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Mechanisms of Desensitization and Resensitization of G Protein-Coupled Neurokinin$_1$ and Neurokinin$_2$ Receptors

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SUMMARY

We compared the desensitization of neurokinin$_1$ and neurokinin$_2$ (NK$_1$ and NK$_2$) receptors expressed in Chinese hamster ovary cells to substance P and neurokinin A, respectively. Substance P and neurokinin A stimulated a rapid increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) for both receptors, which was due to release of Ca^{2+} from intracellular stores. This was followed by a plateau in $[Ca^{2+}]_i$, which was due to influx of extracellular Ca^{2+}, and was more sustained for the NK$_2$ receptor. When Ca^{2+} was present in the extracellular solution, the Ca^{2+} response of the NK$_1$ receptor, but not of the NK$_2$ receptor, rapidly desensitized and slowly resensitized to two exposures to agonist. In contrast, the $[Ca^{2+}]_i$ response, measured in Ca^{2+}-free solution, and inositol triphosphate generation desensitized and resensitized similarly for the NK$_1$ and NK$_2$ receptors. Thus, differences in desensitization between the NK$_1$ receptor and the NK$_2$ receptor may be related to differences in entry of extracellular Ca^{2+}. We compared endocytosis of the NK$_1$ and NK$_2$ receptors to determine whether disparities could account for differences in desensitization. Fluorescent and radiolabeled substance P and neurokinin A were internalized similarly by cells expressing NK$_1$ and NK$_2$ receptors. Thus, disparities in internalization cannot account for differences in desensitization. We used inhibitors to examine the contribution of endocytosis, recycling, and phosphatases to desensitization and resensitization of the NK$_1$ receptor. Desensitization did not require endocytosis. However, resensitization required endocytosis, recycling, and phosphatase activity. This suggests that the NK$_1$ receptor desensitizes by phosphorylation and resensitizes by dephosphorylation in endosomes and recycling.

SP and NKA are members of the tachykinin family of neuropeptides, which are widely distributed in the peripheral and central nervous systems (1). They are neurotransmitters in the enteric nervous system, spinal cord, and brain; stimulate smooth muscle contraction and exocrine gland secretion; and mediate neurogenic inflammation. These actions are mediated by three neurokinin receptors (NK$_1$, NK$_2$, and NK$_3$) that are anchored to the plasma membrane with seven hydrophobic domains and are coupled to G proteins (2). SP has the highest affinity for the NK$_1$ receptor, and NKA has the highest affinity for the NK$_2$ receptor. Ligand binding activates phospholipase C, which generates I(1,4,5)P$_3$ and increases $[Ca^{2+}]_i$, and stimulates adenylyl cyclase and phospholipase A$_2$ (1, 3, 4).

Physiological responses to repetitive applications of SP rapidly desensitize in tissues that naturally express the NK$_1$ receptor and in transfected cells and then gradually resensitize (5–7). Other G protein-coupled receptors similarly desensitize, including the β_2-adrenergic receptor (8) and the gastrin-releasing peptide receptor (9). In contrast, responses to NKA in cells expressing the NK$_2$ receptor are sustained and do not desensitize to the same extent (4, 10, 11). The reason for this difference in desensitization between the NK$_1$ receptor and the NK$_2$ receptor is unknown, but it may relate to differences in agonist-induced receptor phosphorylation or receptor endocytosis.

Many receptors desensitize by phosphorylation and uncoupling from G proteins. This has been thoroughly studied for the β_2-adrenergic receptor. G protein receptor kinases 2 and 3 (β-adrenergic receptor kinases 1 and 2) phosphorylate the activated β_2-adrenergic receptor, which then binds β-arrestins 1 and 2 (8). This interdicts interaction between the receptor and G proteins and causes desensitization. The thrombin receptor similarly desensitizes (12). G protein receptor kinases 2 and 3 also phosphorylate the activated NK$_1$ receptor when it is reconstituted in phospholipid vesicles (13). There are multiple consensus sites for phosphorylation by G protein receptor kinases and protein kinase C in intra-

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ABBREVIATIONS: SP, substance P; NK, neurokinin; I(1,4,5)P$_3$, inositol-1,4,5-trisphosphate; CHO, Chinese hamster ovary; BSA, bovine serum albumin; Cy3-SP, cyanine 3-labeled substance P; Cy3-NKA, cyanine 3-labeled neurokinin A; KNRK, rat kidney epithelial cells; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

cellular loop 3 and carboxyl-tail of the NK_1 receptor but not of the NK_2 receptor (14). This may account for the differences in desensitization of these receptors. However, the role of phosphorylation in desensitization of tachykinin receptors in cells has not been directly studied.

Agonist-induced endocytosis may contribute to desensitization by depleting the cell surface of high affinity receptors. The NK_1 receptor rapidly internalizes after binding SP and efficiently recycles (7, 15, 16). The β_2-adrenergic receptor and gastrin-releasing peptide receptor also internalize after agonist binding and recycle (17, 18). However, the β_2-adrenergic receptor desensitizes normally, but does not resensitize, when endocytosis is inhibited (19, 20). This suggests that desensitization occurs by receptor phosphorylation rather than by internalization. Resensitization of the β_2-adrenergic receptor may require receptor endocytosis, dephosphorylation in endosomes, and recycling. Indeed, the β_2-adrenergic receptor shows reduced phosphorylation in endosomes and does not resensitize if recycling is inhibited (21, 22). We do not know whether endocytosis of the NK_1 receptor contributes to its desensitization or whether dephosphorylation and recycling mediate resensitization. We also do not know whether the NK_2 receptor, which does not desensitize significantly, is internalized after agonist binding.

In the current study, we examined how the NK_1 and NK_2 receptors, stably expressed in cell lines, desensitize and resensitize. We (i) compared desensitization and resensitization of $[Ca^{2+}]_i$ responses and $I(1,4,5)P_3$ generation; (ii) examined endocytosis of fluorescent and radiolabeled agonists; and (iii) investigated the effects of inhibitors of endocytosis, recycling, and phosphatases on desensitization and resensitization.

