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# Neurokinin-1 Receptor Resensitization Precedes Receptor Recycling

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## ABSTRACT

Following agonist binding, neurokinin-1 receptors undergo rapid desensitization followed by internalization and recycling. Desensitization requires receptor phosphorylation but does not require internalization, whereas resensitization is thought to require internalization and recycling. Our previous data, however, have suggested that, following activation and desensitization, the return of responsiveness to the neurokinin-1 agonist substance P (termed "resensitization") occurs hours before internalized receptors are recycled back to the plasma membrane. To further investigate this novel mechanism of neurokinin-1 receptor resensitization, we have studied the time courses of neurokinin-1 receptor responsiveness, recycling, and dephosphorylation by measuring cellular $Ca^{2+}$ responses, ligand-receptor binding, and receptor phosphorylation, respectively. Concentration-response curves and competition binding curves were obtained at various times following desensitization. The effects of the nonhydrolyzable GTP analog Gpp(NH)p on substance P binding were also studied to assess receptor-G protein coupling. After receptor activation and desensitization, $Ca^{2+}$ signaling in response to substance P occurred within 90 min, whereas the return of receptor binding required 240 min. Receptor dephosphorylation was greater than 90% complete 20 min after agonist washout. In addition, the return of substance P responsiveness coincided with a return in sensitivity of substance P binding to Gpp(NH)p, indicating a return in receptor-G protein coupling. These data show that the resensitization of responsiveness to substance P precedes receptor recycling. This may result from a conversion of nonfunctional neurokinin-1 receptors to functional receptors at the plasma membrane.

The neurokinin-1 receptor (NK1R) is a member of the G protein-coupled receptor (GPCR) family of membrane proteins. The NK1R typically couples to the $G_q$ family of G proteins. Thus, activation of the receptor leads to activation of phospholipase C and mobilization of intracellular $Ca^{2+}$. Many studies have investigated the mechanism of NK1R desensitization (e.g., Garland et al., 1996; Sanders and LeVine, 1996; Vigna, 1999), but few have probed the mechanism of resensitization. Desensitization is a decrease in receptor responsiveness following prolonged or repeated exposure to an agonist. Likewise, resensitization is the return of responsiveness following desensitization.

Following activation of the NK1R, the receptor undergoes rapid desensitization, and the receptors are internalized within minutes (Garland et al., 1996; Bennett et al., 2002). Phosphorylation and G protein uncoupling are thought to mediate desensitization and internalization of most GPCRs (reviewed by Ferguson, 2001). Grady et al. (1995) used cyclohexamide to show that internalized rat NK1Rs (rNK1Rs) are recycled back to the plasma membrane rather than undergoing receptor degradation followed by de novo synthesis. Thus, GPCR resensitization is thought to be a result of receptor dephosphorylation (Pippig et al., 1995; Ferguson, 2001) and receptor recycling following internalization (Garland et al., 1996).

While studying the effects of monensin and concanavalin A on rNK1R desensitization, internalization, recycling, and resensitization, we found that, following agonist-induced rNK1R desensitization and internalization, there was an increase in rNK1R-mediated $Ca^{2+}$ signaling following a 60-min recovery period without agonist, but there was not an equivalent return of plasma membrane receptor binding during the same recovery period (Bennett et al., 2002). Using a

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**ABBREVIATIONS:** NK1R, neurokinin-1 receptor; GPCR, G protein-coupled receptor; rNK1R, rat NK1R; CHO, Chinese hamster ovary; ES, extracellular solution; ANOVA, analysis of variance; 5-HT, 5-hydroxytryptamine.

fluorescent substance P analog, we also showed that internalization corresponds with a decrease in plasma membrane binding of radiolabeled substance P following receptor activation, and recycling corresponds with an increase in binding following internalization. Thus, we hypothesized that rNK1R resensitization precedes recycling. To further investigate this novel mechanism of rNK1R resensitization, we examined the return of $Ca^{2+}$ signaling to the rNK1R agonist, substance P, recycling of receptors back to the plasma membrane, receptor dephosphorylation, and receptor-G protein coupling.

Changes in agonist potency and efficacy during desensitization and resensitization were assessed by determining concentration-response curves for $Ca^{2+}$ activation. The amount of desensitization of the phospholipase C pathway following rNK1R activation was also measured. Competition binding curves were obtained during desensitization and resensitization. Finally, we studied the effects of a nonhydrolyzable GTP analog, Gpp(NH)p, on radiolabeled substance P binding. Gpp(NH)p induces receptor-G protein uncoupling (Luber-Narod et al., 1990; Takeda et al., 1992; Tota et al., 1994), so we were able to determine changes in G protein coupling after agonist activation and receptor resensitization. Based on the results of these studies, we suggest that rNK1R resensitization in Chinese hamster ovary (CHO) cells is due to an increase in G protein coupling to noninternalized plasma membrane receptors following dephosphorylation and is not the result of receptor recycling or changes in the phospholipase C pathway.

## Materials and Methods

**$Ca^{2+}$ Measurements.** CHO cells stably expressing the rNK1R were kindly provided by Dr. James Krause (Neurogen, Inc., Branford, CT) and maintained as previously described (Takeda et al., 1992). The receptor expression level is ~200,000 high-affinity rNK1R binding sites per cell (Takeda et al., 1992).

