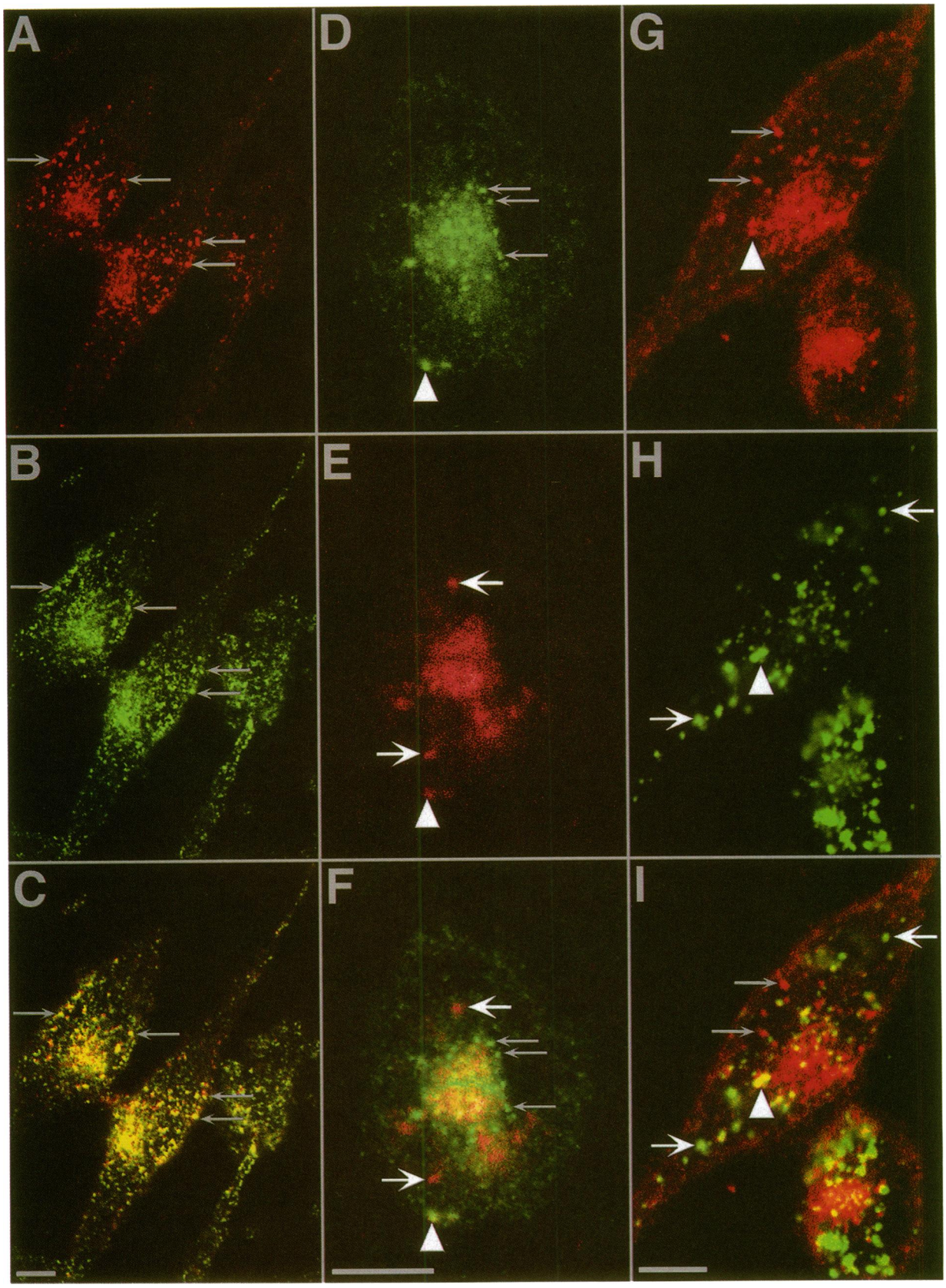
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Flag antibody) and Tf-R. However, close examination by confocal microscopy indicated that these receptors were usually in different vesicles at both time points. At 60 min, the Flag immunoreactivity was concentrated in numerous small perinuclear vesicles (Figure 2D, small arrows), which were distinct from the larger, more diffusely stained structures containing CI MP-R immunoreactivity (Figure 2E, large arrows). A rare instance of colocalization is shown by an arrowhead in Figure 2, D–F.

The GM10 antibody stained spherical and oval lysosomes that were randomly distributed both in the periphery and the center of KNRK Flag NK1-R cells. At 30–60 min after warming to 37°C to induce endocytosis, NK1-R immunoreactivity was located in small perinuclear vesicles (Figure 2G, small arrows). These were distinct from the larger vesicles stained with the GM10 antibody (Figure 2H, large arrows). A rare instance of colocalization is shown by the arrowhead in Figure 2, G–I. Colocalization of the NK1-R immunoreactivity with lysosomes was rare even when cells were treated with 10 mM NH_4Cl, to prevent possible receptor degradation.

The NK1-R and Cy3-SP Were Colocalized with Clathrin at Early Time Points

To determine whether SP internalization is associated with clathrin-coated pits, KNRK Flag NK1-R cells were incubated with cy3-SP at 4°C, washed, warmed to 37°C, and processed for immunofluorescence using an antibody to clathrin. Clathrin immunoreactivity was detected at the plasma membrane, and in vesicles in a central region. At 4°C, cy3-SP was uniformly distributed at the cell surface. After 30 s to 2 min at 37°C, cy3-SP was clustered in patches at the cell surface and also localized in small vesicles beneath the plasma membrane (Figure 3, A and D). The patches and some of the vesicles were associated with clathrin immunoreactivity (Figure 3, B and E). Superimposition of these images gave a yellow signal in areas of colocalization (Figure 3, C and F). After 5 min, few of the vesicles containing cy3-SP were stained by the clathrin antibody. Similar results were obtained when cells were incubated with unlabeled SP and then reacted with antibodies to the NK1-R and clathrin. After 10 and 30 min at 37°C, vesicles in peripheral and central regions of the cell containing NK1-R immunoreactivity were not stained by the clathrin antibody.