Materials and Methods

Reagents. SP and NKA were obtained from Peninsula Laboratories (San Carlos, CA) and Bachem Bioscience (King of Prussia, PA). Bolton-Hunter ^{125}I-SP and ^{125}I-NKA (2000–2200 Ci/mmol) were obtained from Amersham Corp. (Chicago, IL). Bafilomycin A_1 was a gift from Dr. Jonathan R. Green (Ciba-Geigy, Basel, Switzerland). Bisfunctional cyanine 3.18 was a gift from Dr. Lauren Ernst (Biological Detection Systems, Pittsburgh, PA). Fura-2/acetoxymethyl ester, pleuronic solution, and Slow Fade were obtained from Molecular Probes (Eugene, OR). $I(1,4,5)P_3$) was purchased from CalBiochem (La Jolla, CA), and [^{3}H]$I(1,4,5)P_3$ (21 Ci/mmol) was purchased from Amersham Corp. A rabbit polyclonal antiserum (No. 11884–5) was raised to a 15-residue peptide (K^{393}TMTESSSFYSNMLA^{407}), corresponding to the intracellular carboxyl terminus of the rat NK_1 receptor (23). A mouse monoclonal antibody to the transferrin receptor was a gift from Dr. Ian Trowbridge (The Salk Institute, San Diego, CA). Affinity-purified fluorescein isothiocyanate-conjugated goat anti-mouse IgG and Texas Red-conjugated goat anti-rabbit IgG were purchased from Cappel Research Products (Durham, NC). Okadaic acid was obtained from GIBCO-BRL (Bethesda, MD). Amiloride, nifedipine, and SK&F 96365 were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell lines. The generation and characterization of CHO cell lines expressing the NK_1 or NK_2 receptor have been described previously (4). The number of high affinity binding sites per cell was 200,000 for the CHO-NK_1 receptor and 200,000 for the CHO-NK_2 receptor. Cells were maintained in minimal essential medium-α supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml G-418 in 5% O_2/95% CO_2 at 37°. Cells were plated onto glass coverslips (for microscopy and measurement of $[Ca^{2+}]_i$) or plastic wells (for binding experiments and measurement of $I(1,4,5)P_3$ generation) for at least 48 hr before the experiments.

Measurement of $[Ca^{2+}]_i$. Cells were washed with a physiological salt solution (137 mM NaCl, 4.7 mM KCl, 0.56 mM $MgCl_2$, 2 mM $CaCl_2$, 1.0 mM Na_2HPO_4, 10 mM HEPES, 2.0 mM L-glutamine, and 5.5 mM D-glucose, pH 7.4) containing 0.1% BSA. They were incubated in this solution with 2.5 μM Fura-2/acetoxymethyl ester and 0.2% pleuronic solution for 30 min at 37° and washed, and fluorescence was measured at 340 and 380 nm excitation and 510 nm emission (23). The ratio of the fluorescence at the two excitation wavelengths, which is proportional to the $[Ca^{2+}]_i$, was calculated.

To examine the contribution of extracellular Ca^{2+} to the change in $[Ca^{2+}]_i$, cells were exposed to a single dose of SP or NKA (10 nM) in Ca^{2+}-free solution. In some experiments, Ca^{2+} was added back to the assay solution at 180 sec to 2 mM. To examine desensitization and resensitization, cells were incubated with a 10 nM desensitizing dose of agonist or to carrier (control) for 1 min at 37°, washed, and then exposed to a 10 nM test dose of agonist at various times after the desensitizing dose. To determine whether intracellular Ca^{2+} pools were intact, cells were exposed to 1 μM thapsigargin (24). The change from the original baseline before any agonist exposure was calculated. The response to the test dose of agonist was compared between agonist- and carrier-treated cells and expressed as a percentage.

Measurement of $I(1,4,5)P_3$. Cells were dispersed with nonenzymatic dissociation buffer and resuspended in Hanks' balanced salt solution containing 0.1% BSA to 3×10^6 cells/ml. To examine desensitization and resensitization, the cell suspension (180 μl/reaction) was incubated with a 10 nM desensitizing dose of agonist or carrier (control) for 1 min at 37°. Cells were centrifuged ($500 \times g$ for 1 min), resuspended in agonist-free medium at 37°, and exposed to a 1 μM test dose of agonist 10–30 min after the first dose. In preliminary experiments, the agonist-induced $I(1,4,5)P_3$ response peaked at 5–10 sec and returned to baseline by 15–20 sec. To measure the initial burst of $I(1,4,5)P_3$ generation, reactions were quenched with an equal volume of 30% trichloroacetic acid 5 sec after the test dose. Cells were placed on ice for 10 min and centrifuged ($14,000 \times g$ for 30 sec) to pellet precipitated protein. The supernatant was removed and extracted four times with 2 volumes of water-saturated ether. An aliquot of the extract (50 μl) was used in a competitive binding assay with rat cerebellar membranes (25); the membranes contain a receptor that is highly specific for $I(1,4,5)P_3$, the active metabolite in the phospholipase C pathway leading to the release of Ca^{2+} from intracellular stores. A standard curve was generated with unlabeled $I(1,4,5)P_3$ (1 pM–100 nM) and [^{3}H]$I(1,4,5)P_3$ (1 nM) to allow calculation of $I(1,4,5)P_3$ levels in the cell extracts. Results were calculated as pmol of $I(1,4,5)P_3/10^6$ cells and were expressed as a percentage of the response in carrier-treated cells.

Drug treatments. Endocytosis of the NK_1 receptor was inhibited by treatment of cells for 5 min with 80 μM phenylarsine oxide at 60 min before the $I(1,4,5)P_3$ assays or with 80 μM phenylarsine oxide plus 5 μM β-mercaptoethanol immediately before $[Ca^{2+}]_i$ measurement (15, 26, 27). Recycling of the NK_1 receptor was inhibited by treatment of cells with 1 μM bafilomycin A_1 for 60 min before $[Ca^{2+}]_i$ measurement (18). Phosphatases were inhibited by treatment of cells with 50 nM okadaic acid for 60 min before $[Ca^{2+}]_i$ measurement. Ca^{2+} channels were blocked by treatment of cells with 10 μM nifedipine, 10 μM amiloride, or 5 μM SK&F 96365 for 60 sec before exposure to agonist. Control cells were treated with carrier.

Examination of endocytosis by fluorescence microscopy. Cy3-SP and Cy3-NKA were used to observe endocytosis of the NK_1 receptor and the NK_2 receptor, respectively (28). Cells were incubated with 100 nM Cy3-SP or Cy3-NKA in Dulbecco's modified Eagle's medium containing 0.1% BSA for 2 hr at 4° for equilibrium binding. They were washed and placed in medium at 37° for 0–30 min for endocytosis. Cells were fixed in 4% paraformaldehyde in 100 mM phosphate-buffered saline, pH 7.4, at 4° for 20 min and mounted in Slow Fade. For immunofluorescence studies to colocalize the NK_1 receptor and the transferrin receptor, cells were incubated with 10

nM SP, washed, warmed to 37°, and fixed (16). They were processed to simultaneously localize the NK_1 receptor and transferrin receptor by immunofluorescence. Cells were examined with a Zeiss Axioplan microscope or a Zeiss Laser Scan Inverted 410 microscope (16).

Quantification of endocytosis with ^{125}I peptides. ^{125}I-SP and ^{125}I-NKA were used to quantify endocytosis of the NK_1 receptor and the NK_2 receptor, respectively. Cells were incubated for 2 hr at 4° with 10 pM ^{125}I-SP or ^{125}I-NKA in Hanks' balanced salt solution containing 0.1% BSA, 0.2 mg/ml bacitracin, 20 μg/ml leupeptin, and 20 μg/ml chymostatin. They were washed and incubated at 37° for 0–60 min. Cells were washed with ice-cold phosphate-buffered saline and incubated in 250 μl of ice-cold 0.2 M acetic acid containing 50 mM NaCl, pH 2.5, on ice for 5 min to separate acid-labile (cell-surface) from acid-resistant (internalized) label (15). Nonspecific binding was measured in the presence of 1 μM SP or NKA and was subtracted to give specific binding. Results are expressed as the percentage of specifically bound label contained in the intracellular fraction.