To determine the time course for the return of rNK1R responses following desensitization, rNK1R-expressing CHO cells were cultured onto six-well plates (Corning Incorporated, Corning, NY) until confluent. The cells were loaded with Fura PE-3 (Texas Fluorescence Labs, Austin TX), a ratiometric $Ca^{2+}$ indicator, for 30 min at 37°C. Unincorporated Fura PE-3 was removed by rinsing the cells two times with $Ca^{2+}$-containing extracellular solution (ES) (Perrine et al., 2000). Desensitization was induced by applying 100 nM substance P (Sigma-Aldrich, St. Louis, MO) for 5 min at 37°C. Substance P was removed by rinsing the cells three times with ES. Cells were allowed to recover for 0, 5, 10, 20, 30, 45, 60, 90, 180, or 240 min at 37°C in the absence of substance P. Substance P (100 nM) was reapplied at these times to measure the amount of receptor responsiveness. A $Ca^{2+}$ ionophore, ionomycin (Sigma-Aldrich), was added (10 $\mu$M) to each well at the end of each experiment to elicit a maximum $Ca^{2+}$ response for normalization between wells and experiments.

$Ca^{2+}$ levels were determined by measuring emission at 510 nm following excitation at 340 and 380 nm of the $Ca^{2+}$-Fura complex. The baseline was subtracted from the peak of each substance P and ionomycin response to determine the amplitude of the $Ca^{2+}$ elevations. Substance P responses were calculated as a percentage of the ionomycin response for that well. To measure the return of $Ca^{2+}$ signaling, the second substance P response was expressed as a percentage of the initial (nondesensitized) substance P response.

**Radioligand Binding of rNK1Rs following Internalization.** The time course for receptor recycling was determined by inducing receptor internalization and then measuring receptor binding at the plasma membrane following various recovery times. To do this, rNK1R-expressing CHO cells were cultured on T-25 flasks (Corning Incorporated) until confluent. The growth medium was removed and replaced with ES. Substance P (100 nM) was added for 5 min at 37°C to induce receptor internalization. Cells were rinsed three times with ES and incubated at 37°C without agonist for 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, or 300 min. Cells from an additional flask were not treated with substance P to serve as a control. Cells were scraped from each flask, pelleted, and resuspended in ice-cold Tris-buffered saline binding buffer as previously described (Bennett and Simmons, 2001). Cells (~100,000 cells/well) from each of the recovery times and from the control flask were added to a prewetted MultiScreen 96-well BV filtration plate (Millipore Corporation, Billerica, MA). Bolton-Hunter $^{125}$I-labeled substance P (50 pM) (PerkinElmer Life and Analytical Sciences, Boston, MA) was added to each well and incubated for 1 h at 4°C to label plasma membrane receptors. At the end of the incubation, the cells were quickly rinsed three times with Tris-buffered saline. The filters were punched from the plate, and the radioactivity was counted using a Packard Cobra II gamma counter (PerkinElmer Life and Analytical Sciences).

A number of steps were taken to ensure that unlabeled substance P was removed from the cells prior to the addition of radiolabeled substance P. First, the cells were washed three times prior to scraping. After scraping, the cells were stored in Tris-buffered saline for about 20 min while being counted. After counting, this medium was removed, and the cells were resuspended in fresh buffer. Thus, the cells experienced four solution changes over a period of at least 30 min before the radioligand was added. Since the dissociation rate constant of substance P is 0.28 $min^{-1}$ (Takeda et al.,1992), the unlabeled substance P should have been removed during the washes, and the solution changes prior to the addition of radiolabeled substance P. We confirmed the effectiveness of these steps to remove unlabeled substance P in previous experiments (Bennett et al., 2002).

Nonspecific binding was defined as the amount of radiolabeled substance P binding in the presence of an excess of unlabeled substance P (1 $\mu$M) and subtracted from the raw data. Data are expressed as a percentage of plasma membrane binding obtained from control cells (those not treated with unlabeled substance P).

**Phosphorylation of rNK1Rs.** Receptor phosphorylation was measured by incubating rNK1R-expressing CHO cells cultured on T-25 flasks until confluent with [$^{32}$P]orthophosphate (MP Biomedicals, Irvine, CA) for 2 to 3 h at 37°C. Substance P (100 nM) was added for 5 min at 37°C to elicit receptor phosphorylation. One flask was not treated with substance P to determine the amount of basal phosphorylation. After a 5-min incubation, excess substance P and unincorporated $^{32}$P were removed by rinsing three times with HEPES/Krebs buffer (Roush et al., 1999). The cells were scraped and immunoprecipitated as previously described (Roush et al., 1999). Briefly, cells were pelleted, rinsed, and then lysed. The supernatant was incubated with a rNK1R antibody followed by incubation with protein A Sepharose beads. The beads were rinsed and then eluted with SDS buffer. The samples were analyzed as previously described (Bennett et al., 2002). Briefly, each sample was boiled in SDS buffer and subjected to nonstacking gel electrophoresis. Two gels were loaded. One gel was exposed and analyzed, and the other gel was stained for protein. Several steps were taken to ensure equal loading of receptors, such as using cells from the same passage and immunoprecipitating the same number of cells.

The intensity of each band of receptors observed on the exposed gel was measured. The amount of basal phosphorylation was set to 1, and the data are expressed as a proportion of the basal phosphorylation.

**Concentration-Response Curves following Desensitization.** Concentration-response curves for $Ca^{2+}$ activation were constructed at various times following desensitization to assess changes in $EC_{50}$ and $E_{max}$. $EC_{50}$ is the concentration of substance P required to elicit 50% of the maximum response, and $E_{max}$ is the maximum effect elicited by substance P. CHO cells expressing the rNK1R were

loaded with Fura PE-3 as described above. Cells were incubated with substance P (100 nM) for 5 min and rinsed three times. Cells were allowed to recover for 0, 10, 20, 45, or 90 min in the absence of agonist. The second concentration of substance P (0.1–1000 nM) was then applied followed by ionomycin.