Hyperosmolar Sucrose, Potassium Depletion, and Phenylarsine Oxide Inhibited Loss of Cell Surface NK1-R

We used an antibody to the extracellular Flag epitope to quantify cell surface receptors based on immunoreactivity rather than function. Cell-surface Flag immunoreactivity was markedly reduced when KNRK Flag NK1-R cells were incubated with SP at 4°C, washed, and warmed to 37°C (Figure 4A). The largest reduction occurred within the first 10 min at 37°C, and after 30 min the surface immunoreactivity had declined to 60.3 ± 2.8% (n = 13 experiments) of control levels (no SP). After 240 min, surface immunoreactivity had returned to 89.9 ± 3.7% (n = 9 experiments) of the control. The return was complete after 8 h. The extent of the loss of surface Flag immunoreactivity at 30 min was dependent on the concentration of SP, and was maximal at 1 μM SP and half maximal at ~0.5 nM SP (Figure 4B).

The immunofluorescence experiments with the NK1-R and Flag antibodies showed that SP induced internalization of the NK1-R at 37°C. Thus, the decline in cell-surface Flag immunoreactivity after incubation with SP and warming probably represents internalization of the NK1-R, and permits direct quantification of receptor endocytosis. The mechanism of this endocytosis was examined by treating cells with hyperosmolar sucrose or phenylarsine oxide, or by potassium depletion. In KNRK Flag NK1-R cells, SP induced a marked decline in surface Flag immunoreactivity after 30 min at 37°C. This decline was significantly attenuated by hyperosmolar sucrose, potassium depletion, and phenylarsine oxide (Table 1). These results, together with the observation that cy3-SP and the NK1-R co-localize with clathrin during the early

Figure 2. Confocal photomicrographs of KNRK Flag NK1-R cells stained to localize the NK1-R, Tf-R, CI MP-R, and lysosomes. Cells were incubated with 10 nM unlabeled SP for 60 min at 4°C, washed, and incubated in SP-free medium at 37°C for 30 min (A–C) or 60 min (D–I), and fixed. (A–C) Cells were incubated with the polyclonal NK1-R antibody and monoclonal Tf-R antibody, followed by Texas Red-labeled goat anti-rabbit IgG and FITC-labeled goat anti-mouse IgG. An optical section through the base of the cells is shown. Panel A shows localization of NK1-R and panel B shows localization of Tf-R. Panel C is formed by superimposing images from A and B, indicating colocalization of receptors in the same yellow vesicles (arrows). (D–F) Cells were incubated with the monoclonal Flag antibody and the polyclonal CI MP-R antibody, followed by FITC-labeled goat anti-rabbit IgG and rhodamine-labeled goat anti-mouse IgG. Panel D shows localization of Flag and panel E shows localization of CI MP-R. Panel F is formed by superimposing images from D and E. Note the small vesicles with Flag immunoreactivity (panel D, small arrows) are distinct from the large vesicles with CI MP-R immunoreactivity (panel E, large arrows). The arrow head in D–F shows a rare instance of co-localization. (G–I) Cells were incubated with the polyclonal NK1-R antibody and the monoclonal GM10 antibody, followed by rhodamine-labeled goat anti-rabbit IgG and FITC-labeled goat anti-mouse IgG. Panel G shows localization of NK1-R and panel H shows localization of GM10. Panel I is formed by superimposing images from G and H. Note the small vesicles with NK1-R immunoreactivity (panel G, small arrows) are distinct from the large vesicles with GM10 immunoreactivity (panel H, large arrows). The arrowhead in G–I shows a rare instance of co-localization. Bar, 10 μm.