Statistical analysis. Results are expressed as mean ± standard error and are compared with the use of analysis of variance and the Student-Newman-Keuls test. A value of $p < 0.05$ was considered significant.

Results and Discussion

Characterization of agonist-induced $[Ca^{2+}]_i$ responses. Responses to SP that are mediated by the NK_1 receptor often rapidly attenuate, even in the continued presence of agonist, and desensitize to repeated application of SP (5–7). In contrast, responses to NKA that are mediated by the NK_2 receptor are more sustained and do not desensitize as markedly (4, 10, 11). We compared attenuation of agonist-induced $[Ca^{2+}]_i$ responses in CHO-NK_1 receptor and CHO-NK_2 receptor cells.

The $[Ca^{2+}]_i$ responses were biphasic for both receptors. When CHO-NK_1 receptor or CHO-NK_2 receptor cells were exposed to 10 nM SP or NKA, respectively, there was a prompt increase in $[Ca^{2+}]_i$, which declined to a plateau (Fig. 1A). The peak response was similar for both receptors, but the plateau was far more sustained for CHO-NK_2 receptor cells, in which it declined to 72.3 ± 3.0% of the peak by 180 sec, than for CHO-NK_1 receptor cells, in which it declined to 43.9 ± 7.4% of the peak by 180 sec (three observations).

To determine which phase of the response was dependent on an influx of extracellular Ca^{2+}, we assayed CHO-NK_1 receptor and CHO-NK_2 receptor cells in Ca^{2+}-free solution. Exposure to 10 nM SP or NKA caused a prompt increase in $[Ca^{2+}]_i$ which was similar to that seen in the presence of extracellular Ca^{2+} (Fig. 1B). However, in both cell lines, $[Ca^{2+}]_i$ rapidly declined to basal levels, and there was no plateau phase in the absence of extracellular Ca^{2+}. When Ca^{2+} was added to the extracellular solution (to 2 mM) at 180–200 sec after exposure to peptide, there was a large and sustained increase in $[Ca^{2+}]_i$ in both cell lines (Fig. 1C). This plateau was far more sustained for the NK_2 receptor than for the NK_1 receptor. There was a large, sustained increase in $[Ca^{2+}]_i$, even when Ca^{2+} was added to the extracellular fluid at 5–10 min after exposure to peptide, in both cell lines.

We used inhibitors to characterize the channel mediating the influx of Ca^{2+}. Treatment of CHO-NK_1 receptor cells or CHO-NK_2 receptor cells with 10 μM nifedipine, to inhibit L-type channels, or 10 μM amiloride, to inhibit N-type channels, had no effect on the influx of Ca^{2+} (Fig. 2). However, treatment with 5 μM SK&F 96365, which blocks a channel

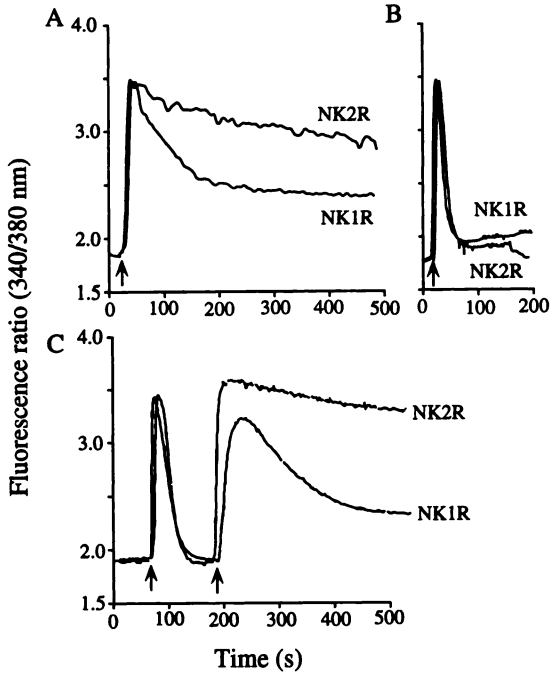

Fig. 1. Changes in $[Ca^{2+}]_i$ in CHO-NK_1 receptor (*NK1R*) cells and CHO-NK_2 receptor (*NK2R*) cells to 10 nM SP or NKA, respectively (*first arrow*). Assays were in the presence (A) or absence (B and C) of Ca^{2+} in the extracellular solution. C, Ca^{2+} was added (*second arrow*) to give 2 mM in extracellular solution. Results are expressed as the ratio of the fluorescence at the two excitation wavelengths.

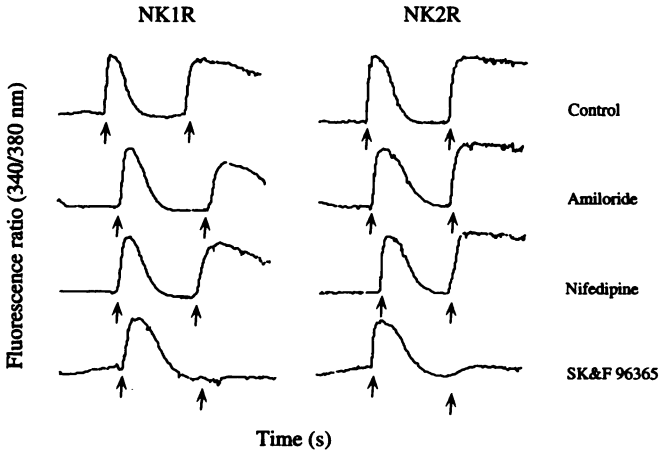

Fig. 2. Effects of Ca^{2+} channel blockers on $[Ca^{2+}]_i$ responses in CHO-NK_1 receptor (*NK1R*) cells and CHO-NK_2 receptor (*NK2R*) cells to 10 nM SP or NKA, respectively (*first arrow*). Cells were assayed in Ca^{2+}-free extracellular solution. Ca^{2+} was added (*second arrow*) to give 2 mM in extracellular solution. Amiloride, 10 μM; nifedipine, 10 μM; SK&F 96365, 5 μM. Scan durations are 200 sec.

that has been linked to receptor-mediated Ca^{2+} influx, abolished or markedly attenuated the influx (Fig. 2).

The results show that the prompt increase in $[Ca^{2+}]_i$ that followed activation of the NK_1 and NK_2 receptors is due to release of Ca^{2+} from intracellular stores. This is rapidly attenuated for the NK_1 and NK_2 receptors, even in the continued presence of agonist. The plateau phase, which is far more sustained for the NK_2 receptor than for the NK_1 receptor, is dependent on Ca^{2+} entry from the extracellular fluid. This influx of Ca^{2+} occurs through a Ca^{2+} channel that is sensitive to SK&F 96365 (29).