**Competition Binding Curves following Desensitization.** For competition binding curves, CHO cells expressing the rNK1R were cultured on T-25 flasks until confluent. Substance P (100 nM) was added for 5 min at 37°C. Cells were rinsed three times and then allowed to recover for 0, 10, 20, 45, 90, or 300 min in the absence of agonist. Cells were scraped, pelleted, and resuspended. Cells (100,000 cells/well) were added to each well of a prewetted 96-well filter plate. Unlabeled substance P ranging from 0.1 to 1000 nM was added to compete with radiolabeled substance P (50 pM) at the rNK1Rs. Cells were incubated for 2 h at 4°C to allow equilibrium binding (Bennett and Simmons, 2001).

Nonspecific binding was defined as the amount of radiolabeled substance P binding in the presence of an excess of unlabeled substance P (1 $\mu$M) and subtracted from the raw data. Data were normalized by calculating $B/B_0$, where $B$ is the counts per minute of radiolabeled substance P specifically bound in the presence of unlabeled substance P, and $B_0$ is the counts per minute of radiolabeled substance P bound in the presence of 100 pM unlabeled substance P.

**Heterologous Desensitization.** We have taken advantage of the fact that CHO cells naturally express a $G_q$-coupled purinergic receptor to measure heterologous desensitization of the phospholipase C pathway following rNK1R activation (Iredale and Hill, 1993). $Ca^{2+}$ responses elicited by ATP were measured by loading cells with Fura PE-3 as described above. A concentration-response curve for ATP was determined by adding graded concentrations of ATP (1 nM–1 mM) to rNK1R-expressing CHO cells. Then, to determine the amount of phospholipase C pathway desensitization, substance P (100 nM) was added for 5 min and washed out following rNK1R activation. Cells were allowed to recover for 0, 5, 10, 20, or 30 min. ATP (10 $\mu$M) was then added to measure the responsiveness of the phospholipase C pathway. This was followed by application of ionomycin (10 $\mu$M) to obtain a maximum.

**Effects of Gpp(NH)p on Radiolabeled Substance P Binding.** CHO cells expressing the rNK1R were cultured on T-75 flasks (Corning Incorporated) until confluent. Unlabeled substance P (100 nM) was added for 5 min at 37°C to induce receptor desensitization and internalization. The cells were rinsed three times with ES and scraped or allowed to recover for 90 min without agonist and then scraped. For controls, cells were not treated with substance P. Membranes were prepared as previously described (Takeda et al., 1992). The amount of protein in each sample was determined by Bradford analysis, and then membranes were resuspended to a concentration of 5 $\mu$g of protein/100 $\mu$l of Tris-buffered saline binding buffer with 3 mM $MnCl_2$ and added to a prewetted MultiScreen 96-well HV filtration plate (Millipore Corporation). Increasing concentrations (0.1–10 nM) of $^{125}$I-substance P were added to the membranes in the presence or absence of Gpp(NH)p (100 $\mu$M) for 1 h at room temperature. Gpp(NH)p was added 15 min prior to the addition of radiolabeled substance P. Filters were rinsed three times with Tris-buffered saline, removed, and counted to determine counts per minute values.

Nonspecific binding was defined as the amount of radiolabeled substance P binding in the presence of an excess of unlabeled substance P (1 $\mu$M) and subtracted from the raw data. Data were normalized to the amount of radiolabeled substance P bound in picomoles per milligram of protein (B) and expressed as a fraction of the concentration of radiolabeled ligand (B/F).

**Statistics.** The statistical significance of the receptor activation, receptor binding, phosphorylation, and Gpp(NH)p data were determined by one-way ANOVA. Two-way ANOVA was performed to test for significant differences between the various concentration-response and competition binding curves. $EC_{50}$ values were obtained from a sigmoidal regression equation (GraphPad Prism; GraphPad Software Inc., San Diego, CA), which is simplified to $E = E_{max} \times [A]/(EC_{50}+[A])$ when the Hill coefficient is set to 1. In this equation, E is the effect and A is the agonist. Two-way ANOVA was used to test significance between the curves generated by control, desensitized, and resensitized membranes in the absence and presence of Gpp(NH)p. $B_{max}$ and $IC_{50}$ values were obtained from one- or two-site binding analysis (GraphPad Prism).

## Results

**rNK1R Signaling and Recycling.** The time courses for the return of $Ca^{2+}$ signaling and rNK1R membrane binding were determined to provide a direct comparison between the two events. A 5-min exposure to substance P (100 nM) induced an 83 ± 14% desensitization of the rNK1R-mediated $Ca^{2+}$ response and a 49 ± 14% loss of $^{125}$I-substance P plasma membrane binding (time 0 on Fig. 1). Compared with the initial substance P response, the substance P response after desensitization was significantly decreased ($p < 0.05$) until the 45-min recovery period. The response returned to 100% of the control level in 90 min. Although there was a complete return of $Ca^{2+}$ signaling during this time period, there was not a significant change in cell-surface binding. A significant ($p < 0.05$) increase in receptor binding at the plasma membrane was not observed until 120 min after agonist washout (Fig. 1), long after the response had resensitized. Furthermore, the return of receptors to the plasma membrane was not complete until 240 min after washout of substance P. These data show that the return of the responsiveness to substance P precedes recycling of receptors to the cell membrane.

