Agonist-induced internalization of the substance P (NK₁) receptor expressed in epithelial cells

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Internalization of the NK₁ receptor (NK₁R) and substance P was observed in cells transfected with cDNA encoding the rat NK₁R by using anti-receptor antibodies and cyanine 3-labelled substance P (cy3-substance P). After incubation at 4 °C, NK₁R immunoreactivity and cy3-substance P were confined to the plasma membrane. Within 3 min of incubation at 37 °C, NK₁R immunoreactivity and cy3-substance P were internalized into small intracellular vesicles located beneath the plasma membrane. Fluorescein isothiocyanate-labelled transferrin and cy3-substance P were internalized into the same vesicles, identifying them as early endosomes. After 60 min at 37 °C, NK₁R immunoreactivity was detected in larger, perinuclear vesicles. Internalization of ¹²⁵I-labelled substance P was studied by using an acid wash to dissociate cell-surface label from that which has been internalized. Binding reached equilibrium after incubation for

60 min at 4 °C with no detectable internalization. After 10 min incubation at 37 °C, 83.5 ± 1.0 % of specifically bound counts were internalized. Hyperosmolar sucrose and phenylarsine oxide, which are inhibitors of endocytosis, prevented internalization of ¹²⁵I-labelled substance P and accumulation of NK₁R immunoreactivity into endosomes. Acidotropic agents caused retention of ¹²⁵I-labelled substance P within the cell and inhibited degradation of the internalized peptide. Continuous incubation of cells with substance P at 37 °C reduced ¹²⁵I-substance P binding at the cell surface. Therefore, substance P and its receptor are internalized into early endosomes within minutes of binding, and internalized substance P is degraded. Internalization depletes NK₁Rs from the cell surface and may down-regulate the response of a cell to substance P.

INTRODUCTION

Many cell-surface receptors are internalized by endocytosis within moments of ligand binding. Ligand-induced internalization is important for ligand uptake (low-density-lipoprotein, asialoglycoprotein and transferrin receptors), for signal transduction (epidermal growth factor and insulin receptors), and for desensitization and resensitization (adrenergic and thrombin receptors) [1–5].

Although internalization of receptors with a single transmembrane domain has been extensively studied [1–3], far less is known about internalization of receptors with seven transmembrane domains. This large family includes the neuropeptide, adrenergic and thrombin receptors. Agonists of the β_2 -adrenergic receptor induce a selective loss of the capacity of the receptor to bind hydrophilic, but not hydrophobic, ligands [6]. This process, termed sequestration, represents either rapid internalization of the receptor or a change in its conformation in the plasma membrane that makes it inaccessible to a hydrophilic ligand. Direct evidence for internalization includes the observations that β_2 -adrenergic receptors redistribute from the cell surface into early endosomes within 2 min of addition of isoprenaline [5], and that β_2 -adrenergic receptors are present in light vesicles separated from the plasma membrane of isoprenaline-treated cells by gradient centrifugation [7]. Inhibition of internalization does not affect desensitization of β_2 -adrenergic receptors to repeated exposure to agonists, but instead prevents recovery or resensitization [6,8]. While thrombin receptors are also internalized within minutes of ligand binding, internalization of these receptors is sufficiently rapid to contribute to their desensitization [4].

We examined ligand-induced internalization of the high-

affinity substance P (or NK₁) receptor (NK₁R). Many of the effects of substance P on neurotransmission, smooth-muscle contraction, exocrine gland secretion, plasma extravasation and regulation of immune and inflammatory processes are mediated through interaction with NK₁R [9–11]. Internalization of NK₁R is potentially important because it could affect the responsiveness of cells to substance P. The internalization of radiolabelled substance P has been examined in anterior pituitary cells [12] and pancreatic acinar cells [13]. However, the lack of receptor antibodies and the low levels of receptors that are endogenously expressed by many cell lines have prevented the direct observation of NK₁R internalization. We used antibodies specific for the intracellular C-terminus of rat NK₁R and an extracellular, N-terminal Flag epitope to observe agonist-induced internalization of the NK₁R directly in transfected epithelial cells by fluorescence microscopy. The internalization of substance P was observed by labelling substance P with the fluorophore cyanine 3 (cy3). Internalization was quantified in binding experiments with radiolabelled substance P, and the degradation of internalized substance P was studied by column chromatography. Our results show that substance P and the NK₁R are internalized into early endosomes within minutes of binding, and that substance P is subsequently degraded in an acidified compartment. Internalization depletes NK₁Rs from the cell surface and may thereby down-regulate the responsiveness of cells to substance P.

MATERIALS AND METHODS

Materials

Epitope-labelled NK₁R comprised the rat NK₁R and an N-terminal Flag peptide (DYKDDDDK) in the neomycin-resistant

Abbreviations used: NK₁R, NK₁ receptor; HBSS, Hanks balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; TFA, trifluoroacetic acid; cy3, cyanine 3; GRP, gastrin-releasing peptide.

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expression vector pcDNA I Neo [14]. This was transfected into Kirsten murine sarcoma virus-transformed rat kidney cells (KNRK cells). For comparison KNRK cells were transfected with cDNA encoding the native rat NK₁R. Cell lines expressing the Flag-NK₁R (KNRK Flag-NK₁R clone 17) or the native NK₁R (KNRK NK₁R clone 9) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 100 ml/l bovine calf serum, 100 units/ml penicillin G, 100 µg/ml streptomycin and 400 µg/ml G418 at 37 °C in 5% CO₂/95% air. The epitope-labelled receptor and the native receptor have been extensively characterized by substance P binding and substance P-induced Ca²⁺ mobilization [14]. The apparent affinities (K_d s) for substance P were determined by Scatchard analysis. The apparent K_d was 5.63 ± 1.54 nM for KNRK Flag-NK₁R cells and 6.15 ± 2.09 nM for KNRK NK₁R cells. There were approx. 80000 binding sites per cell. The EC₅₀ for substance P-induced Ca²⁺ mobilization was 0.66 nM for KNRK Flag-NK₁R cells and 0.76 nM for KNRK NK₁R cells.