Desensitization and resensitization of $[Ca^{2+}]_i$ responses. We compared desensitization and resensitization of $[Ca^{2+}]_i$ responses in CHO-NK_1 receptor and CHO-NK_2 receptor cells to verify that the receptors behave differently, as they do in other cells and tissues. Cells were exposed to a 10 nM desensitizing dose of agonist or carrier, washed, and challenged with a 10 nM test dose of agonist. Initially, we measured the $[Ca^{2+}]_i$ responses in the presence of extracellular Ca^{2+}. When a test dose of SP was given to CHO-NK_1 receptor cells 5 min after the desensitizing dose, the peak response was 33.5 ± 2.0% of carrier-treated cells. By 30 min, the response was 69.7 ± 1.3% of that of the carrier-treated cells, and by 60 min it was 84.0 ± 4.1% (Fig. 3). In CHO-NK_2 receptor cells, the increase in $[Ca^{2+}]_i$ to NKA was sustained even after the cells were washed. When a test dose of NKA was given 5 min after the desensitizing dose, the $[Ca^{2+}]_i$ increased to a peak, which was 87.4 ± 4.8% of that of the carrier-treated cells (Fig. 3). We used thapsigargin, a stimulant of intracellular Ca^{2+} release by an $I(1,4,5)P_3$-independent manner (24), to verify that intracellular Ca^{2+} stores were intact after exposure to SP. CHO-NK_1 receptor cells were exposed to 10 nM SP or carrier for 1 min, washed, and treated with 1 μM thapsigargin 5 min after SP. The $[Ca^{2+}]_i$ response of SP-treated cells was 105 ± 18.6% (three observations) of carrier-treated cells, indicating that the intracellular Ca^{2+} stores were restored.

Therefore, the $[Ca^{2+}]_i$ response mediated by the NK_1 receptor rapidly desensitizes and slowly resensitizes. Desensitization is not due to depletion of intracellular stores of Ca^{2+} because the cells responded normally to thapsigargin. In contrast to the NK_1 receptor, the $[Ca^{2+}]_i$ response of the NK_2 receptor does not desensitize.

The rapid increase in $[Ca^{2+}]_i$ that was measured in CHO-NK_1 receptor or CHO-NK_2 receptor cells after exposure to SP or NKA in Ca^{2+}-free solution was due to release of Ca^{2+} from intracellular stores. This response was rapidly attenuated for both receptors. Therefore, we compared desensitization and resensitization of this portion of signaling by measurement of $[Ca^{2+}]_i$ in Ca^{2+}-free solution. When a test dose of SP was given to CHO-NK_1 receptor cells 5 min after a desensitizing dose, the peak response was 29.5 ± 11.1% of that of the carrier-treated cells (Fig. 4A). When a test dose of NKA was given to CHO-NK_2 receptor cells 5 min after a desensitizing dose, the peak response was 41.2 ± 8.6% of the carrier-treated cells (Fig. 4A). The responses resensitized with a similar time course in both cell lines. Again, thapsigargin was used to verify the integrity of the stores of intracellular Ca^{2+}. Even when assayed in Ca^{2+}-free medium, the $[Ca^{2+}]_i$ response to thapsigargin in cells expressing the NK_1 receptor was 96.7 ± 7.0% of that of the carrier-treated cells after 5 min.

The results show that the mobilization of Ca^{2+} from intra-

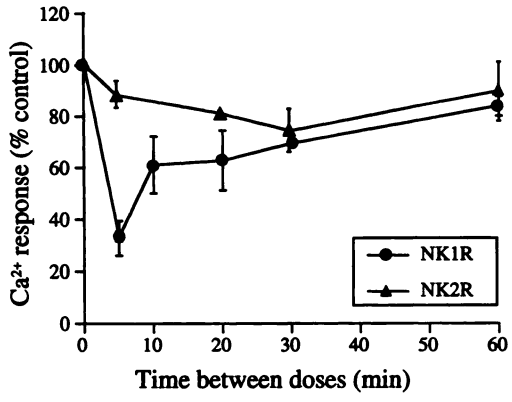

Fig. 3. Desensitization and resensitization of agonist-induced $[Ca^{2+}]_i$ responses in CHO-NK_1 (*NK1R*) receptor and CHO-NK_2 (*NK2R*) receptor cells to two sequential exposures to SP or NKA, respectively. Assays were in the presence of Ca^{2+} in the extracellular solution. Cells were exposed to a 10 nM desensitizing dose of agonist or to carrier (control), washed after 1 min, and then exposed to a 10 nM test dose of agonist at various times after the desensitizing dose. Results are expressed as the percent response to the test dose of agonist compared with the carrier-treated cells at each time point. The change from basal of the 340/380 ratio in carrier-treated cells (100%) was 1.92 ± 0.36 for CHO-NK_1 receptor cells and 2.48 ± 0.46 for CHO-NK_2 receptor cells. Data are from three observations.

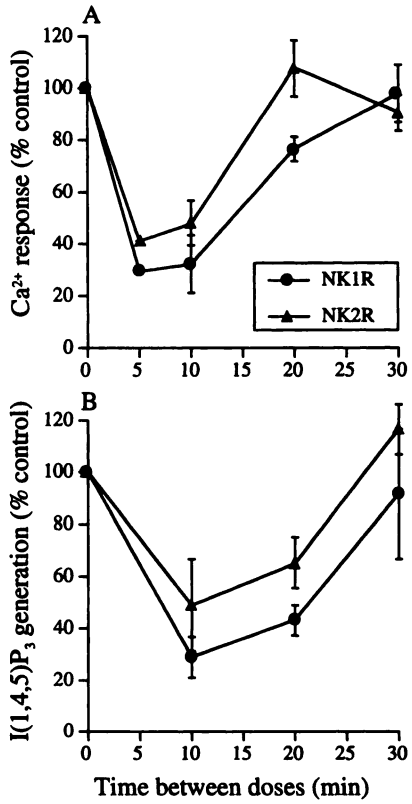

Fig. 4. Desensitization and resensitization of agonist-induced $[Ca^{2+}]_i$ responses (A) and $I(1,4,5)P_3$ generation (B) in CHO-NK_1 receptor (*NK1R*) and CHO-NK_2 (*NK2R*) receptor cells to two sequential exposures to SP or NKA, respectively. Assays of $[Ca^{2+}]_i$ were in Ca^{2+}-free extracellular fluid. To examine desensitization of $[Ca^{2+}]_i$ responses, cells were exposed to a 10 nM desensitizing dose of agonist or to carrier (control), washed after 1 min, and then exposed to a 10 nM test dose of agonist at various times after the desensitizing dose. To examine desensitization of $I(1,4,5)P_3$ generation, cells were exposed to a 10 nM desensitizing dose of agonist or to carrier (control), washed after 1 min, and then exposed to a 1 μM test dose of agonist at various times after the desensitizing dose. Results are expressed as the percent response to the test dose of agonist compared with the carrier-treated cells at each time point. The change from basal of the 340/380 ratio measured in Ca^{2+}-free medium in carrier-treated cells (100%) was 1.30 ± 0.18 for CHO-NK_1 receptor cells and 1.18 ± 0.10 for CHO-NK_2 receptor cells. The basal level of $I(1,4,5)P_3$ was 3.91 ± 1.79 pmol/10^6 cells for CHO-NK_1 receptor cells and 4.10 ± 0.92 pmol/10^6 cells for CHO-NK_2 receptor cells. The stimulated level (100%) of $I(1,4,5)P_3$ was 33.49 ± 5.79 pmol/10^6 cells for CHO-NK_1 receptor cells and 35.89 ± 5.06 pmol/10^6 cells for CHO-NK_2 receptor cells. Data are from three observations.

cellular stores that results from activation of the NK_1 or NK_2 receptor desensitizes similarly to repetitive challenge with agonists. Therefore, differences in Ca^{2+} entry from extracellular fluid may account for most of the differences in desensitization that have been observed between these receptors.