**rNK1R Dephosphorylation.** To determine whether the return of $Ca^{2+}$ signaling correlated with the phosphorylation state of rNK1Rs, the time course for rNK1R dephosphorylation following exposure to substance P was examined. A 5-min exposure to substance P (100 nM) induced a 3-fold increase ($p < 0.05$) in rNK1R phosphorylation over basal levels (Fig. 2). A slight decrease in phosphorylation was ob-

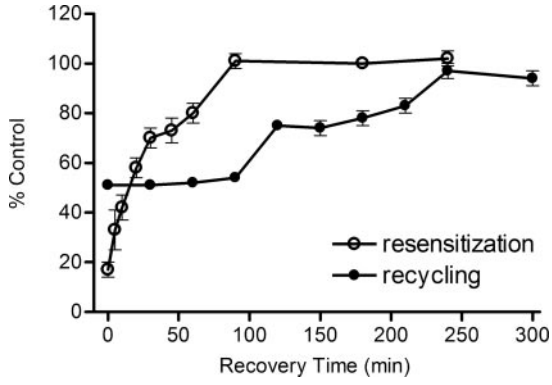


**Fig. 1.** Time courses of rNK1R resensitization and recycling. Desensitization and internalization of rNK1Rs were elicited by incubation with substance P (100 nM) for 5 min. The ability of substance P to elicit a $Ca^{2+}$ response and plasma membrane binding of $^{125}$I-substance P were measured at various times to assess resensitization of the response and recycling of the receptors, respectively. For resensitization (○), the data are expressed as a percentage of the initial substance P response of nondesensitized cells (control) and represent a sample size of at least six. For recycling (●), the data are expressed as a percentage of the maximum amount of binding of untreated cells (control), and the sample size was at least 17 for each point. Each symbol represents the mean ± S.E.M. A significant increase ($p < 0.05$) in rNK1R responsiveness was evident 10 min after agonist washout; however, a significant increase ($p < 0.05$) in plasma membrane binding was not detected until 120 min after agonist washout.

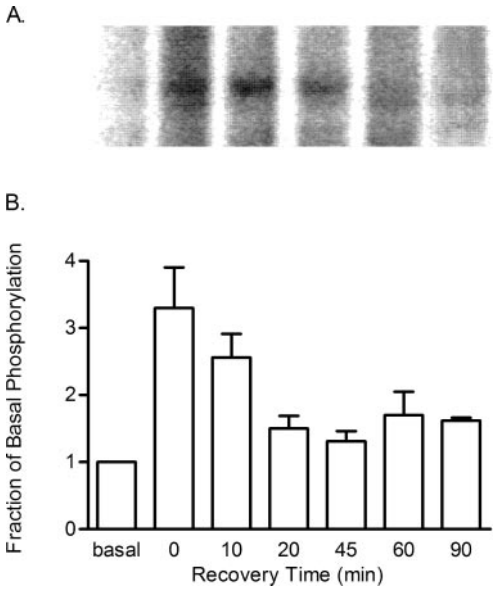


**Fig. 2.** rNK1R dephosphorylation time course. rNK1R-expressing CHO cells were incubated with [$^{32}$P]orthophosphate, and substance P was applied to induce receptor phosphorylation. Cells were then allowed to recover for 0 (lane 2), 10 (lane 3), 20 (lane 4), 45 (lane 5), or 90 min (lane 6) at 37°C without agonist. Basal phosphorylation was measured from cells not treated with substance P (lane 1). A representative gel is shown in A. The graph in B is the average from four experiments expressed as a fraction of basal phosphorylation for that particular experiment. Each column represents the mean ± S.E.M.

served following a 10-min recovery period without agonist. A significant ($p < 0.05$) decrease in phosphorylation was observed 20 min after agonist washout. The receptors were further dephosphorylated following a 45-min recovery period, with no further change at 90 min. Thus, rNK1R dephosphorylation occurs during the period from 10 to 45 min after agonist washout.

**Concentration-Response Curves following Desensitization.** The finding that the return of $Ca^{2+}$ signaling precedes receptor recycling could be accounted for by an accompanying increase in either the potency ($EC_{50}$) or efficacy

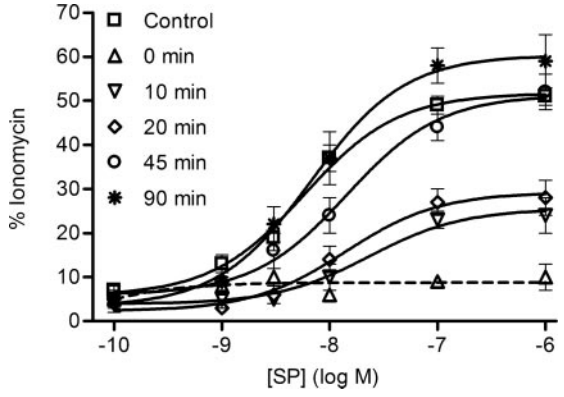


**Fig. 3.** Concentration-response curve for $Ca^{2+}$ activation during rNK1R resensitization. rNK1Rs were desensitized with substance P (100 nM) and allowed to recover without agonist. Graded concentrations of substance P (0.1–1000 nM) were applied at different times during the recovery period to establish concentration-response curves. Graded concentrations were also added to nondesensitized cells to serve as a control. The substance P responses are expressed as a percentage of the ionomycin response to show differences in $E_{max}$. The dashed line represents data displaying a linear regression for which an $EC_{50}$ value could not be determined.