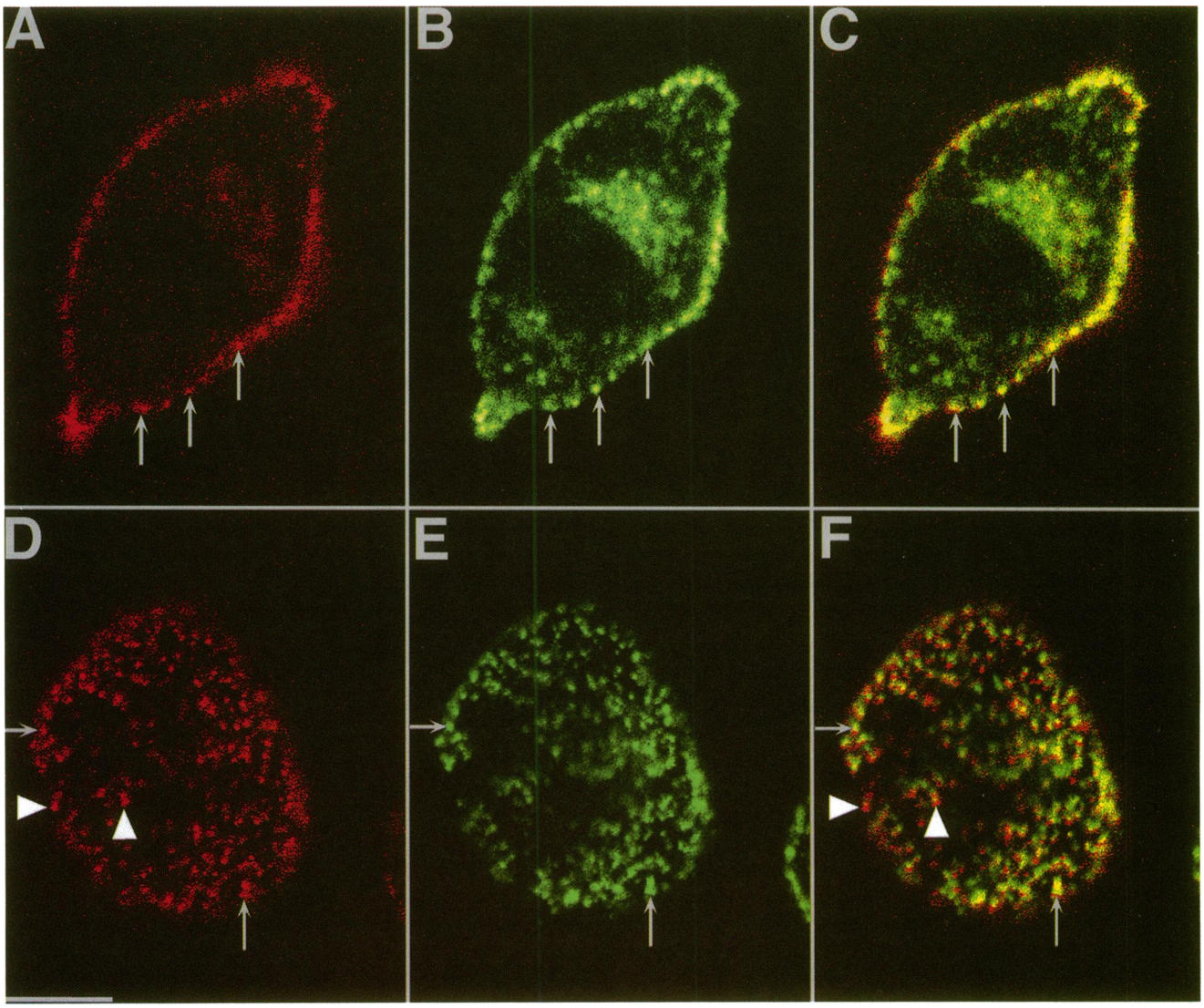

Figure 3. Confocal photomicrographs of KNRK Flag-NK1R showing localization of cy3-SP and clathrin. Cells were incubated with 100 nM cy3-SP for 60 min at 4°C, washed, and incubated in SP-free medium at 37°C for 30 s (A–C) or 2 min (D–F). Cells were fixed and reacted with the monoclonal clathrin antibody, followed by FITC-labeled goat anti-mouse IgG. Panels A and D show cy3-SP clustered in patches at the plasma membrane and internalized into small vesicles beneath the cell surface, and panels B and E show clathrin immunoreactivity. Panels C and F are formed by superimposing images from A and B, and D and E, respectively. Note the colocalization of cy3-SP and clathrin immunoreactivity in patches at the cell surface and some vesicles (arrows). The arrowheads in panels D–E show a nonimmunoreactive vesicle containing cy3-SP. Bar, 10 μm.

stages of endocytosis, indicate that endocytosis of SP and the NK1-R mainly occurs via clathrin-coated pits. A clathrin-independent mechanism cannot be excluded because potassium depletion did not abolish the loss of surface Flag immunoreactivity.

Acidotropic Agents, but not Cycloheximide, Inhibited Recovery of Cell Surface NK1-R

The recovery of cell-surface Flag immunoreactivity after incubation with SP at 4°C and warming to 37°C for 240 min may represent the recycling of internalized receptors to the cell surface, or the migration of preformed or newly synthesized receptors from the Golgi apparatus to the plasma membrane. To determine the contribution of newly synthesized protein to this recovery, we treated cells with cycloheximide. Recovery was unaffected by cycloheximide, and is thus independent of new protein synthesis (Table 2). To examine the importance of correct sorting of SP and the NK1-R in acidified endosomes to the recovery

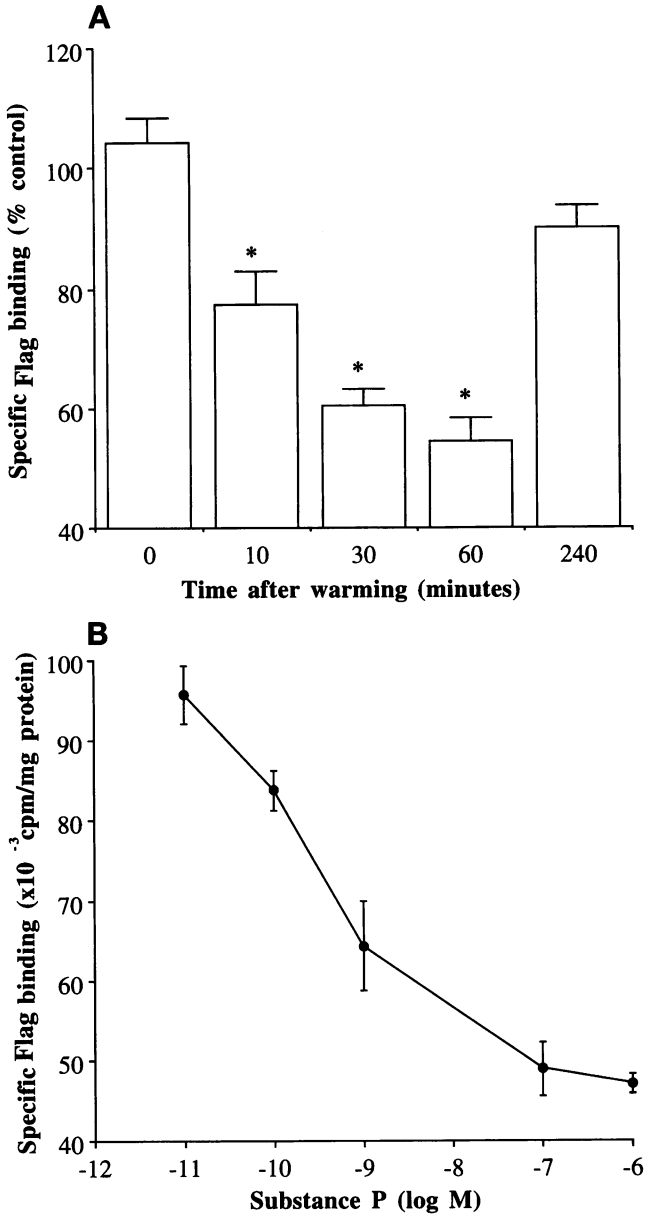

Figure 4. Quantification of surface Flag immunoreactivity in KNRK Flag NK1-R cells. (A) Cells were incubated with 10 nM SP for 60 min at 4°C, washed, and incubated in SP-free medium at 37°C for 0–240 min. Cells were incubated at 4°C with the Flag antibody followed by ^{125}I-sheep anti-mouse Ig. At each time point, specific Flag binding is expressed as a percentage of binding to control cells not treated with SP. Results are from n = 5–13 experiments. *, $p < 0.05$ compared with the 0 min. (B) Cells were incubated with graded concentrations of SP for 60 min at 4°C, washed, and incubated in SP-free medium at 37°C for 30 min. Cells were incubated at 4°C with the Flag antibody followed by ^{125}I-sheep anti-mouse Ig. Specific Flag binding is expressed as cpm per mg of protein. Results are from n = 3 experiments.