Desensitization and resensitization of $I(1,4,5)P_3$ responses. Release of Ca^{2+} from intracellular stores is regulated by the $I(1,4,5)P_3$ receptor (30). Therefore, we also examined desensitization and resensitization of $I(1,4,5)P_3$ generation by using a radioreceptor assay that measures transient changes in $I(1,4,5)P_3$ levels rather than the accumulation of radioactive products from the phospholipase C pathway. This allowed measurement of the response to a test dose of agonist without interference by the desensitizing dose because $I(1,4,5)P_3$ returned to basal levels by 20 sec in CHO cells. Cells were exposed to a 10 nM desensitizing dose of agonist or carrier, washed, and challenged with a 1 μM test dose of agonist. When a test dose of SP was given to CHO-NK_1 receptor cells 10 min after a desensitizing dose, the response was 28.8 ± 7.9% of that of carrier-treated cells (Fig. 4B). When a test dose of NKA was given to CHO-NK_2 receptor cells 10 min after a desensitizing dose, the response was 48.6 ± 17.8% of that of carrier-treated cells (Fig. 4B). The responses resensitized with a similar time course in both cell lines.

Therefore, the generation of $I(1,4,5)P_3$ and the release of Ca^{2+} from intracellular stores desensitize similarly for the NK_1 receptor and the NK_2 receptor, although the extent of desensitization of the NK_1 receptor is greater than that of the NK_2 receptor. The receptors resensitize with the same time course.

Agonist-induced endocytosis of NK_1 and NK_2 receptors. We examined whether differences in agonist-induced internalization of the NK_1 and NK_2 receptors could account for differences in desensitization. We previously used receptor antibodies, Cy3-SP, and confocal microscopy to observe endocytosis of the NK_1 receptor expressed in KNRK cells (7, 15, 16). In these cells, Cy3-SP and the NK_1 receptor are internalized by a clathrin-mediated process into early endosomes containing the transferrin receptor, a marker of early endosomes. SP and the NK_1 receptor are sorted in acidified perinuclear endosomes into distinct pathways. SP is degraded in lysosomes, and the NK_1 receptor recycles to the plasma membrane.

To determine whether SP induced internalization of the NK_1 receptor into a recycling endosomal compartment in a different transfected cell line, we incubated CHO-NK_1 receptor cells with SP and compared the distributions of the NK_1 and transferrin receptors by using immunofluorescence and confocal microscopy. When cells were exposed to 10 nM SP for 2 hr at 4°, the NK_1 receptor was at the plasma membrane, whereas the transferrin receptor was in peripheral and perinuclear endosomes. After 10 min at 37°, the NK_1 receptor was in superficial endosomes, and there was reduced surface staining (Fig. 5A). These endosomes also contained the transferrin receptor (Fig. 5B), as determined by superimposition of confocal images to give a yellow signal at sites of colocalization (Fig. 5C). Therefore, SP induces internalization of the

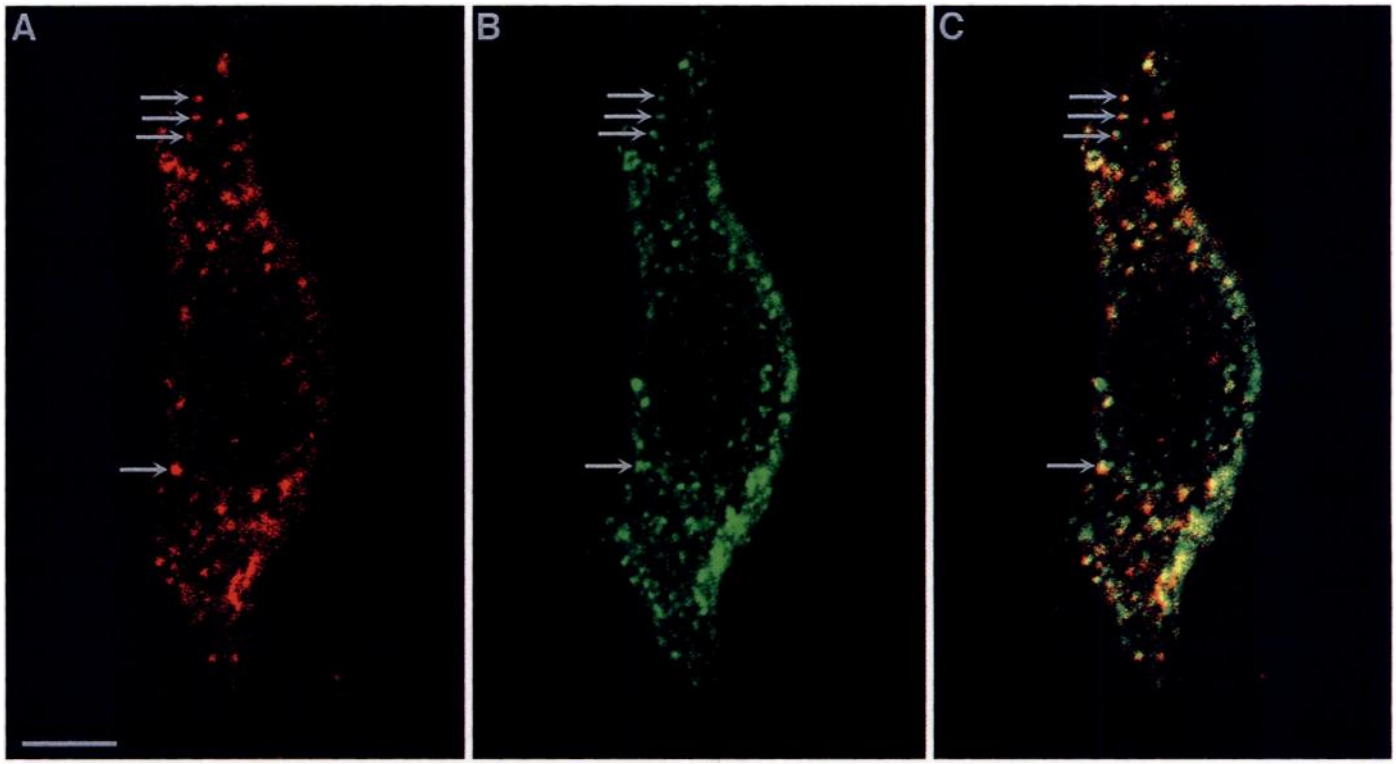

Fig. 5. Confocal photomicrographs showing internalization of the NK_1 receptor and the transferrin receptor by CHO-NK_1 receptor cells. Cells were incubated with 10 nM SP for 2 hr at 4°, washed, and then incubated for 10 min at 37°. Cells were fixed and processed by double-antibody immunofluorescence with the use of a polyclonal antibody to the NK_1 receptor with a Texas Red-labeled secondary antibody, and a monoclonal antibody to the transferrin receptor with a fluorescein-labeled secondary antibody. Cells were observed with confocal microscopy to detect the NK_1 receptor (A) and the transferrin receptor (B). C, Superimposition of images from A and B. *Arrows pointing to the yellow vesicles*, colocalization of the NK_1 receptor and the transferrin receptor. *Scale bar*, 10 μm.