An antiserum to rat NK₁R (no. 11884-5) was raised by immunization of rabbits with a peptide (KMTESSEFYNSMLA) corresponding to the C-terminal 15 amino acid residues of the rat NK₁R (residues 393–407) coupled to bovine thyroglobulin [14]. A monoclonal antibody (M2), raised in mice to the Flag peptide, was obtained from International Biotechnologies, New Haven, CT, U.S.A. The use of these antibodies for immunocytochemical localization of the Flag-NK₁R has been described [14]. Affinity-purified fluorescein- or rhodamine-conjugated goat anti-(mouse IgG) and goat anti-(rabbit IgG) were from Cappel Research Products, Durham, NC, U.S.A. Bis-functional cyanine 3.18 was a gift from Biological Detection Systems (Pittsburgh, PA, U.S.A.) and fluorescein isothiocyanate (FITC)-labelled human transferrin was from Molecular Probes (Eugene, OR, U.S.A.).

Monensin, chloroquine, leupeptin, colchicine and phenylarsine oxide were from Sigma Chemical Co., St. Louis, MO, U.S.A. Bolton-Hunter-labelled ¹²⁵I-substance P (specific radioactivity 2000 Ci/mmol) was from Amersham Corporation. G418 was from Gibco/BRL, Gaithersburg, MD, U.S.A. Substance P and neurokinin A were from Peninsula Laboratories, San Carlos, CA, U.S.A. CP 96,345 was from Pfizer, Groton, CT, U.S.A.

Internalization of NK₁R

Antibodies to the C-terminus of rat NK₁R and to the Flag epitope were used to examine ligand-induced internalization of NK₁R by immunocytochemistry. KNRK Flag-NK₁R cells on poly(lysine)-coated glass coverslips were washed twice with Hanks balanced salt solution (HBSS) or DMEM (pH 7.4) containing 1 g/l BSA. Cells were incubated in medium (HBSS or DMEM) with agonist (substance P or neurokinin A, 1×10^{-11} – 1×10^{-7} M) for 1 h at 4 °C. This allowed binding to reach equilibrium without internalization [14]. The cells were then washed with peptide-free medium and incubated at 37 °C to allow internalization to proceed. Cells were fixed for microscopy at 0–16 h after raising the temperature. In experiments to examine the effects of the NK₁R antagonist CP 96,345 on substance P-induced internalization, cells were preincubated with the antagonist (1 µM) for 10 min before incubating with substance P. Internalization experiments were repeated in the presence of hyperosmolar sucrose (0.45 M). Cells were also pretreated with phenylarsine oxide (80 µM) for 5 min at 37 °C and washed three times with medium before the internalization experiment.

At the specified times cells were washed with 100 mM PBS (pH 7.4) and fixed in 40 g/l formaldehyde in PBS (pH 7.4) for 10 min at room temperature. Cells were washed three times in

PBS containing 10 g/l normal goat serum and 1 g/l saponin. Cells were incubated with the primary antibodies (anti-NK₁R serum no. 11884-5, 1:1000 or anti-Flag M2 antibody, 10 µg/ml) in the same buffer for 4 h at 37 °C. Cells were washed and incubated with the secondary antibodies [rhodamine-conjugated goat anti-(rabbit IgG) or fluorescein-conjugated goat anti-(mouse IgG), 1:200] for 2 h at room temperature. For co-localization of NK₁R and the Flag epitope, cells were simultaneously incubated with a mixture of the primary antibodies followed by a mixture of the secondary antibodies [14]. Controls for antibody specificity included preabsorption of the antibodies with the fragment of the NK₁R or the Flag peptide (10 mM, overnight at 4 °C) and staining untransfected KNRK cells.

Cells were examined using a Zeiss Axioplan microscope with fluorescein (Zeiss 487910, excitation wavelength 450–490 nm, emission wavelength 515–565 nm, chromatic beam splitter 510 nm) and rhodamine (Zeiss 487915, excitation wavelength 546 nm, emission wavelength 590 nm, chromatic beam splitter 580 nm) filters. Cells were also observed by confocal microscopy using a Zeiss Laser Scan Inverted 410 microscope with an argon-krypton laser with fluorescein (line selection 485/20 nm, main dichroic 510 nm, emission LP 515 nm) and rhodamine (line selection 530–585 nm, main dichroic 580 nm, emission LP 568 nm) filters.

Internalization of cy3-substance P and FITC-labelled transferrin

Substance P was labelled with the fluorophore cy3 to allow the direct observation of the internalized peptide. Substance P (0.65 mM) was incubated in 0.1 M NaH₂CO₃ (pH 9.3) with 0.3 mM cyanine 3.18 for 30 min at room temperature. The reaction was stopped by addition of a one-tenth volume of 0.1 M glycine (pH 9.3), and the products were separated by reverse-phase h.p.l.c. using a C-18 column (Vydac, 5 µm, 4.6 mm × 250 mm; Separations Group, Hesperia, CA, U.S.A.). The column was equilibrated in 1 ml/l trifluoroacetic acid (TFA) in water and peptides were eluted with a linear gradient of 1 ml/l TFA in acetonitrile (0% acetonitrile, 5 min; 0–30% acetonitrile, 5 min; 30% acetonitrile, 5 min; 30–60% acetonitrile, 30 min) with a flow rate of 1 ml/min. The absorbance of the eluent was monitored at 214 nm and fractions were collected for quantification by amino acid composition analysis. Unlabelled substance P eluted from the column at 18.1 min, and cy3-substance P was eluted at 19.4 min. Mass spectrometry indicated that cy3-substance P had a molecular mass of 2028 Da and suggested that Lys³ was labelled with a single cy3 molecule. The cy3-substance P was fully functional as assessed by measurement of Ca²⁺ mobilization in KNRK Flag-NK₁R cells [14]. The Ca²⁺ response was abolished by pretreatment of cells with the NK₁R antagonist CP-96,345 (1 µM).