TABLE 1
Parameters for rNK1R binding and function during resensitization

| Recovery Time | $EC_{50}$ | $E_{max}$ | $IC_{50}$ | Maximum $B/B_0$ ($B_0$ from Control) |
|---|---|---|---|---|
| | *nM* | % *ionomycin* | *nM* | |
| Control | 5.5 ± 0.1 | 51 ± 2 | 0.70 ± 0.04 | 1.00 ± 0.02 |
| 0 min | | 10 ± 3* | 0.84 ± 0.03 | 0.51 ± 0.04* |
| 10 min | 23.1 ± 0.3* | 24 ± 4* | 0.60 ± 0.04 | 0.41 ± 0.01* |
| 20 min | 13.4 ± 0.2* | 29 ± 4* | 0.59 ± 0.03 | 0.32 ± 0.03* |
| 45 min | 14.8 ± 0.2* | 51 ± 5 | 0.49 ± 0.04 | 0.41 ± 0.01* |
| 90 min | 6.7 ± 0.2 | 60 ± 6 | 0.55 ± 0.03 | 0.46 ± 0.03* |
| 300 min | | | 0.68 ± 0.06 | 0.90 ± 0.08 |

* Significant differences ($p < 0.01$) compared with control.

($E_{max}$) of substance P. If either of these were the case, substance P could produce a greater effect acting via fewer receptors. To assess this, concentration-response curves for $Ca^{2+}$ activation by substance P were established at various times following desensitization. The curves are shown in Fig. 3. The $EC_{50}$ and $E_{max}$ values obtained from the curves are shown in Table 1. As expected since the substance P response is desensitized at the early time points, a significant decrease ($p < 0.05$) in $E_{max}$ was seen 0, 10, and 20 min after agonist washout compared with control. There was a return in efficacy to control levels 45 and 90 min after agonist washout. An $EC_{50}$ value could not be established for the 0-min recovery curve due to the small amplitude of the responses. A significant increase ($p < 0.05$) was observed in the $EC_{50}$ values obtained 10, 20, and 45 min after agonist washout compared with the control and 90 min curves. Thus, there was a return of both SP efficacy and potency 90 min after agonist washout.

**Competition Binding Curves following Desensitization.** To assess changes in substance P binding following desensitization, the maximum amount of binding and the concentration of substance P required to inhibit 50% of the maximum amount of binding, the $IC_{50}$, were determined by constructing competition binding curves at various times following desensitization (Fig. 4). The $IC_{50}$ values and the maximum binding levels obtained from the curves are summarized in Table 1. Consistent with a decrease in the number of membrane receptors following desensitization shown in Fig. 1, a significant decrease ($p < 0.05$) in the maximum amount of binding for each recovery time, with the exception of the 300-min recovery, was seen compared with control. There was no significant difference in $IC_{50}$ values between any of the curves compared with control.

**Heterologous Desensitization.** Thus far, we have been examining the return of the responsiveness to substance P after desensitization to substance P, i.e., homologous desensitization. Since the return of $Ca^{2+}$ signaling could be due to changes in the phospholipase C pathway downstream of the receptor, we have also measured the amount of heterologous desensitization that occurs after rNK1R activation. As shown in Fig. 5A and by Iredale and Hill (1993), when applied to CHO cells, ATP mobilizes intracellular $Ca^{2+}$ in a concentration-dependent manner. ATP exhibits an $EC_{50}$ value of 1.4 $\mu$M and elicits a near-maximal effect at 10 $\mu$M. Thus, 10 $\mu$M ATP was used to determine the amount of heterologous desensitization of the purinergic receptor pathway following rNK1R activation. A 5-min exposure to 100 nM substance P caused a significant ($p < 0.05$) 34 ± 5% decrease in the ability of ATP, applied immediately following substance P washout,

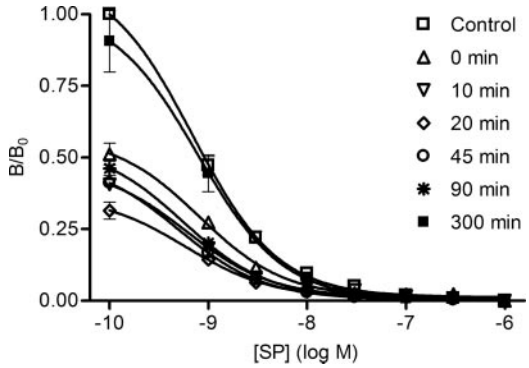


**Fig. 4.** Competition binding curves during resensitization. rNK1Rs expressed in CHO cells were internalized with substance P (100 nM) and allowed to recover without agonist for various times. Competition binding was performed with $^{125}$I-substance P (50 pM) and graded concentrations (0.1–1000 nM) of unlabeled substance P. Each symbol is the mean ± S.E.M. and represents an $n$ of at least six. The $B_0$ value for each point was that obtained from control cells.