NK_1 receptor in CHO and KNRK cells into early endosomes containing a recycling receptor.

To determine whether differences in desensitization are related to differences in receptor endocytosis and trafficking, we localized the NK_1 and NK_2 receptors by using Cy3-SP and Cy3-NKA. We have shown that NK_1 receptor and Cy3-SP colocalize in KNRK cells up to 30 min after internalization (7, 15, 16). At 4°, Cy3-SP and Cy3-NKA were confined to the plasma membrane of CHO-NK_1 receptor and CHO-NK_2 receptor cells, respectively (Fig. 6, A and C). After 5 min at 37°, there was minimal surface labeling, and Cy3-SP and Cy3-NKA were in superficial endosomes (Fig. 6, B and D). After 30 min, Cy3-SP and Cy3-NKA were in larger, perinuclear endosomes. Thus, the NK_2 receptor is internalized similarly to the NK_1 receptor, and differences in internalization are unlikely to account for differences in desensitization. However, these studies did not allow quantification of minor differences in the rate or extent of endocytosis.

We quantified endocytosis of the NK_1 and NK_2 receptors by using ^{125}I-SP or ^{125}I-NKA with an acid wash to separate cell-surface from internalized peptide. At 4°, 13.9 ± 1.4% of specifically bound ^{125}I-SP was internalized in CHO-NK_1 receptor cells, and 0.7 ± 0.3% of the specifically bound ^{125}I-NKA was internalized in CHO-NK_2 receptor cells (triplicate observations, four experiments). After 10 min at 37°, 90.0 ± 1.9% of ^{125}I-SP was internalized in CHO-NK_1 receptor cells, and 81.8 ± 1.0% of ^{125}I-NKA was internalized in CHO-NK_2 receptor cells (Fig. 7). Thus, there were no qualitative or quantitative differences in agonist-induced endocytosis that could account for the differences in desensitization of the $[Ca^{2+}]_i$ responses of the NK_1 and NK_2 receptors.

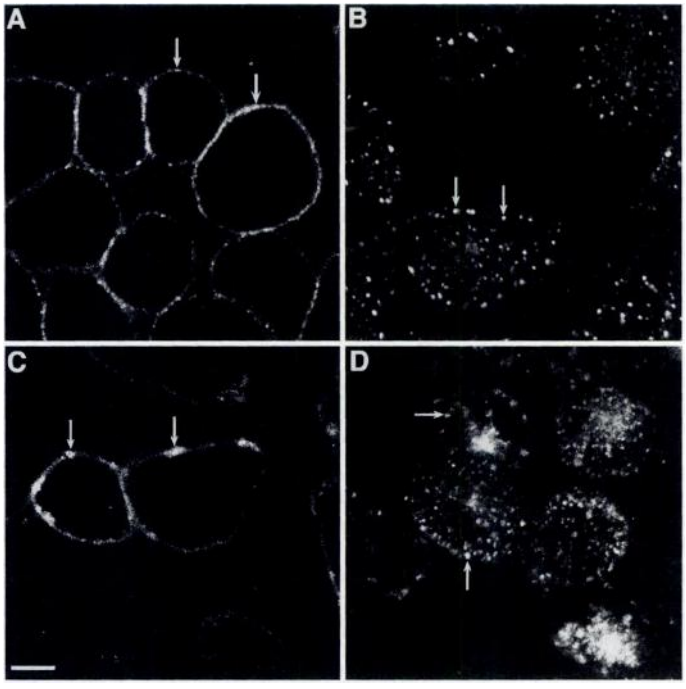

Fig. 6. Confocal photomicrographs showing internalization of Cy3-SP and Cy3-NKA by CHO-NK_1 receptor and CHO-NK_2 receptor cells. CHO-NK_1 receptor cells (A and B) or CHO-NK_2 receptor cells (C and D) were incubated with 100 nM Cy3-SP or Cy3-NKA, respectively, for 2 hr at 4°, washed, and incubated for 0 min (A and C) or 5 min (B and D) at 37°. Cells were fixed and observed with confocal microscopy. *Scale bar*, 10 μm.

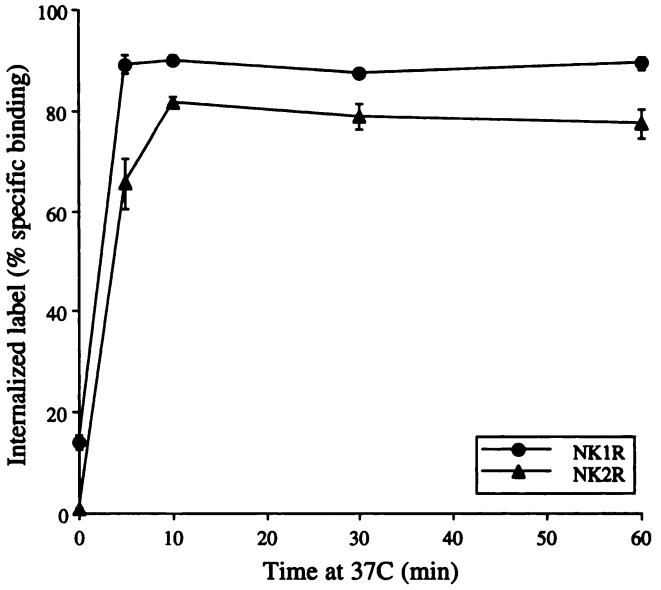

Fig. 7. Internalization of ^{125}I-SP or ^{125}I-NKA by CHO-NK_1 receptor (*NK1R*) and CHO-NK_2 receptor (*NK2R*) cells. CHO-NK_1 receptor and CHO-NK_2 receptor cells were incubated with 10 pM ^{125}I-SP or ^{125}I-NKA, respectively, for 2 hr at 4°. Cells were washed and incubated at 37° for the specified times. An acid wash was used to separate acid-sensitive (cell-surface) and acid-resistant (internalized) fractions, and results are expressed as the percentage of specifically bound label contained in the intracellular fraction. Data are based on four experiments (triplicate observations).