To examine internalization of cy3-substance P, KNRK Flag-NK₁R cells were washed and incubated in HBSS or DMEM-BSA containing cy3-substance P (10 nM) for 1 h at 4 °C. The cells were then washed with peptide-free medium and incubated at 37 °C to allow internalization to proceed. Cells were washed with ice-cold PBS and fixed in 40 g/l formaldehyde for 20 min at 4 °C. To co-localize cy3-substance P and the NK₁R, fixed cells were washed three times in PBS containing 10 g/l normal goat serum and 0.025 g/l saponin, and incubated with anti-NK₁R serum 11884-5, (1:1000 dilution) for 2 h at room temperature, followed by fluorescein-conjugated goat anti-(rabbit IgG). Cells were post-fixed in formaldehyde.

To examine internalization of cy3-substance P and FITC-labelled transferrin, a marker for early endosomes [4,5], simultaneously, KNRK Flag-NK₁R cells were washed and incubated

as described with cy3-substance P (10 nM) and FITC-transferrin (17 μ M) for 1 h at 4 °C. The cells were then washed with peptide-free medium and incubated at 37 °C to allow internalization to proceed. Cells were washed with ice-cold PBS and fixed in 40 g/l formaldehyde for 20 min at 4 °C.

Controls for specificity of cy3-substance P binding included pre-incubation of cells with CP 96,345 or unlabelled substance P (1 μ M) for 30 min at 4 °C before incubation with cy3-substance P, and incubation of cy3-substance P with KNRK cells that were transfected with vector without the NK₁R insert [14].

Internalization of ¹²⁵I-substance P

KNRK Flag-NK₁R cells or KNRK NK₁R cells on poly(lysine)-coated plastic wells [(1.5–2.0) × 10⁵ cells/well] were washed with HBSS-BSA and incubated in this medium for 1 h at 37 °C. Cells were incubated with ¹²⁵I-substance P (10 pM) in 250 μ l of HBSS-BSA for 1 h at 4 °C. The temperature was raised to 37 °C for 0–60 min. At specified times the cells were washed three times in ice-cold PBS. An acid-wash procedure was used to separate acid-sensitive (cell-surface) label from acid-resistant (internalized) label. Cells were incubated in 250 μ l of ice-cold 0.2 M acetic acid/50 mM NaCl (pH 2.5) on ice for 5 min. Acid-stripped cells were lysed in 250 μ l of 0.5 M NaOH for 30 min at room temperature, and the acid-sensitive and acid-resistant pools were counted. In separate experiments non-stripped cells were lysed as described and counted to determine total binding (acid-sensitive plus acid-resistant). Binding studies were repeated with leupeptin (20 μ M), colchicine (50 μ M), monensin (50 μ M), chloroquine (500 μ M), NH₄Cl (10 mM) or hyperosmolar sucrose (0.45 M). Cells were also pretreated with phenylarsine oxide (80 μ M) for 5 min at 37 °C, washed three times with HBSS-BSA and allowed to recover for 60 min before the binding assay. Non-specific binding, determined by incubating cells with 1 μ M unlabelled substance P, was subtracted to determine the specific binding.

To determine whether continuous exposure of cells to substance P resulted in a reduction of NK₁R at the cell surface, KNRK Flag-NK₁R cells were incubated with unlabelled substance P (1 × 10⁻¹¹–1 × 10⁻⁷ M) for 30 min to 8 h at 37 °C. For time points of longer than 30 min, the medium was replaced with medium containing fresh substance P every 30 min, because substance P was rapidly degraded by the cells with a half-life of about 30 min. The acid-wash procedure was used to strip peptide from the cell surface. Cells were washed three times in HBSS-BSA and total binding to equilibrium was examined by incubating cells with ¹²⁵I-labelled substance P (10 pM) for 1 h at 4 °C. Specific binding at each time point was compared with that of control cells which were treated with the substance P carrier solution (HBSS-BSA) before the acid wash and binding.

Degradation of internalized ¹²⁵I-substance P

The metabolic fate of ¹²⁵I-substance P was examined by analysis of the medium, acid-sensitive and acid-resistant fractions by h.p.l.c. KNRK Flag-NK₁R cells on poly(lysine)-coated plastic wells [(1.5–2.0) × 10⁵ cells/well] were washed with HBSS-BSA. Cells were incubated with ¹²⁵I-substance P (100 pM) in 250 μ l of HBSS-BSA for 1 h at 4 °C and then for 30 min at 37 °C. The medium was aspirated, acidified with 1 vol of 5 ml/l TFA in water and saved for analysis. The cells were washed three times with ice-cold PBS, and the acid-sensitive fraction was collected by incubation with the acid-stripping solution, as described. The acid-resistant fraction was obtained by lysing the stripped cells with 5 ml/l TFA in water. The acidified medium, acid-sensitive

samples and acid-resistant samples were immediately boiled for 10 min to stop degradation and centrifuged (10000 g, 2 min). The supernatant was analysed by reverse-phase h.p.l.c. using a C-18 column (Vydac, 5 μ m, 4.6 mm × 250 mm). The column was equilibrated in 1 ml/l TFA in water and peptides were eluted with a linear gradient of acetonitrile containing 1 ml/l TFA (0%, 5 min; 0–80%, 35 min) with a flow rate of 1 ml/min. The radioactivity of the eluent was monitored using a flow-through liquid-scintillation counter and peak areas were measured by integration. Degradation studies were repeated with leupeptin (20 μ M), colchicine (50 μ M), monensin (50 μ M), chloroquine (500 μ M), NH₄Cl (10 mM) or hyperosmolar sucrose (0.45 M). Cells were also pretreated with phenylarsine oxide (80 μ M) for 5 min at 37 °C, washed three times with HBSS-BSA and allowed to recover for 60 min before the degradation assay.

Statistical analysis

Results are expressed as mean ± S.E.M. Differences between groups were examined by ANOVA and the Student–Newman–Keuls test. A *P* < 0.05 was considered statistically significant.