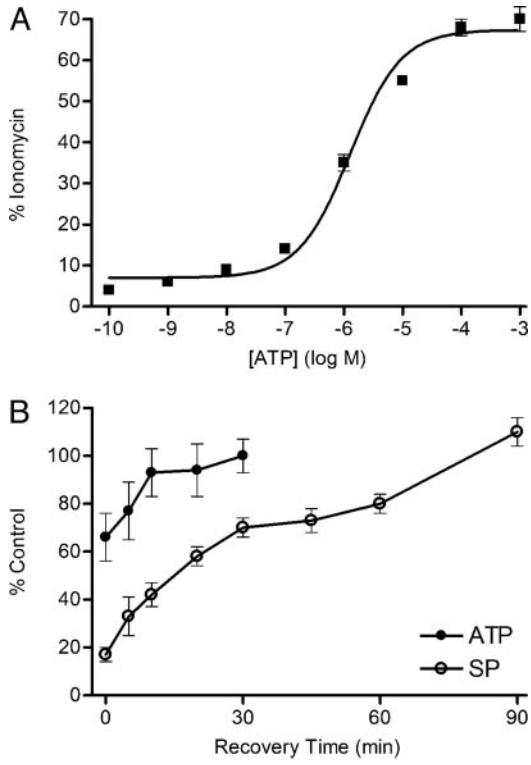


**Fig. 5.** Effects of ATP before and after rNK1R activation. A, concentration-response relationship for ATP-induced $Ca^{2+}$ elevations. Graded concentrations of ATP (1 nM–1 mM) were added to rNK1R-expressing CHO cells, followed by ionomycin. Data are expressed as a percentage of the ionomycin response. Each symbol represents an $n$ of at least eight. B, heterologous desensitization of the ATP-induced $Ca^{2+}$ response following rNK1R activation. CHO cells were treated with 100 nM substance P for 5 min and allowed to recover for 0, 5, 10, 20, or 30 min without agonist. ATP (10 $\mu$M) was then added, followed by ionomycin to normalize between experiments (●). A significant decrease ($p < 0.05$) in the ability of ATP to elicit a $Ca^{2+}$ response was observed immediately following rNK1R activation and after a 5-min recovery period. The time course for rNK1R resensitization from Fig. 1 is also plotted here (○) to demonstrate differences in rNK1R and phospholipase C pathway desensitization and resensitization. The amount of phospholipase C pathway desensitization is not as great as the amount of rNK1R desensitization. In addition, resensitization of the phospholipase C pathway was complete after a 10-min recovery period, whereas 90 min was required for rNK1R resensitization.

to elicit a $Ca^{2+}$ response (Fig. 5B). Following a 10-min recovery period, a significant difference was no longer observed. Thus, the responsiveness to ATP returned to control levels within 10 min of substance P washout. This shows that substance P does cause some heterologous desensitization of the ATP response, but this heterologous phase is over in 10 min.

**Effects of Gpp(NH)p on Radiolabeled Substance P Binding.** To determine changes in receptor-G protein coupling following rNK1R activation and desensitization, saturation binding studies were conducted with membranes from rNK1R-expressing CHO cells under various conditions. Previous membrane binding studies of the neurokinin-1 receptor have shown that, in the presence of Gpp(NH)p, high-affinity receptors are converted to a low-affinity state (Tota et al., 1994). Sensitivity of agonist binding to Gpp(NH)p suggests the existence of high- and low-affinity receptor states, with the high-affinity state representing the ligand-receptor-G protein ternary complex. The effect of Gpp(NH)p on radiolabeled substance P binding was determined to observe changes in G protein coupling following receptor activation.

Scatchard analysis (Fig. 6) of substance P binding to plasma membrane rNK1Rs under control conditions revealed a curvilinear relationship consistent with two binding affinities with $K_{d_1} = 0.04$ nM and $K_{d_2} = 2.23$ nM. A curvilinear fit was also obtained following a 90-min recovery period (recovered membranes) with $K_{d_1} = 0.09$ nM and $K_{d_2} = 8.92$ nM. However, Scatchard analysis of membrane receptors obtained following a 0-min recovery period (desensitized membranes) displayed a linear fit with only one binding site of lower affinity ($K_d = 19.50$ nM). In the presence of Gpp(NH)p, the Scatchard analysis was linear under all three conditions, indicating the presence of only low-affinity receptors. There was not a significant difference among the three different conditions when Gpp(NH)p was present. Furthermore, there was not a significant difference between the curves generated by control and recovered membranes in the absence of Gpp(NH)p. Conversely, in the absence of Gpp(NH)p, there was a significant difference ($p < 0.001$) between the curve generated by desensitized membranes compared with the curve generated by both control and recovered membranes. The presence of Gpp(NH)p caused a significant decrease ($p < 0.001$) in plasma membrane binding under control and recovered conditions but not when rNK1Rs were desensitized. The $K_d$ and $B_{max}$ values for the various experimental conditions are summarized in Table 2.

## Discussion

We have previously reported that resensitization of the response of rNK1Rs to substance P precedes the return of internalized receptors to the cell membrane (Bennett et al., 2002). In our initial study, a 5-min substance P exposure caused a significant decrease (90%) in rNK1R responsiveness (desensitization) along with a decrease (50%) in the maximum amount of plasma membrane binding (internalization). Using a fluorescent analog of substance P, we showed that the loss of plasma membrane binding was a result of internalization. Following agonist washout and a 60-min recovery period, there was a significant increase in receptor responsiveness (resensitization) without an increase in plasma membrane binding (recycling). Resensitization was evident, but not complete, after 60 min. In the present study, we have examined the time courses for the return of $Ca^{2+}$ signaling