in surface Flag immunoreactivity, we treated cells with acidotropic agents. Bafilomycin A_1, monensin, chloroquine, and NH_4Cl significantly reduced the recovery of Flag immunoreactivity at the cell surface (Table 2). Brefeldin A slightly inhibited the recovery of surface Flag immunoreactivity. We have previously shown that monensin, chloroquine, and NH_4Cl do not affect internalization of SP by KNRK Flag NK1-R cells (Garland *et al.*, 1994), and none of the drugs tested prevented the decline in surface Flag immunoreactivity measured at 30 min after warming the cells (our unpublished results). Together, these experiments indicate that the internalized NK1-R recycles back to the cell surface.

To identify the site at which these drugs altered the endocytic and recycling pathway, we incubated KNRK Flag NK1-R cells with SP at 4°C, washed the cells, warmed them to 37°C for 240 min, and localized the NK1-R by immunofluorescence. In controls, NK1-R immunoreactivity had returned to the cell surface with only residual NK1-R in vesicles in a perinuclear region and below the plasma membrane (Figure 5A). This distribution was unaffected by cycloheximide (our unpublished results), indicating that the recovery of surface NK1-R was independent of new protein synthesis. Bafilomycin A_1, monensin, chloroquine, NH_4Cl and brefeldin A markedly reduced the return of cell surface NK1-R and caused its retention in vesicles (Figure 5, B–F). Thus, the agents that attenuated the recovery of surface Flag immunoreactivity also cause retention of the NK1-R immunoreactivity in perinuclear vesicles.

Internalized SP Was Intact in Early Endosomes but Degraded at Later Time Points: Degradation Was Inhibited by Bafilomycin A_1

To examine whether SP was intact at different points in the endocytic pathway, KNRK Flag NK1-R cells were incubated with ^{125}I-SP at 4°C, warmed to 37°C, and washed with acid to separate cell surface (acid labile) from internalized (acid resistant) fractions. At 4°C, when cy3-SP was co-localized with the NK1-R at the plasma membrane (Figure 1, A and E), analysis of the cell surface fractions by HPLC showed that 88.9 ± 1.9% (n = 4 observations) of the radioactivity eluted as intact ^{125}I-SP at 27.5 min (Figure 6A). After 10 min at 37°C, when cy3-SP was co-localized with the NK1-R in early endosomes (Figure 1, B and F), 90.9 ± 1.5% of the label in the internalized fractions eluted as intact ^{125}I-SP (Figure 6A). After 30 min, the last time when cy3-SP was partially co-localized with the NK1-R (Figure 1, C and G), 62.0 ± 2.4% of the internalized label was undegraded (Figure 3A). After 60 min, when cy3-SP was no longer co-localized with the NK1-R, only 53.6 ± 2.1% of the internalized label was intact (Figure 6A). The degradation of internalized ^{125}-I SP was accompanied by the formation of metabolites (Figure 6, A and B). The main metabolite (Figure 6,

Table 1. Effects of hyperosmolar sucrose, potassium depletion, and phenylarsine oxide on surface Flag immunoreactivity in KNRK Flag NK1-R cells at 30 min

Treatment	Carrier (no sucrose)	Sucrose (0.45 M)	Carrier (potassium replete)	Potassium depletion	Carrier (DMSO)	Phenylarsine oxide (80 μM)
Specific flag binding (% control)	55.3 ± 1.0	100.3 ± 3.8*	64.7 ± 1.2	81.3 ± 2.6*	52.8 ± 1.9	99.5 ± 12.9*

KNRK Flag NK1-R cells were treated with sucrose, phenylarsine oxide, or depleted of potassium. Control cells were treated with appropriate carrier solutions (DMEM-BSA for sucrose and DMSO for phenylarsine oxide) or were potassium replete. Cells were incubated with 10 nM SP for 60 min at 4°C, washed, and incubated in SP-free medium at 37°C for 30 min. Cells were incubated with the Flag antibody followed by ^{125}I-sheep anti-mouse Ig. Specific Flag binding is expressed as a percentage of binding to corresponding control cells that were not treated with SP. Triplicate observations from n = 3 experiments. *, $p < 0.05$ compared to the corresponding control.

peak A) eluted from the HPLC column at 21.3 min, and accounted for 25.5 ± 1.5% of the recovered radioactivity after 30 min and 38.0 ± 0.6% after 60 min of incubation at 37°C. The minor metabolites (Figure 6, peaks B and C) eluted at 23.3 and 25.6 min. Therefore, SP is intact at the cell surface and in early endosomes when it is colocalized with the NK1-R. After sorting in a perinuclear region, SP is slowly degraded with the formation of several metabolites.

We have previously shown that acidotropic reagents inhibit the degradation of internalized ^{125}I-SP in KNRK Flag NK1-R cells (Garland *et al.*, 1994). To determine whether a selective inhibitor of vacuolar H^+-ATPase also inhibited degradation, we treated cells with bafilomycin A_1. After 30 min of incubation at 37°C, the proportion of recovered counts that eluted as intact SP was 56.7 ± 1.9% in control cells, 85.2 ± 3.6% in bafilomycin A_1-treated cells, and 58.2 ± 4.0% in brefeldin A-treated cells. Therefore, bafilomycin A_1 but not brefeldin A significantly inhibited SP degradation ($p < 0.05$).