RESULTS

Internalization of NK₁R

Internalization of NK₁R in KNRK Flag-NK₁R cells was examined by double-antibody immunocytochemistry using the anti-NK₁R and anti-Flag antibodies. Staining of NK₁R by both antibodies was specific as it was abolished by pre-incubation with the peptide antigen and was not observed in control cells that were transfected with a vector without the NK₁R insert [14]. Both antibodies stained the same structures, although the plasma membrane was more intensely stained by the anti-NK₁R serum than by the anti-Flag antibody.

In cells not exposed to substance P, both antibodies stained the plasma membrane and there was minimal intracellular staining. When cells were incubated with substance P (10 nM) for 60 min at 4 °C, to allow binding to reach equilibrium without internalization [14], the NK₁R immunoreactivity was confined to the plasma membrane and there was no detectable internalization (Figure 1a). The cells were washed in substance P-free medium and the temperature was raised to 37 °C to allow internalization to occur. After 1 min incubation at 37 °C, NK₁R immunoreactivity was confined to the cell surface. However, after incubation for 3, 5 or 10 min at 37 °C, there was a reduction in the intensity of cell-surface staining and both antibodies stained numerous small vesicles located beneath the plasma membrane (Figure 1b). After 30 or 60 min incubation at 37 °C, larger more centrally located vesicles were stained (Figure 1c). After 2, 4 or 8 h of incubation at 37 °C, the NK₁R immunoreactivity was concentrated in diffusely stained perinuclear structures (Figure 1d). After 16 h, there was minimal intracellular staining and the plasma membrane was strongly stained. There was no detectable internalization of the receptor in the absence of substance P.

Confocal microscopy confirmed that NK₁R immunoreactivity was located in intracellular vesicles, rather than aggregated in the plasma membrane. Figure 2 shows a series of optical sections through three cells that were stained with the anti-Flag antibody after 30 min of incubation at 37 °C. At this time the immunoreactive NK₁R was localized in vesicles in a perinuclear region and there was minimal staining of the plasma membrane. Similar results were obtained when cells were stained with the anti-NK₁R serum (results not shown).

Internalization of NK₁R immunoreactivity was observed after

Figure 1 Internalization of NK₁R examined by double-antibody immunocytochemistry using anti-NK₁R and anti-Flag antibodies and observed by conventional fluorescence microscopy

KNRK Flag-NK₁R cells were exposed to substance P (10 nM) for 60 min at 4 °C. The cells were washed and incubated in substance P-free medium at 37 °C. Cells were fixed and stained as described in the Materials and methods section after 0 min (a), 3 min (b), 60 min (c) or 4 h (d) incubation at 37 °C. Cells were photographed using rhodamine filters to detect the anti-NK₁R serum (left-hand panels) and fluorescein filters to detect the anti-Flag antibody (right-hand panels) in the same fields. The arrows indicate that the same structures were stained by both antibodies. Scale bar = 10 μ m.

exposure of cells to 0.1–100 nM substance P, but internalization was less pronounced or undetectable with lower concentrations of peptide (0.01 nM). Incubation of KNRK Flag-NK₁R cells with the specific NK₁R antagonist CP 96,345 (1 μ M) for 60 min at 4 °C and then 30 min at 37 °C did not induce internalization of the NK₁R (Figure 3b). However, pre-incubation with CP 96,345 prevented substance P-induced internalization of this receptor (Figure 3c).

Hyperosmolar sucrose and phenylarsine oxide inhibit agonist-induced internalization of the β_2 -adrenergic receptor [6,15]. Therefore, the effects of these agents on substance P-induced internalization of NK₁R were examined by immunocytochemistry using the anti-NK₁R serum. In control experiments, substance P induced rapid internalization of the immunoreactive NK₁R into vesicles (Figures 1 and 3a). Internalization was prevented by hyperosmolar sucrose or phenylarsine oxide (Figures 3d and 3e).

Neurokinin A is also an agonist of NK₁R [9]. Incubation of cells with neurokinin A (100 nM) induced internalization similar to that observed with substance P (Figure 3f).

Internalization of cy3–substance P and FITC–transferrin

To determine whether substance P and the NK₁R were internalized together into the same compartment, KNRK Flag-NK₁R cells were incubated with cy3–substance P, fixed and then the NK₁R was localized using the receptor antiserum and a fluorescein-conjugated secondary antiserum. Cells were examined by confocal microscopy. When cells were fixed and stained for the receptor after 60 min incubation with cy3–substance P at 4 °C, both cy3–substance P and the immunoreactive receptor were confined to the cell surface (results not shown). However, after 5 or 10 min incubation at 37 °C, there was a reduction in the intensity of cell-surface staining for both cy3–substance P and NK₁R, and both molecules were localized in numerous small vesicles located just beneath the plasma membrane (Figures 4a and 4b). Co-localization was confirmed by superimposing images of the same field for cy3–substance P and the fluorescein-labelled NK₁R (Figure 4c). Cy3–substance P binding was specific since it was blocked by pretreatment of cells with CP 96,345 or unlabelled substance P, and there was no detectable binding of cy3–

Figure 2 Internalization of NK₁R examined by immunocytochemistry using the anti-Flag antibody and observed by confocal fluorescence microscopy

KNRK Flag-NK₁R cells were exposed to substance P (10 nM) for 60 min at 4 °C. The cells were washed and incubated in substance P-free medium for 30 min at 37 °C. Cells were fixed and stained as described in the Materials and methods section. The figure shows a series of optical sections at 1 μ m intervals through three cells. Scale bar = 5 μ m.

Figure 3 Internalization of NK₁R examined by immunocytochemistry using the anti-NK₁R serum and observed by conventional fluorescence microscopy

KNRK Flag-NK₁R cells were incubated with peptide or drug for 60 min at 4 °C, washed and then incubated in medium for 30 min at 37 °C. Cells were fixed and stained as described in the Materials and methods section. (a) Substance P (SP) (10 nM). (b) CP 96,345 (1 μM). (c) CP 96,345 (1 μM) and SP (10 nM). (d) SP (10 nM) and hyperosmolar sucrose (0.45 M). (e) SP (10 nM) after pretreatment with phenylarsine oxide (80 μM). (f) Neurokinin A (100 nM). Scale bar = 10 μm.

substance P to KNRK cells transfected with a vector lacking the NK₁R insert.