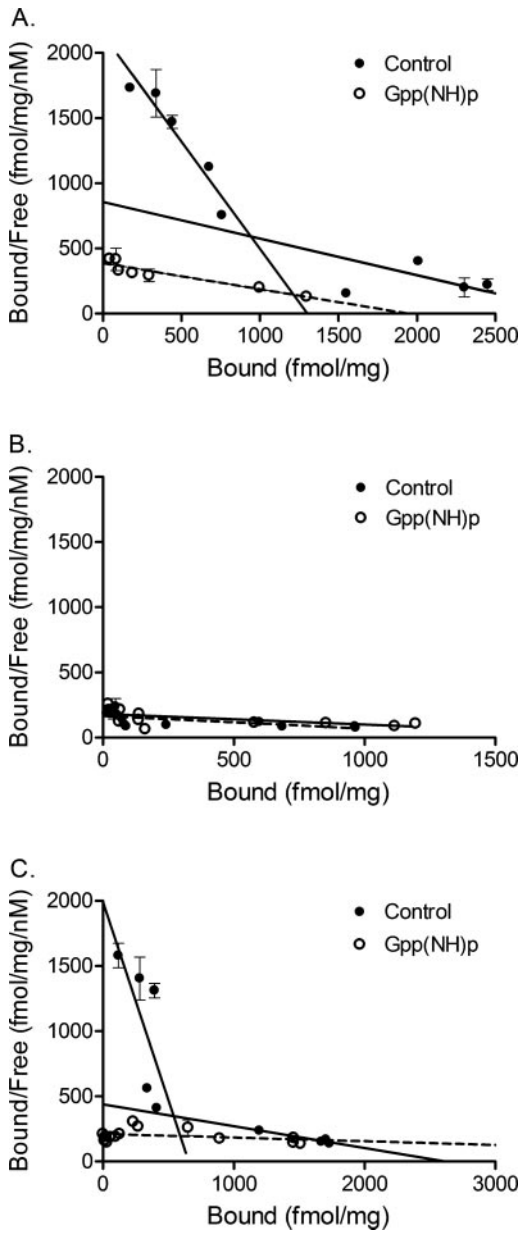


**Fig. 6.** Sensitivity of substance P binding to Gpp(NH)p. CHO cells expressing the rNK1R were treated with 100 nM unlabeled substance P for 5 min at 37°C, rinsed, and either scraped (B) or allowed to recover for 90 min (C). Control cells were not treated with substance P (A). Membranes were prepared and incubated with $^{125}$I-substance P (0.1–10 nM) in the presence (○) or absence (●) of 100 $\mu$M Gpp(NH)p. Each symbol represents an $n$ of at least six, combined from two separate experiments. Binding in the presence of Gpp(NH)p is represented by a dashed line. Scatchard analysis demonstrates curvilinear regression of the binding isotherms for control and resensitized receptors, whereas linear regression is shown for desensitized receptors. In the presence of Gpp(NH)p, Scatchard analysis shows linear regression under all three conditions.

and rNK1R recycling. In addition, we have investigated this novel mechanism of rNK1R resensitization with respect to receptor responsiveness, agonist binding, receptor phosphorylation, heterologous desensitization, and G protein coupling.

The time courses of the return of $Ca^{2+}$ signaling and receptor recycling, shown in Fig. 1, clearly show that the return of $Ca^{2+}$ signaling occurs 2.5 h before receptor recycling. There is a complete return of $Ca^{2+}$ signaling within 90 min, but a significant increase in radioligand binding at the plasma membrane is not observed until 120 min and is not complete until 240 min. After exposure to a desensitizing concentration of substance P, some, but not all, of the plasma membrane receptors are internalized. The receptors that remain at the plasma membrane are able to bind agonist with a low affinity, but they are not functional in that they do not elicit a $Ca^{2+}$ response. Over the next 90 min, there is a gradual return of the $Ca^{2+}$ response, whereas the maximum amount of receptor binding to plasma membrane receptors does not significantly change. This indicates that there is an underlying change in responsiveness to substance P that is independent of receptor recycling.

TABLE 2
Binding parameters of radiolabeled substance P to control, desensitized, and resensitized rNK1Rs in the presence and absence of Gpp(NH)p

| Recovery Time | $B_{max1}$ | $K_{d1}$ | $B_{max2}$ | $K_{d2}$ |
|---|---|---|---|---|
| | *fmol/mg* | *nM* | *fmol/mg* | *nM* |
| Control (no GppNHp) | 137 ± 54 | 0.04 ± 0.01 | 2471 ± 248 | 2.23 ± 0.18 |
| Control (GppNHp) | | | 2024 ± 79 | 5.43 ± 0.26 |
| 0 min (no GppNHp) | | | 2540 ± 553 | 19.50 ± 6.31 |
| 0 min (GppNHp) | | | 3296 ± 862 | 22.22 ± 8.41 |
| 90 min (no GppNHp) | 242 ± 72 | 0.09 ± 0.03 | 2631 ± 521 | 8.92 ± 1.81 |
| 90 min (GppNHp) | | | 2702 ± 265 | 8.65 ± 1.69 |

Ligand-activated NK1Rs are phosphorylated by G protein-coupled receptor kinases (Kwatra et al., 1993). Phosphorylation and subsequent $\beta$-arrestin binding induce receptor internalization and halt receptor signaling, leading to desensitization (McConalogue et al., 1999; Martini et al., 2002). It has thus been hypothesized that receptor dephosphorylation precedes resensitization (Pippig et al., 1995; Ferguson, 2001). The finding that inhibition of phosphatase activity by okadaic acid prevents NK1R resensitization further supports this hypothesis (Garland et al., 1996). In agreement with these findings, we show that dephosphorylation of rNK1Rs occurs between 10 and 45 min of agonist washout, which coincides with resensitization.