To determine whether SP was degraded in lysosomes, KNRK Flag NK1-R cells were incubated with cy3-SP at 4°C, washed, warmed to 37°C for 30–60 min, and processed to localize lysosomes by immunofluorescence using the GM10 antibody. Cy3-SP was usually localized in vesicles that were distinct from lysosomes, even in the presence of 10 or 15 mM NH_4Cl to inhibit degradation (Figure 7). Only rare instances of co-localization were observed (Figure 7, small arrows).

DISCUSSION

The NK1-R and SP Are Rapidly Internalized by a Clathrin-dependent Mechanism into Early Endosomes

In the steady state in the absence of SP, the NK1-R was found at the plasma membrane. When cells were exposed to SP, washed, and warmed to 37°C, the receptor and its ligand clustered at the cell surface, and moved together into early endosomes underneath the plasma membrane, which contained the Tf-R (Dautry-Varsat *et al.*, 1983; Hopkins and Trowbridge, 1983). The results agree with previous studies showing that cy3-SP and FITC-transferrin are internalized into the same vesicles (Garland *et al.*, 1994). Other receptors with seven-transmembrane domains that are internalized into endosomes containing the Tf-R include the β_2-adrenergic receptor and the thrombin receptor (von Zastrow and Kobilka, 1992; Hoxie *et al.*, 1993; Brass *et al.*, 1994).

SP and the NK1-R were internalized by a clathrin-dependent mechanism, because cy3-SP and the NK1-R

Table 2. Effects of acidotropic reagents, bafilomycin A_1, and brefeldin A on surface Flag immunoreactivity in KNRK Flag NK1-R cells at 240 min

Treatment	Carrier	Cycloheximide (70 μM)	Bafilomycin A_1 (1 μM)	Monensin (50 μM)	Chloroquine (500 μM)	NH_4Cl (10 mM)	Brefeldin A (10 μg/ml)
Specific flag binding (% control)	88.3 ± 4.2 (9)	87.8 ± 3.2 (6)	63.6 ± 3.4* (5)	64.7 ± 5.0* (5)	67.1 ± 9.8* (3)	72.5 ± 8.2 (6)	76.9 ± 2.8 (5)

KNRK Flag NK1-R cells were treated with drugs or appropriate carrier solutions. Cells were incubated with 10 nM SP for 60 min at 4°C, washed, and incubated in SP-free medium at 37°C for 30 min. Cells were incubated with the Flag antibody followed by ^{125}I-sheep anti-mouse Ig. Specific Flag binding is expressed as a percentage of binding to control cells that were not treated with SP. Triplicate observations from n = 3–9 experiments (parentheses). *, $p < 0.05$ compared to the control.