To determine whether substance P was internalized into early endosomes, KNRK Flag-NK₁R cells were incubated with both cy3-substance P and FITC-labelled transferrin, fixed and examined by confocal microscopy. When cells were examined after 60 min incubation with cy3-substance P and FITC-transferrin at 4 °C, both cy3-substance P and FITC-transferrin were confined to the cell surface (results not shown). However, after 5 or 10 min incubation at 37 °C, there was a reduction in the intensity of cell-surface staining for both cy3-substance P and FITC-transferrin and both molecules were localized in numerous small granules located just beneath the plasma membrane (Figures 4d and 4e). Co-localization was confirmed by superimposing images of the same field for cy3-substance P and the fluorescein-labelled transferrin (Figure 4f).

Internalization of ¹²⁵I-substance P

The binding of ¹²⁵I-substance P to KNRK Flag-NK₁R cells and KNRK NK₁R cells is specific, temperature-dependent, and reversible [14]. KNRK Flag-NK₁R cells were incubated with ¹²⁵I-substance P (10 pM) for 60 min at 4 °C to allow binding to reach equilibrium. When cells were washed with the acid-

stripping solution immediately after this binding period, $92.3 \pm 0.9\%$ (triplicate observations, $n = 4$ experiments) of the bound label was recovered in the acid-sensitive fraction. Only $3.0 \pm 0.6\%$ of the bound label was in the acid-resistant fraction, indicating that minimal internalization had occurred. Non-specific binding after 60 min at 4 °C was less than 5% of the total bound counts.

After 60 min of incubation of KNRK Flag-NK₁R cells with ¹²⁵I-substance P (10 pM) at 4 °C, $25.9 \pm 1.3\%$ of the added counts were specifically bound to the cells. At this time, $23.6 \pm 2.4\%$ of added counts were in the acid-sensitive fraction and $3.0 \pm 3.4\%$ were in the acid-resistant fraction. Rapid internalization of ¹²⁵I-substance P occurred when the temperature was raised to 37 °C (Figure 5). At 10 min, when internalization was maximal, $22.0 \pm 1.8\%$ of added counts were associated with the cells. Now $3.3 \pm 0.2\%$ of added counts were in the acid-sensitive fraction and $17.2 \pm 1.7\%$ in the acid-resistant fraction. The total binding of ¹²⁵I-substance P to the cells was measured in separate experiments and found to be the approximate sum of the acid-sensitive and acid-resistant fractions (Figure 5).

Treatment of cells with hyperosmolar sucrose or with phenylarsine oxide slightly reduced the equilibrium binding measured after 60 min incubation at 4 °C. At equilibrium, $25.9 \pm 1.3\%$ of added counts were specifically bound to untreated cells, $19.3 \pm 2.5\%$ were bound to cells treated with hyperosmolar

Figure 4 Internalization of fluorescein-labelled NK₁R and cy3-substance P (panels a–c) and FITC-transferrin and cy3-substance P (panels d–f) observed by confocal microscopy

The cells in panels (a–c) were incubated with cy3-substance P (100 nM) for 60 min at 4 °C, washed and then incubated in medium for 10 min at 37 °C. Cells were then incubated with the NK₁R antiserum and a fluorescein-conjugated secondary antiserum, as described in the Materials and methods section. Cells were observed using fluorescein filters to detect NK₁R immunoreactivity (a) and rhodamine filters to detect cy3-substance P (b). The image in (c) is formed by superimposing images from panels (a) and (b). Co-localization of NK₁R immunoreactivity and cy3-substance P is indicated by arrows pointing to the yellow granules. The cells in panels (d–f) were incubated with cy3-substance P and FITC-transferrin for 60 min at 4 °C, washed and then incubated in medium for 10 min at 37 °C. Cells were observed using fluorescein filters to detect FITC-transferrin (d) and rhodamine filters to detect cy3-substance P (e). The image in (f) is formed by superimposing images from panels (d) and (e). Co-localization of FITC-transferrin and cy3-substance P is indicated by arrows pointing to the yellow granules. Scale bar = 5 µm.

Figure 5 Binding of ^{125}I -substance P to KNRK Flag-NK₁R cells

Cells were incubated with ^{125}I -substance P (10 pM) for 60 min at 4 °C to allow binding to reach equilibrium without internalization. The temperature was raised to 37 °C for the specified times and the acid-sensitive (○), acid-resistant (▲) and total binding (acid-sensitive plus acid-resistant) (●) were measured as described in the Materials and methods section. Binding is expressed as a percentage of the counts added to the cells normalized to a cell count of 100 000 and is the mean of triplicate observations of $n = 3$ experiments.

sucrose, and $18.3 \pm 3.6\%$ were bound to cells treated with phenylarsine oxide. These treatments almost eliminated internalization of ^{125}I -substance P (Figure 6a). After incubation for 10 min at 37 °C, only $1.3 \pm 0.7\%$ of added counts were in the acid-resistant fraction of cells treated with hyperosmolar sucrose, $2.0 \pm 0.9\%$ were in the acid-resistant fraction of cells treated with phenylarsine oxide, compared with $17.2 \pm 1.7\%$ in the acid-resistant fraction of untreated cells. In cells treated with hyperosmolar sucrose or phenylarsine oxide, the acid-sensitive fraction remained high throughout the experiment (Figure 6b). The time course of substance P internalization and the effects of hyperosmolar sucrose and phenylarsine oxide were similar in cells expressing the native and the epitope-labelled receptor (results not shown).