Contributions of receptor endocytosis, receptor recycling, and phosphatases to desensitization and resensitization of the NK_1 receptor. The NK_1 receptor desensitized and resensitized in CHO-NK_1 receptor cells as it does in other tissues and cell lines (4–6). After binding SP, the NK_1 receptor is phosphorylated by G protein receptor kinases, which may mediate desensitization, and dephosphorylation may be required for resensitization to occur (13). SP binding also triggers internalization and recycling of the NK_1 receptor, which may also contribute to desensitization and resensitization (16). We used inhibitors of endocytosis, recycling, and phosphatases and examined their effects on desensitization and resensitization of the NK_1 receptor. Endocytosis of the NK_1 receptor was inhibited with 80 μM phenylarsine oxide and 5 μM β-mercaptoethanol (15). Recycling of the NK_1 receptor was inhibited with 1 μM bafilomycin A_1, which inhibits vacuolar H^+/ATPase (16). Phosphatases were inhibited with 50 nM okadaic acid. Desensitization and resensitization of SP-induced $[Ca^{2+}]_i$ responses and $I(1,4,5)P_3$ generation were examined in CHO-NK_1 receptor cells as described above.

SP-induced $I(1,4,5)P_3$ generation and $[Ca^{2+}]_i$ responses desensitized similarly in cells treated with phenylarsine oxide and in carrier-treated cells (Fig. 8, A and B). However, these responses did not resensitize after 30–60 min in cells treated with phenylarsine oxide. This suggests that endocytosis is not required for desensitization but is required for resensitization of the NK_1 receptor. SP-induced $[Ca^{2+}]_i$ responses desensitized similarly in cells treated with bafilomycin A_1 and in carrier-treated controls (Fig. 8B). However, $[Ca^{2+}]_i$ responses did not resensitize after 60 min in cells treated with bafilomycin A_1. This suggests that recycling is required for resensitization of the NK_1 receptor. SP-induced $[Ca^{2+}]_i$

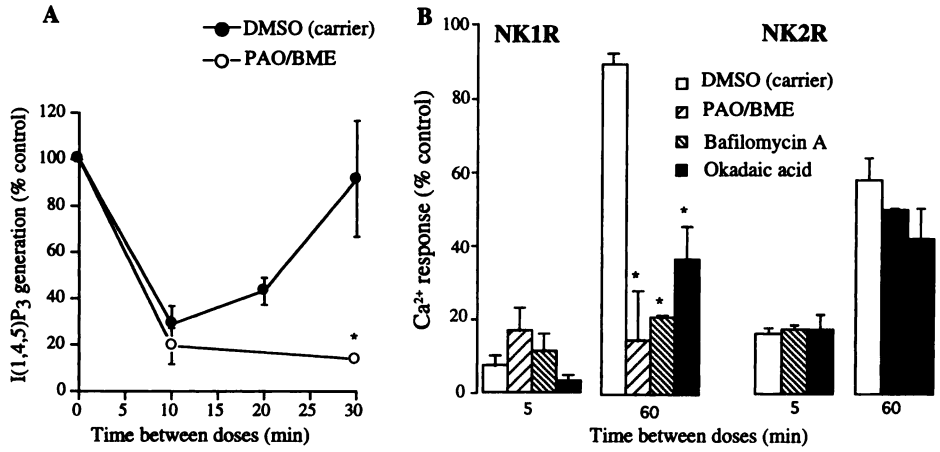

Fig. 8. Effects of drugs on desensitization and resensitization of $I(1,4,5)P_3$ generation in CHO-NK_1 receptor cells (A) and $[Ca^{2+}]_i$ responses (B) in CHO-NK_1 receptor (*NK1R*) and CHO-NK_2 receptor (*NK2R*) cells to two sequential exposures to SP (NK_1 receptor) or NKA (NK_2 receptor). To examine desensitization of $I(1,4,5)P_3$ generation, cells were exposed to a 10 nM desensitizing dose of agonist or to carrier [dimethylsulfoxide (*DMSO*), control], washed after 1 min, and then exposed to a 1 μM test dose of agonist at various times after the desensitizing dose. To examine desensitization of $[Ca^{2+}]_i$ responses, cells were exposed to a 10 nM desensitizing dose of agonist or to carrier (control), washed after 1 min, and then exposed to a 10 nM test dose of agonist at various times after the desensitizing dose. Assays of $[Ca^{2+}]_i$ in CHO-NK_1 receptor cells were in the presence of extracellular Ca^{2+}. Assays of $[Ca^{2+}]_i$ in CHO-NK_2 receptor cells were in the absence of extracellular Ca^{2+}. Results are expressed as the percent response to the test dose of agonist compared with the carrier-treated cells at each time point. *PAO/BME*, 80 μM phenylarsine oxide and 5 μM β-mercaptoethanol; bafilomycin A_1, 1 μM; okadaic acid, 50 nM. Data are based on three experiments (triplicate observations). *, $p < 0.05$ compared with carrier control.

responses desensitized similarly in cells treated with okadaic acid and in carrier-treated control cells (Fig. 8B). However, $[Ca^{2+}]_i$ responses did not resensitize after 60 min in cells treated with okadaic acid. This suggests that phosphatases are important for resensitization of the NK_1 receptor.

Because the NK_2 receptor does desensitize and resensitize in Ca^{2+}-free medium, we examined the effects of some of these inhibitors on desensitization and resensitization of the Ca^{2+} response to repeated application of NKA, measured in Ca^{2+}-free medium. So our observations would be comparable to those obtained with the NK_1 receptor, we examined desensitization of the NK_2 receptor at 5 min and resensitization at 60 min after the initial challenge with NKA. In carrier-treated control cells, there was marked desensitization at 5 min and considerable resensitization at 60 min (Fig. 8B). However, resensitization measured at 60 min was slightly less than that observed at 30 min, probably because the maintenance of cells in Ca^{2+}-free medium for this time depleted intracellular stores of Ca^{2+}. Similarly to the NK_1 receptor, bafilomycin A_1 and okadaic acid had no effect on desensitization (Fig. 8B). In contrast to the NK_1 receptor, for which bafilomycin A_1 and okadaic acid markedly inhibited resensitization, these agents only slightly reduced resensitization of the NK_2 receptor. These results suggest that recycling and phosphatases are less important for resensitization of the NK_2 receptor than of the NK_1 receptor. Although we do not know whether the NK_2 receptor recycles (because we lack a suitable antibody to examine recycling), it internalizes similarly to the NK_1 receptor. In addition, we do not know whether the NK_2 receptor is phosphorylated after activation. However, there are far fewer consensus sites for phosphorylation of the C-tail and third intracellular loop of the NK_2 receptor than of the NK_1 receptor, which suggests that kinases are less important for desensitization of the NK_2 receptor. Therefore, it would be expected that phosphatases are less important for NK_2 receptor resensitization than for NK_1 receptor resensitization. We could not determine the importance of internalization of the NK_2 receptor to desensitization and resensitization, as we could not maintain the viability of cells treated with phenylarsine oxide in Ca^{2+}-free medium.