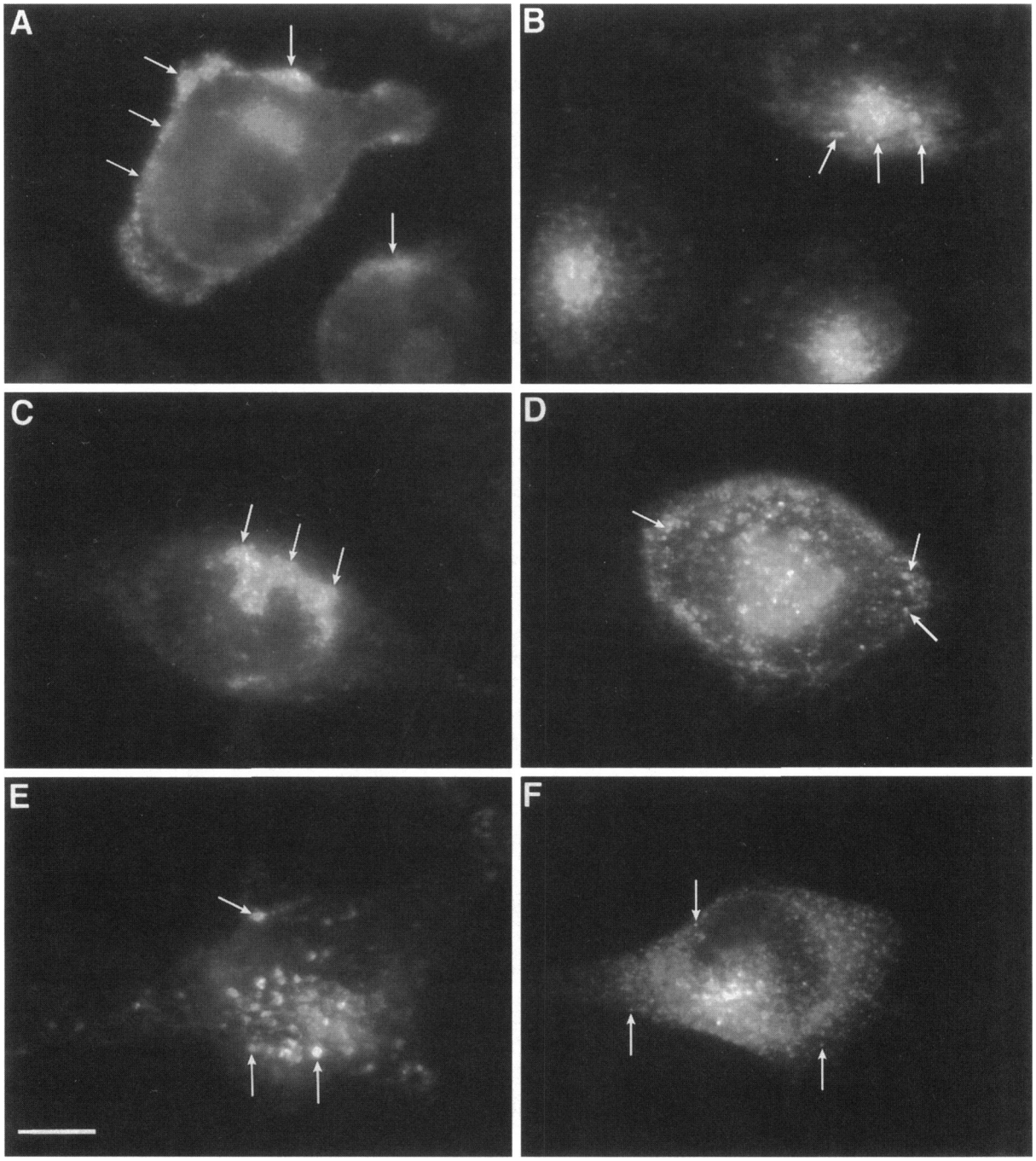

Figure 5. The effects of drug treatments on the distribution of NK1-R immunoreactivity in KNRK Flag NK1-R cells. Cells were incubated with 10 nM SP for 60 min at 4°C, washed, and incubated in SP-free medium at 37°C for 240 min. Cells were fixed and incubated with NK1-R antibody and a Texas Red-labeled secondary antibody. A, control; B, 1 μM bafilomycin A_1; C, 50 μM monensin; D, 500 μM chloroquine; E, 10 mM NH_4Cl; F, 10 μg/ml brefeldin A. Bar, 10 μm.

colocalized with clathrin in clusters at the cell surface and in the first-formed vesicles, and conditions interfering with formation of clathrin-coated pits, such as hyperosmolar sucrose and potassium depletion (Larkin *et al.*, 1983; Heuser and Anderson, 1989; Hansen *et al.*, 1993a), blocked endocytosis of SP and the NK1-R (Garland *et al.*, 1994). Other receptors internalized by a clathrin-dependent mechanism include the constitutive recycling nutrient receptors, such as the Tf-R (Pearse, 1982) and the low density lipoprotein receptor

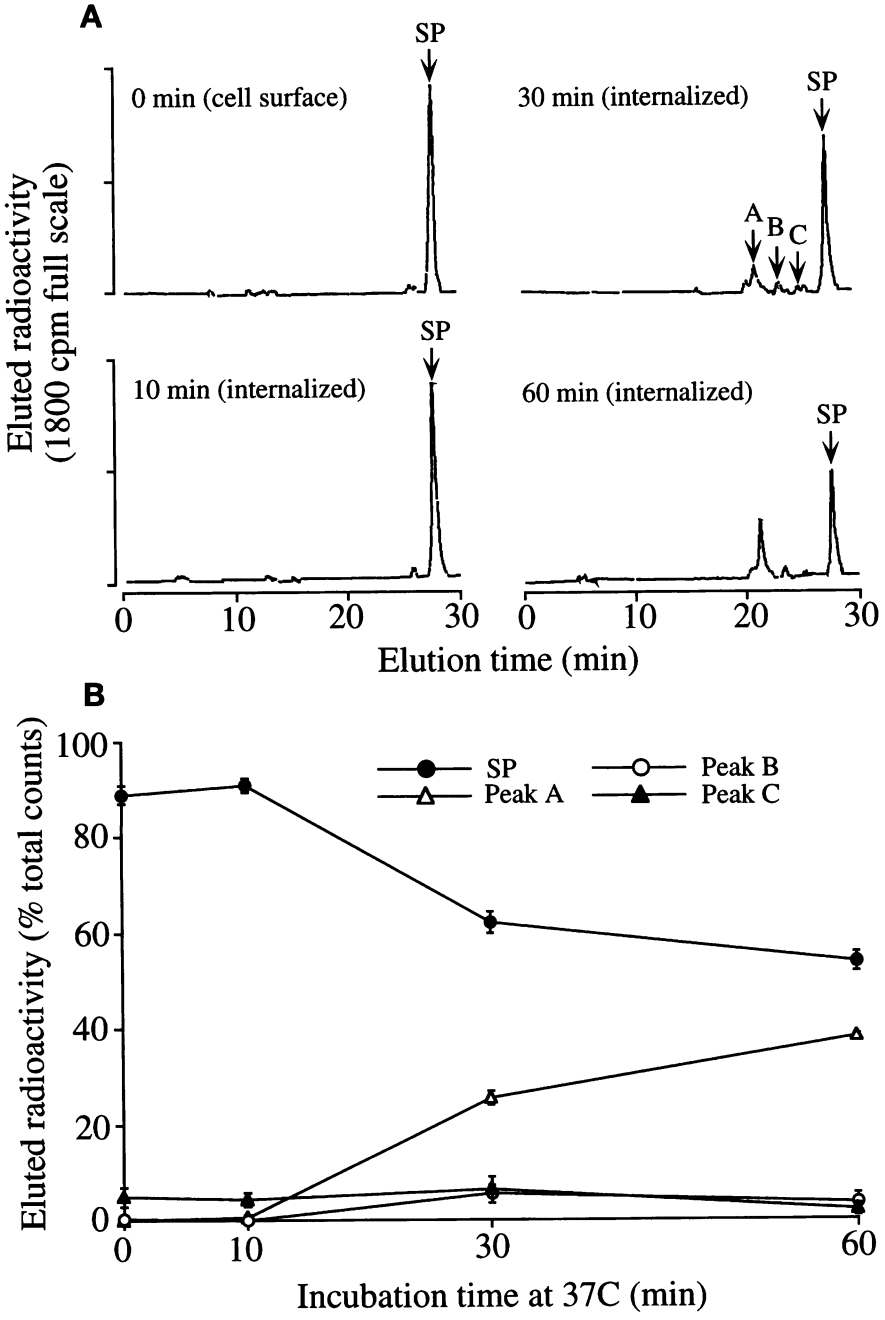

Figure 6. Degradation of internalized ^{125}I-SP by KNRK Flag NK1-R cells. Cells were incubated with 0.5 nM ^{125}I-SP plus 9.5 nM of unlabeled SP for 60 min at 4°C, washed, and incubated in SP-free medium at 37°C for 0–60 min. Cell surface and internalized fractions were obtained by an acid wash and analyzed by HPLC. (A) Chromatograms of cell surface (0 min) and internalized (10, 30, and 60 min) label. (B) Time course of degradation of ^{125}I-SP and the appearance of ^{125}I-labeled metabolites (n = 4 observations).