The acidotropic agents NH_4Cl , monensin and chloroquine did not significantly affect equilibrium binding measured after 60 min incubation at 4 °C (results not shown). These agents also did not affect internalization of ^{125}I -substance P, since the rate of generation of acid-resistant material was indistinguishable (Figure 7b). However, after the temperature had been raised to 37 °C for 60 min, they caused a retention of the label in the cells that was statistically significant when compared with untreated cells (Figure 7a). At this time, $21.8 \pm 4.2\%$ of added counts were in the acid-resistant fraction of cells treated with NH_4Cl , $20.5 \pm 0.9\%$ with monensin and $16.7 \pm 3.2\%$ with chloroquine, compared with $6.1 \pm 1.7\%$ in untreated cells. This experiment also clearly shows that the disappearance of acid-resistant and total cell-associated radioactivity essentially reflects intracellular degradation. Treatment of cells with the protease inhibitor leupeptin and the inhibitor of microtubule function colchicine did not affect total binding or internalization of ^{125}I -substance P (results not shown).

Continuous exposure of cells to unlabelled substance P reduced the subsequent binding of ^{125}I -substance P. Cells were incubated with substance P (1×10^{-11} – 1×10^{-7} M) for 30 min to 8 h at 37 °C, acid-washed, and then incubated with ^{125}I -substance P (10 pM) for 1 h at 4 °C to measure binding at equilibrium. After 30 min incubation at 37 °C, there was a concentration-dependent reduction in specific binding compared with control cells that

Figure 6 The effects of hyperosmolar sucrose (0.45 M; ▲) and phenylarsine oxide (80 μM ; △) on internalization of ^{125}I -substance P (10 pM) in KNRK Flag-NK₁R cells

This shows (a) the acid-resistant fraction and (b) the acid-sensitive fraction of the bound label. Binding is expressed as a percentage of the counts added to the cells normalized to a cell count of 100 000 and is the mean of triplicate observations of $n = 3$ experiments. * $P < 0.05$ compared with control (●).

were incubated with substance P carrier before the acid wash and binding experiment (Figure 8). Binding was reduced with longer pre-incubation times with unlabelled substance P. The maximum reduction in ^{125}I -substance P binding was $48 \pm 13\%$ of control cells after 8 h incubation at 37 °C with 10 nM substance P.

Degradation of internalized ^{125}I -substance P

In control experiments, ^{125}I -substance P (100 pM) was incubated in medium without cells for 60 min at 4 °C. At this time, approx. 85% of the radioactivity recovered from the h.p.l.c. column eluted with intact substance P and was thus undegraded (results not shown). When ^{125}I -substance P was incubated with KNRK Flag-NK₁R cells for 60 min at 4 °C, to allow binding to continue to equilibrium without internalization, $89.4 \pm 1.2\%$ of the recovered radioactivity in the acid-sensitive fraction eluted with intact substance P (Figure 9a). There was too little radioactivity in the acid-resistant fraction to analyse by h.p.l.c. When ^{125}I -substance P (100 pM) was incubated in medium without cells for 60 min at 4 °C followed by 30 min at 37 °C, $65.0 \pm 5.6\%$ of the

Figure 7 The effects of monensin (50 μ M; $\circ$), chloroquine (500 μ M; $\blacktriangle$) and NH_4Cl (10 mM; $\triangle$) on internalization of ^{125}I -substance P (10 pM) in KNRK Flag-NK₁R cells

This shows (a) the acid-resistant fraction and (b) the acid-sensitive fraction of the bound label. Binding is expressed as a percentage of the counts added to the cells normalized to a cell count of 100 000 and is the mean of triplicate observations of $n = 3$ experiments. * $P < 0.05$ compared with control ($\bullet$).

radioactivity recovered from the h.p.l.c. column was eluted with intact substance P. When ^{125}I -substance P was incubated with KNRK Flag-NK₁R cells for 30 min at 37 $^{\circ}\text{C}$, $28.6 \pm 7.4\%$ of the recovered radioactivity in the medium was eluted as intact substance P. This was not consistently affected by hyperosmolar sucrose, phenylarsine, NH_4Cl , monensin, chloroquine, colchicine or leupeptin. Indeed, ^{125}I -substance P is also degraded by untransfected KNRK cells, possibly by secreted enzymes [16].

The metabolic fate of internalized ^{125}I -substance P was examined by h.p.l.c. analysis of the acid-resistant fractions. When ^{125}I -substance P (100 pM) was incubated with KNRK Flag-NK₁R cells for 30 min at 37 $^{\circ}\text{C}$, only $54.4 \pm 0.6\%$ of the recovered radioactivity in the acid-resistant fraction was eluted as intact substance P (Table 1, Figure 9b). There was too little radioactivity to analyse in the acid-resistant fraction from cells treated with hyperosmolar sucrose or phenylarsine oxide because these agents prevented internalization. There was a significant increase in the proportion of intact substance P in the acid-resistant fraction when cells were incubated with NH_4Cl , monensin and chloro-

Figure 8 Effects of pre-incubation of cells with graded concentrations of unlabelled substance P for 30 min at 37 $^{\circ}\text{C}$ on subsequent binding of ^{125}I -substance P

Cells were incubated with unlabelled substance P at 37 $^{\circ}\text{C}$, washed with acid to remove surface peptide, and incubated with ^{125}I -substance P (10 pM) for 60 min at 4 $^{\circ}\text{C}$ to allow binding to reach equilibrium, as described in the Materials and methods section. Binding is expressed as a percentage of that observed in control cells that were incubated with substance P carrier solution before the acid wash and binding, and is the mean of triplicate observations of $n = 4$ experiments.

quine (Table 1, Figures 9c–9e). The proportion of intact substance P in the acid-resistant fraction was slightly increased by incubation of cells with leupeptin or colchicine, but this was not statistically different from untreated cells (Table 1, Figure 9f).