Our data suggest that desensitization and resensitization to substance P seem to occur at the level of receptor-G protein coupling and are not the result of changes in the function of the phospholipase C pathway or $Ca^{2+}$ handling machinery downstream of the receptor. ATP causes intracellular $Ca^{2+}$ mobilization via a $G_q$-coupled purinergic receptor naturally expressed in CHO cells. By activating this pathway with substance P and then assessing the $Ca^{2+}$ response elicited by ATP, we were able to determine the contribution of heterologous desensitization of the phospholipase C pathway to rNK1R-specific desensitization and resensitization. Exposure to substance P did decrease the ability of ATP to elicit a $Ca^{2+}$ response (34% heterologous desensitization), but this effect was much less than the degree to which it decreased the ability of a second application of substance P to elicit a response (85% homologous desensitization). Furthermore, the return of $Ca^{2+}$ signaling induced by ATP occurred within 10 min, much faster than the 90-min recovery period required for the return of $Ca^{2+}$ signaling induced by substance P. Thus, the return of $Ca^{2+}$ signaling that we see is not

predominately due to changes in the phospholipase C pathway or in the $Ca^{2+}$ handling machinery, although these changes may play a role during the first 10 min of recovery from desensitization.

By conducting membrane binding studies in the presence of either GTP or the nonhydrolyzable GTP analog GppNHp, we were able to demonstrate two receptor states in membranes from control and recovered cells, but only a single, low-affinity binding site in the membranes was obtained from desensitized cells. These data are consistent with two receptor states, a G protein-coupled and non-G protein-coupled state, being present under control and recovered conditions, whereas only a single, non-G protein-coupled state is present when the response is desensitized. These data show that, although there is not an increase in the number of receptors at the plasma membrane during a 90-min recovery, there is an increase in G protein coupling to plasma membrane receptors. In addition, because there was not a change in binding at the plasma membrane between the time of internalization and the increase in G protein coupling, these data also suggest that the increase in coupling was likely due to receptors that were not internalized following receptor activation.

Resensitization of many GPCRs has been shown to require internalization, endosome acidification, receptor dephosphorylation, and receptor recycling. Inhibition of internalization prevents resensitization of NK1Rs (Garland et al., 1996; Schmidlin et al., 2001; Bennett et al., 2002) and $\beta_2$-adrenergic receptors (Pippig et al., 1995). The dependence of resensitization on internalization has led to the suggestion that resensitization requires recycling. This suggestion has been strengthened by the finding that inhibition of recycling by acidotropes, such as monensin and bafilomycin A, also inhibits resensitization of NK1Rs (Garland et al., 1996; Bennett et al., 2002) and $\beta_2$-adrenergic receptors (Pippig et al., 1995). Unfortunately, few studies have directly inhibited recycling to study its effects on resensitization. Acidotropes are not specific inhibitors of recycling in that they also prevent dephosphorylation and agonist degradation (Grady et al., 1995). Our study is the first to provide a direct comparison between rNK1R resensitization and recycling.

The data presented here suggest that recycling is not necessary for the return of $Ca^{2+}$ signaling but that this resensitization can occur at the cell surface. Our data show that agonist activation causes rNK1Rs phosphorylation and internalization. While internalized receptors are being dephosphorylated and recycled, the receptors at the plasma membrane undergo an increased ability to couple to G proteins that brings about a return of $Ca^{2+}$ signaling.

The results suggest that there are two types of receptors at the plasma membrane. Initially, one of these types is able to be activated, couple to G proteins, and elicit a $Ca^{2+}$ response, whereas the other type is not. Following exposure to substance P, the ligand-activated, G protein-coupled plasma membrane receptors elicit a $Ca^{2+}$ response, become phosphorylated, and are internalized. These receptors are dephosphorylated and recycled back to the membrane over 4 h. Over a shorter time period, the second type of receptor, which remained at the plasma membrane, becomes able to be activated and mediates the more rapid phase of resensitization.

Others have also reported that GPCRs can undergo resensitization without recycling. 5-$HT_{2A}$ receptor resensitization has also been shown to occur without recycling (Gray et al., 2001). In that study, 5-$HT_{2A}$ receptor, but not $\beta_2$-adrenergic receptor, resensitization was shown to be independent of receptor internalization and thus recycling. In addition, the cell-surface mechanism for 5-$HT_{2A}$ receptor resensitization that was shown in human embryonic kidney 293 cells was not seen in C6 glioma cells. These findings support that GPCRs are not all regulated in the same manner and that there is cell-specific regulation of GPCRs.

Based on our data, we also suggest a unique mechanism of rNK1R resensitization in CHO cells expressing the receptor. Since our results were obtained with an expression system, it is unclear whether this mechanism of rNK1R resensitization exists in vivo. NK1R recycling in rat dorsal horn neurons (Wang and Marvizon, 2002) and guinea pig myenteric neurons (Southwell et al., 1998) has been shown to be complete between 60 to 90 min after agonist washout, but resensitization was not measured prior to recycling in either study. In the rat pancreas, NK1R resensitization was complete within 30 min of agonist washout, but recycling was not measured (Maa et al., 2000). Based on our findings and the lack of a direct correlation between resensitization and recycling in vivo, measuring the time courses of resensitization and recycling in vivo would be beneficial in elucidating the mechanism of NK1R resensitization. Since NK1Rs have been implicated in depression (Kramer et al., 1998; Duffy et al., 2002), emesis (Harrison et al., 2001), and asthma (Rumsey et al., 2001), understanding the mechanisms of desensitization and resensitization of this receptor could lead to novel therapeutics.

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