(Carpentier *et al.*, 1982; Larkin *et al.*, 1983; Heuser and Anderson, 1989), and signaling receptors that are internalized after ligand binding, exemplified by the epidermal growth factor receptor (Carpentier *et al.*, 1982; Larkin *et al.*, 1983), the thrombin receptor (Hoxie *et al.*, 1993), the β_2-adrenergic receptor (Yu *et al.*, 1993), and the receptor for gastrin-releasing peptide (Slice *et al.*, 1994; Grady *et al.*, 1995). Our results cannot exclude a contribution of a clathrin-independent mechanism, such as one involving caveolin (Rothberg *et al.*, 1990; Anderson *et al.*, 1992). Ligands that are internalized by clathrin-dependent or -independent mechanisms enter endosomes containing the Tf-R, and follow a common pathway (Tran *et al.*, 1987; Hansen *et al.*, 1993b).

Cy3-SP was not detected at the cell surface within minutes of warming. At this time, >80% of ^{125}I-SP is internalized by transfected KNRK cells (Garland *et al.*, 1994), and a large portion of bound SP is also internalized by anterior pituitary cells (Larsen *et al.*, 1989). In contrast, after incubation with SP, residual surface

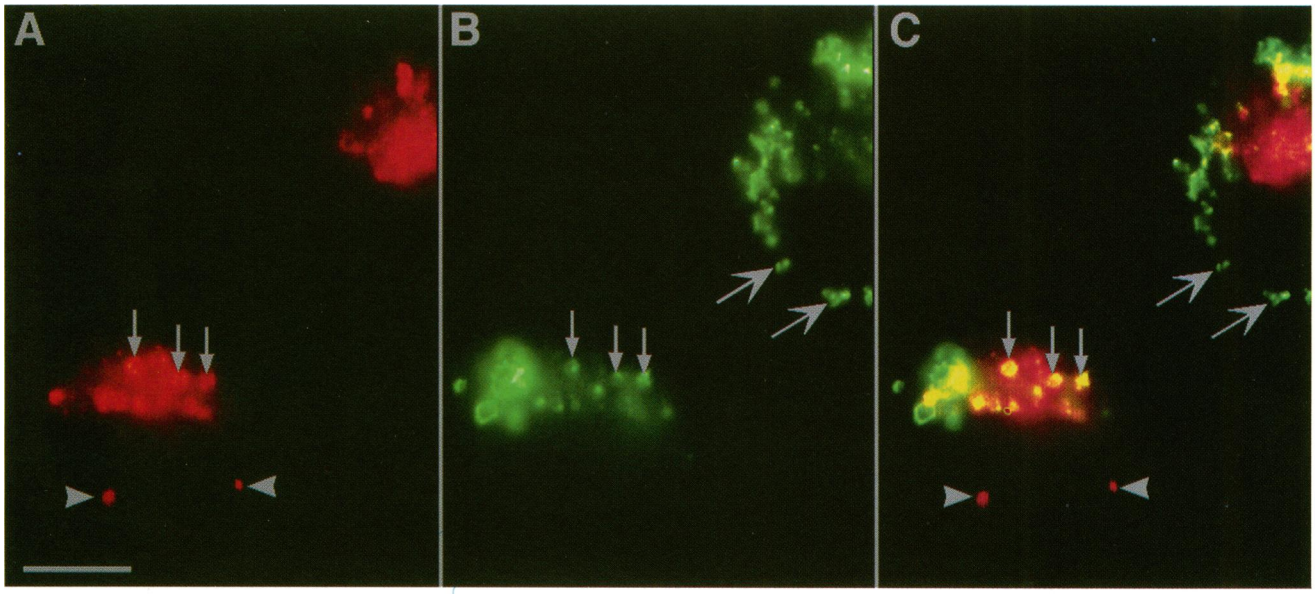

Figure 7. Simultaneous detection of cy3-SP (A) and GM10 immunoreactivity (B) in KNRK Flag NK1-R cells. Cells were incubated with 100 nM cy3-SP for 60 min at 4°C, washed, and incubated in SP-free medium at 37°C for 60 min. All solutions contained 10 mM NH_4Cl. The cells were fixed and incubated with the GM10 antibody and a FITC-labeled secondary antibody. Image C is formed by superimposing images A and B. Note the colocalization (designated by the small arrows) of cy3-SP and GM10 immunoreactivity in some vesicles (A–C). Other vesicles containing cy3-SP were not stained by the GM10 antibody (arrowheads, A and C). Most of the GM10-stained vesicles did not contain cy3-SP (large arrows, B and C). Bar = 10 μm.

receptor was consistently detected by microscopy, and by quantification using the Flag antibody. The discrepancy between the almost complete internalization of ligand and the partial internalization of receptor may be related to the affinity state of the receptor. Internalization of neuropeptide receptors has previously been quantified by using radioactively-labeled ligand at pM concentrations. Only high affinity receptors are detected by this method. By using antibodies, we identified and quantified surface NK1-R independent of affinity state. Because internalization of the NK1-R is accompanied by a decrease in high affinity ^{125}I-SP binding (Bowden *et al.*, 1994), it is likely that only the high affinity, ligand-bound receptors are internalized. Alternatively, the receptor may be continuously internalized and recycled, so that a dynamic pool of receptors exists at the plasma membrane rather than a static pool incapable of endocytosis.

The NK1-R and SP Are Sorted into Recycling and Degradative Pathways in a Perinuclear Region

Cy3-SP and the NK1-R were colocalized in perinuclear vesicles and subsequently sorted into distinct compartments. Like the superficial endosomes, the central vesicles containing the NK1-R also contained the Tf-R. Thus, the NK1-R was co-localized with a well known recycling receptor at many steps of the endocytic pathway. During recycling of the Tf-R, iron dissociates from transferrin in early endosomes acidified to a pH of <6.2 (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983; Salzman and Maxfield, 1993), suggesting that SP dissociates from the NK1-R at this pH.