DISCUSSION

Our results show that after binding both substance P and its receptor are internalized into early endosomes within minutes of raising the temperature to 37 $^{\circ}\text{C}$. This depletes receptors from the cell surface and may contribute to a down-regulation in the response of a cell to substance P. Once internalized substance P is degraded, probably in an acidic compartment. Substance P and the NK₁R were internalized into the same granules within minutes of raising the temperature of the cells to 37 $^{\circ}\text{C}$. Although these granules could represent aggregation of the receptor in the plasma membrane, examination by confocal microscopy confirmed that the receptor was indeed located in intracellular vesicles. FITC–transferrin was internalized into the same vesicles as cy3–substance P within 5 and 10 min of raising the temperature to 37 $^{\circ}\text{C}$, identifying the vesicles as early endosomes. Others have used receptor antibodies to show that the β_2 -adrenergic and thrombin receptors are also internalized into early endosomes within minutes of binding an agonist [4,5]. At later times, NK₁R immunoreactivity was observed in larger, perinuclear vesicles, possibly indicating entry of NK₁R into other organelles of the endocytic pathway. Additional experiments using markers for intracellular organelles or electron microscopy will be required to unequivocally identify these organelles. The eventual disappearance of the internalized NK₁R may indicate that the receptor is recycled back to the plasma membrane or degraded [1–3].

Neurokinin A, a low-affinity agonist of NK₁R [9], also induced

Figure 9 H.p.l.c. chromatograms of the degradation products of ^{125}I -substance P

(a) Acid-sensitive fraction from KNRK Flag-NK₁R cells incubated with ^{125}I -substance P (100 pM) for 60 min at 4 °C. (b–f) KNRK Flag-NK₁R cells were incubated with ^{125}I -substance P for 60 min at 4 °C. The temperature was raised to 37 °C to allow internalization to proceed for 30 min and the acid-resistant fractions were analysed by h.p.l.c. (b) Control. (c) NH_4Cl (10 mM). (d) Monensin (50 μM). (e) Chloroquine (500 μM). (f) Leupeptin (20 μM), 30 min at 37 °C. Representative chromatograms of duplicate observations from $n = 4$ experiments are shown. The intervals on the vertical axis are 500 c.p.m. and the horizontal axis are 10 min.

Table 1 Degradation of ^{125}I -substance P by KNRK Flag-NK₁R cells

KNRK Flag-NK₁R cells were incubated with ^{125}I -substance P (100 pM) for 60 min at 4 °C. The temperature was raised to 37 °C to allow internalization to proceed for 30 min. The acid-resistant fractions were collected and fractionated by reverse-phase h.p.l.c., as described in the Materials and methods section. Approx. 20000 counts were loaded on the h.p.l.c. The percentage of radioactivity recovered from the h.p.l.c. column that was contained in the peak corresponding to intact ^{125}I -substance P was measured. The results are expressed as the percentage of the total counts eluted from the column and represent the mean of duplicate observations from $n = 4$ experiments. * $P < 0.05$ compared with control of untreated cells at 37 °C.

Treatment	Intact ^{125}I -substance P (% recovered from h.p.l.c.)
Control 37 °C	54.40 ± 0.55
NH_4Cl (10 mM)	83.08 ± 0.55*
Monensin (50 μM)	88.19 ± 0.54*
Chloroquine (500 μM)	89.17 ± 0.93*
Leupeptin (20 μM)	68.49 ± 0.21
Colchicine (500 μM)	62.81 ± 3.58

internalization. However, CP 96,345, which binds to the NK₁R with high affinity but acts as an antagonist [9], did not induce internalization. Therefore, internalization may require binding and signal transduction. The binding experiments showed that ^{125}I -substance P was rapidly internalized after interaction with NK₁R, with an initial time course similar to that observed for the receptor by immunocytochemistry. Therefore, it is probable that the internalization of ^{125}I -substance P reflects internalization of the ligand–receptor complex. In support of our findings are the observations that ^{125}I -substance P undergoes rapid, temperature-dependent internalization in anterior pituitary cells [12] and pancreatic acinar cells [13]. The related neuropeptide bombesin/gastrin-releasing peptide (GRP) is also rapidly internalized by pancreatic acini [17,18] and Swiss 3T3 cells [19,20].

Phenylarsine oxide and hyperosmolar sucrose inhibited substance P-induced internalization of the NK₁R, as determined by immunocytochemistry with receptor antibodies and by binding experiments. Phenylarsine oxide, a tri-arsenical compound, blocks endocytosis of macromolecules by cross-linking proteins that have sulphur groups [21], and hyperosmolar sucrose blocks endocytosis by clathrin-coated pits [22]. Internalization of β_2 -adrenergic receptors is inhibited by phenylarsine oxide [15] and by hyperosmolar sucrose [6]. Therefore, coated-pit endocytosis appears to be a common mechanism involved in the endocytosis of NK₁R in KNRK cells and other receptors belonging to this family.

Blocking acidification of organelles with the acidotropic agents monensin, NH_4Cl and chloroquine caused the retention of the internalized ^{125}I -substance P in KNRK Flag-NK₁R cells, and markedly inhibited its intracellular degradation. Acidification of early endosomes is important for the dissociation of ligands from single transmembrane receptors, and interference with this dissociation can block recycling of receptors to the cell surface [1]. GRP/bombesin, a ligand of a seven transmembrane domain receptor, is also retained in pancreatic acini [18] and Swiss 3T3 cells [19] in the presence of acidotropic agents. In addition, chloroquine inhibits the intracellular degradation of radiolabelled bombesin by pancreatic acini [18]. It is not clear how acidotropic agents inhibit the degradation of internalized substance P. One possibility is that they inhibit the activity of lysosomal enzymes that are active under acidic conditions. However, leupeptin, a lysosomal protease inhibitor, did not affect substance P degradation, possibly because the enzyme active in degradation is resistant to leupeptin. Alternatively, acidotropic agents may interfere with dissociation of the receptor–ligand complex earlier in the endocytic process and thereby decrease the delivery of substance P to lysosomes. Further studies of the steady-state distribution of substance P and NK₁R in the presence of these agents may establish how they affect endocytosis of substance P and its receptor.