The results of these and related studies support the following model for desensitization and resensitization of the NK_1 receptor (Fig. 9). The NK_1 receptor desensitizes by phosphorylation and uncoupling from G proteins. Indeed, G protein receptor kinases 2 and 3 phosphorylate the activated NK_1 receptor when it is reconstituted in phospholipid vesicles (13). The NK_1 receptor also has many consensus sites for phosphorylation by G protein receptor kinases and protein kinase C in the third intracellular loop and C-tail, and truncation of the C-tail at the 338 position, which removes some potential phosphorylation sites, diminishes desensitization (31). The phosphorylated NK_1 receptor may bind to arrestin-like molecules and uncouple from G proteins as inositol pentakisphosphate, which disrupts the interactions of arrestins with receptors, attenuates desensitization of the NK_1 receptor (32). The NK_1 receptor resensitizes when these steps are reversed. Resensitization requires endocytosis, suggesting that reversal occurs in an intracellular compartment. Endosomal acidification is necessary for dissociation of SP and the NK_1 receptor and for sorting into degradative and recycling pathways, respectively (16). Resensitization requires recycling because the NK_1 receptor does not resensitize after inhibition of vacuolar H^+/ATPase. Resensitization also requires phosphatases, which may act in endosomes to dephosphorylate the NK_1 receptor, because the NK_1 receptor does not resensitize if phosphatases are inhibited. NKA-induced generation of $I(1,4,5)P_3$ and mobilization of Ca^{2+} from intracellular stores in cells expressing the NK_2 receptor desensitize and resensitize, but the mechanisms are not fully understood. We do not know whether the NK_2 receptor is phosphorylated, although our results with phosphatase inhibitors indicate that phosphorylation and dephosphorylation are not of critical importance for desensitization and

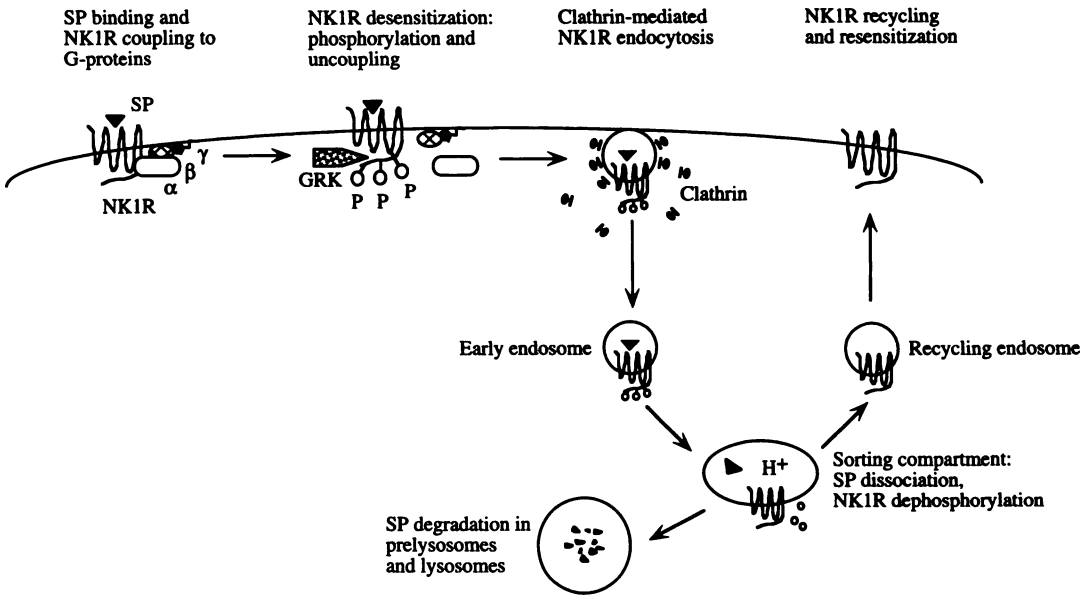

Fig. 9. Model for desensitization and resensitization of the NK_1 receptor (*NK1R*). *GRK*, G protein receptor kinase; α, β, and γ, G protein subunits; *P*, phosphate.

resensitization of the NK_2 receptor. The roles of endocytosis and recycling in desensitization and resensitization of the NK_2 receptor require further investigation.

The β_2-adrenergic receptor desensitizes and resensitizes similarly to the NK_1 receptor, which supports our results. G protein receptor kinases 2 and 3 phosphorylate the activated β_2-adrenergic receptor, which binds β-arrestins 1 and 2 (8). The receptor no longer interacts with G proteins and desensitizes. The β_2-adrenergic receptor internalizes after binding agonists and recycles (17). Although it still desensitizes if internalization is blocked by drugs or receptor mutation, it does not resensitize (19, 20). Therefore, resensitization may require receptor dephosphorylation by endosomal phosphatases, dissociation of β-arrestins and agonists in acidified endosomes, and recycling. Indeed, the β_2-adrenergic receptor in endosomes is less phosphorylated than at the plasma membrane and does not resensitize if recycling is blocked (19–22). Therefore, the model may be applicable for desensitization and resensitization of other G protein-coupled receptors. An alternate mechanism is responsible for resensitization of the thrombin receptor, which is cleaved after binding thrombin. G protein receptor kinases phosphorylate the thrombin receptor and mediate desensitization (12). Resensitization is associated with movement of vesicles containing presynthesized receptor to the plasma membrane (33).

The use of inhibitors of endocytosis, recycling, and phosphatases has broadened our understanding of desensitization and resensitization of G protein-coupled receptors. However, some caution is necessary in interpreting the results because drugs may have nonspecific actions. It will be necessary to examine directly phosphorylation of the NK_1 receptor at the plasma membrane and in endosomes and to study its interactions with kinases, arrestins, and G proteins to unequivocally demonstrate that these proposed mechanisms mediate desensitization and resensitization.

Conclusions. Based on the above results, we conclude that (i) the rapid increase in $[Ca^{2+}]_i$, which is mediated by the generation of $I(1,4,5)P_3$ and release of Ca^{2+} from intracellular stores, desensitizes and resensitizes similarly for the NK_1 receptor and the NK_2 receptor; (ii) the sustained plateau in $[Ca^{2+}]_i$, which is more pronounced for the NK_2 receptor than for the NK_1 receptor, is due to influx of extracellular Ca^{2+}, and differences in this phase of signaling account for the differences in desensitization between the NK_1 receptor and NK_2 receptor; (iii) the NK_2 receptor, like the NK_1 receptor, rapidly internalizes after agonist binding, and differences in internalization cannot explain differences in desensitization; (iv) endocytosis is not necessary for desensitization but is required for resensitization of SP-induced $I(1,4,5)P_3$ generation and $[Ca^{2+}]_i$ responses of the NK_1 receptor; and (v) recycling and phosphatases are required for resensitization of SP-induced $[Ca^{2+}]_i$ responses of the NK_1 receptor but are less important for resensitization of the NK_2 receptor.

Acknowledgments

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