Within 4 h, the steady-state distribution of the NK1-R returned to the basal state at the plasma membrane, as shown by microscopy and by quantification of surface Flag immunoreactivity. There are three theoretical possibilities for the recovery of surface NK1-R: synthesis of new receptors, insertion of pre-synthesized receptors into the plasma membrane, or recycling of internalized receptors. Arguing against the first possibility was the failure of cycloheximide to affect the recovery of the NK1-R at the cell surface. The possibility that presynthesized receptors were inserted into the plasma membrane cannot be totally excluded. However, vesicles containing the NK1-R were not detected in the basal state, suggesting that there were no large intracellular pools of pre-formed NK1-R. This is unlike the complement receptors C3b and C3b1 where movement of vesicles containing presynthesized receptor to the plasma membrane is responsible for increased receptor expression due to stimulation (O'Shea *et al.*, 1985). The possibility that the NK1-R recycles is supported by results obtained with agents that prevent organelle acidification. Bafilomycin A_1, monensin, chloroquine, and NH_4Cl markedly reduced the reappearance of the NK1-R at

the cell surface, and resulted in the aggregation of the receptor in perinuclear vesicles. Therefore, the sorting of SP and the NK1-R in an acidified perinuclear compartment is required for return of the receptor to a basal distribution. For several receptors, including the transferrin receptor, exit from this perinuclear sorting compartment is the rate-limiting step in return to the cell surface (Mayor *et al.*, 1993).

Like the NK1-R, other seven-transmembrane domain receptors are also sorted into recycling pathways. The β_2-adrenergic receptor reappears at the cell surface within 30 min of internalization (von Zastrow and Kobilka, 1992). In contrast, only about 25% of internalized thrombin receptors recycle to the plasma membrane (Hoxie *et al.*, 1993). The gastrin-releasing peptide receptor expressed in KNRK cells is also internalized after ligand binding, and the return to the steady-state distribution at the cell surface occurs fourfold more quickly than the NK1-R in the same cell line (Grady *et al.*, 1995). Thus, the rate of recycling and the proportion of the internalized receptor that is recycled rather than degraded varies from one receptor to another and possibly from cell to cell. Although specific domains of several seven-transmembrane domain receptors that are involved in internalization have been identified by mutagenesis (Valiquette *et al.*, 1990; Benya *et al.*, 1993; Barak *et al.*, 1994), the identification of domains involved in intracellular sorting and recycling has received less attention.

Two observations support the conclusion that few NK1-Rs are degraded in any one recycling event. First, 4 h after exposure to SP and warming, surface Flag immunoreactivity had returned to 90% of original values even in the presence of cycloheximide. Second, internalized NK1-R was rarely colocalized with the CI MP-R, which identifies late endosomes or prelysosomes (Griffiths *et al.*, 1988), or with a lysosomal marker. In contrast, the epidermal growth factor receptor and 75% of internalized thrombin receptors are degraded in lysosomes (Dunn *et al.*, 1986; Hoxie *et al.*, 1993; Brass *et al.*, 1994).

Internalized ligand was not recycled but was instead degraded, because internalized cy3-SP did not reappear at the plasma membrane and ^{125}I-SP was degraded. This is in contrast to asialoglycoproteins, which return to the cell surface in the intact state bound to their receptor (Weigel and Oka, 1984). At early time points, when cy3-SP and the NK1-R were colocalized at the cell surface and in endosomes, ^{125}I-SP was mostly intact. Although we do not know whether SP was bound to its receptor in endosomes, it is possible that receptor-bound peptide is resistant to enzymatic degradation. It is unknown if the internalized SP/NK1-R complex is able to signal, as has been shown for certain growth factor receptors (Kay *et al.*, 1986). At later times, when cy3-SP and the NK1-R were present in distinct compartments, ^{125}I-SP was degraded. Degradation was inhibited by bafilomycin A_1, which also prevented recycling of the NK1-R, and degradation is inhibited by acidotropic reagents (Garland *et al.*, 1994). Thus, acidification of endosomes may be required for correct sorting of SP into a degradative pathway.

Physiological Relevance of Endocytosis of the NK1-R

SP causes internalization of the NK1-R in cells that naturally express the receptor, including endothelial cells of post-capillary venules (Bowden *et al.*, 1994) and enteric neurons in primary culture (our unpublished results). Endocytosis and recycling of the NK1-R coincide with desensitization and resensitization of SP-induced plasma extravasation from post-capillary venules in the intact rat, and with calcium mobilization in transfected KNRK cells (Bowden *et al.*, 1994). Depletion of the plasma membrane of high affinity NK1-R may contribute to desensitization, similar to the desensitization of the thrombin receptor (Hoxie *et al.*, 1993). An alternative mechanism is exemplified by the β_2-adrenergic receptor, which is desensitized by phosphorylation by β-adrenergic receptor kinase and protein kinase A, and uncoupling from second messenger systems (Lohse *et al.*, 1990; Roth *et al.*, 1991). Thus, mutant β_2-adrenergic receptors that do not internalize still desensitize (Barak *et al.*, 1994). The NK1-R is a substrate for β-adrenergic receptor kinase (Kwatra *et al.*, 1993), but the role of phosphorylation in desensitization of the NK1-R is unknown. Resensitization of the β_2-adrenergic receptor requires internalization and dephosphorylation (Yu *et al.*, 1993). Recycling of the NK1-R to the plasma membrane may contribute to resensitization of cellular responses to SP.

Conclusions

SP induces clathrin-dependent internalization of the NK1-R into early endosomes containing the Tf-R. SP and the NK1-R dissociate in acidified perinuclear vesicles. The NK1-R recycles to the plasma membrane and is only rarely degraded. In contrast, SP is degraded within the cell. Internalization and recycling of the NK1-R may modulate the cellular response to SP.

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