Colchicine, an inhibitor of microtubule assembly, had no effect on the internalization and degradation of ^{125}I -substance P by KNRK Flag-NK₁R cells. In contrast, colchicine inhibits internalization of ^{125}I -substance P by pancreatic acinar cells [13], and both internalization and degradation by isolated anterior pituitary cells [12].

The functional significance of NK₁R internalization has yet to be established, but it may be related to desensitization and resensitization to substance P. Internalization, by removing receptors from the cell surface, could contribute to desensitization. Indeed, exposure of cells to substance P caused a marked reduction in subsequent substance P binding, which may down-regulate NK₁R. Thrombin receptors undergo very rapid and marked internalization upon ligand binding (85% internalized on erythroleukaemia cells in 1 min), and this is fast enough to

account for desensitization [4]. The NK₁R is also rapidly desensitized to repeated exposure to substance P [23], and internalization may be sufficiently rapid to contribute to desensitization. However, phosphorylation of NK₁R may also account for its desensitization. Phosphorylation of the β_1 - and β_2 -adrenergic receptors by cyclic AMP-dependent protein kinase A and by a specific β_2 -adrenergic receptor kinase is responsible for acute homologous desensitization [8,24–26]. NK₁R contains 31 serine and threonine residues in the third intracellular loop and the intracellular C-terminus that are potential phosphorylation sites possibly involved in desensitization [10,11] and, like the β_2 -adrenergic receptor, NK₁R is phosphorylated by β_2 -adrenergic receptor kinase [27]. Internalization of the β_2 -adrenergic receptor may be required for dephosphorylation and resensitization because the internalized β_2 -adrenergic receptor is less phosphorylated than the cell-surface receptor [7], and inhibition of internalization interferes with resensitization [6]. It is not known whether internalization of NK₁R is required for receptor resensitization.

The present experiments demonstrate agonist-induced internalization of NK₁R in epithelial cells. Within minutes of binding, both substance P and its receptor are internalized into early endosomes and internalized substance P is degraded.

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REFERENCES

- Wileman, T., Harding, C. and Stahl, P. (1985) *Biochem. J.* **232**, 1–14
- Forgacs, M. (1988) in *The Liver: Biology and Pathobiology* (Arias, I. M., Jakoby, W. B., Popper, H., Schachter, D. and Shafritz, D. A., eds.), pp. 207–225, Raven Press, New York
- Hopkins, C. R., Gibson, A., Shipman, M. and Miller, K. (1990) *Nature (London)* **346**, 335–339
- Hoxie, J. A., Ahuja, M., Belmonte, E., Pizarro, S., Parton, R. and Brass, L. F. (1993) *J. Biol. Chem.* **268**, 13756–13763
- von Zastrow, M. and Kobilka, B. K. (1991) *J. Biol. Chem.* **267**, 3530–3538
- Yu, S. S., Lefkowitz, R. J. and Hausdorff, W. P. (1993) *J. Biol. Chem.* **268**, 337–341
- Sibley, D. R., Strasser, R. H., Benovic, J. L., Daniel, K. and Lefkowitz, R. J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9408–9412
- Hausdorff, W. P., Caron, M. G. and Lefkowitz, R. J. (1990) *FASEB J.* **4**, 2881–2889
- Otsuka, M. and Yoshioka, K. (1993) *Physiol. Rev.* **73**, 229–309
- Yokota, Y., Sasai, Y., Tanaka, K., Fujiwara, T., Tsuchida, K., Shigemoto, R., Kakizuka, A., Ohkubo, H. and Nakanishi, S. (1989) *J. Biol. Chem.* **264**, 17649–17652
- Hershey, A. D. and Krause, J. E. (1990) *Science* **247**, 958–962
- Larsen, P. J., Mikkelsen, J. D., Mau, S. and Saermark, T. (1989) *Mol. Cell. Endocrinol.* **65**, 91–101
- Sjodin, L. (1992) *J. Receptor Res.* **12**, 323–350
- Vigna, S. R., Bowden, J. J., McDonald, D. M., Fisher, J., Okamoto, A., McVey, D. C., Payan, D. G. and Bunnett, N. W. (1994) *J. Neurosci.* **14**, 834–845
- Feldman, R. D., McArdle, W. and Lai, C. (1986) *Mol. Pharmacol.* **30**, 459–462
- Okamoto, A., Lovett, M., Payan, D. G. and Bunnett, N. W. (1994) *Biochem. J.* **299**, 683–693
- Lee, P. C., Jensen, R. T. and Gardner, J. D. (1980) *Am. J. Physiol.* **238**, G213–G218
- Zhu, W.-Y., Goke, B. and Williams, J. A. (1991) *Am. J. Physiol.* **261**, G57–G64
- Zachary, I. and Rozengurt, E. (1987) *EMBO J.* **6**, 2233–2239
- Wang, L.-H., Mantey, S. A., Lin, J.-T., Frucht, H. and Jensen, R. T. (1993) *Biochim. Biophys. Acta* **1175**, 232–242
- Frost, S. C. and Lane, M. D. (1985) *J. Biol. Chem.* **260**, 2646–2652
- Heuser, J. E. and Anderson, R. G. W. (1989) *J. Cell Biol.* **108**, 389–400
- McMillian, M. K., Soltoff, S. P. and Talmo, B. R. (1987) *Biochem. Biophys. Res. Commun.* **148**, 1017–1024
- Lohse, M. J., Benovic, J. L., Caron, M. G. and Lefkowitz, R. J. (1990) *J. Biol. Chem.* **265**, 3202–3209
- Roth, N. S., Campbell, P. T., Caron, M. G., Lefkowitz, R. J. and Lohse, M. J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6201–6204
- Zhou, X.-M. and Fishman, P. H. (1991) *J. Biol. Chem.* **266**, 7462–7468
- Kwatra, M. M., Schwinn, D. A., Schreurs, J., Blank, J. L., Kim, C. M., Benovic, J. L., Krause, J. E., Caron, M. G. and Lefkowitz, R. J. (1993) *J. Biol. Chem.* **268**, 9161–9164