Functional Selectivity of NK<sub>1</sub> Receptor Signaling: Peptide Agonists Can Preferentially Produce Receptor Activation or Desensitization

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ABSTRACT

A cascade of events follows binding of an agonist ligand to the tachykinin NK<sub>1</sub> receptor. These events include activation of multiple signal transduction pathways as well as cellular modulation of receptor function by the process of desensitization. This study examines the differences in the abilities of naturally occurring peptide agonist ligands of the tachykinin NK<sub>1</sub> receptor to preferentially direct signaling through the receptor to produce signal activation versus receptor desensitization. The differential effects of tachykinin peptides with respect to ligand competition binding, receptor-G protein coupling, intracellular Ca<sup>2+</sup> elevations, and receptor desensitization have been measured. In relation to its potency in competition binding studies, substance P produces desensitization at lower concentrations, whereas higher concentrations are required to elicit a Ca<sup>2+</sup> response. In contrast to this, a related peptide, ranatachykinin C, is more effective at activating a Ca<sup>2+</sup> response relative to its ability to produce desensitization. This difference in functional selectivity is conserved for an amphibian and two mammalian ortholog tachykinin receptors. The present study demonstrates that peptide agonist ligands of NK<sub>1</sub> receptors can preferentially produce signal activation or desensitization.

Over the years, the interactions of ligands with receptors have been classified as agonist interactions or antagonist interactions. Recently, it has become clear that a multitude of effects can follow binding of a ligand to a receptor (Kenakin, 2002, 2003). These effects involve activation of multiple signal transduction pathways as well as cellular modulation of receptor function.

One way that receptor function is modulated is by the process of desensitization. Desensitization occurs when the response to an agonist wanes in the continued presence of that agonist. Studies from our lab (Perrine et al., 2000) and from other labs (Vigna, 2001) have shown that the ability of a tachykinin receptor agonist ligand to produce signal activation and its ability to produce desensitization are not necessarily correlated.

Our initial findings in this area showed that four different tachykinin peptides, substance P (SP), ranatachykinin (RTK) A, RTKB, and RTKC, could each activate the signal transduction pathways of the bullfrog SP receptor. For SP and RTKA, the concentrations required to produce desensitization of the receptor were two to four times less than the concentrations required to produce receptor activation. In contrast to this, the concentrations of RTKB and RTKC that produced desensitization were two times greater than the concentration needed to activate the signal transduction pathway.

These data suggested that agonist ligands of the NK<sub>1</sub> receptor may exhibit functional selectivity. That is, agonists with the highest affinity and efficacy to activate a given signal transduction pathway do not also show the greatest degree of desensitization. This possibility seems particularly conceivable with respect to peptide ligands of the NK<sub>1</sub> receptor because these ligands are relatively large molecules that span and interact with multiple extracellular and transmembrane domains of the receptor (Fong et al., 1995; Pellegrini et al., 2001).

Materials and Methods

Whole-Cell Electrophysiology. Single neurons were dissociated from bullfrog sympathetic ganglia as described previously (Simmons and Mather, 1992). Whole-cell recordings of isolated neurons were conducted at room temperature, −23°C, with a patch-clamp amplifier (Warner PC-501; Warner Instruments, Hamden, CT). Peptides were dissolved in extracellular solution and applied by single-cell superfusion. Single neurons were perfused with control extracellular solution before and after drug application. The bath was constantly perfused separately with extracellular solution. The M-current (I<sub>M</sub>)

ABBREVIATIONS: SP, substance P; RTK, ranatachykinin; CHO, Chinese hamster ovary; GTPγS, guanosine 5′-3-O-(thio)triphosphate.
was monitored by 500-ms pulses from a holding potential of −30 to −50 mV every 8 s. The recordings were filtered at 1 kHz. I_R relaxations were sampled at 2.4 kHz. I_Na was measured as the amplitude of the current decrease following a voltage step from −50 to −30 mV (Simmons et al., 1994). Concentration-response curves for I_Na inhibition were generated by applying a range of concentrations (0.1 nM–1 μM). The maximum inhibition during each response was measured, and the proportion of inhibition of I_Na was calculated. The composition of solutions used for these experiments was as follows: extracellular solution, 118 mM NaCl, 2.4 mM KCl, 1.8 mM CaCl_2, 1.8 mM MgCl_2, 5 mM glucose, 15 mM sodium pyruvate, 10 mM HEPES, and 300 nM tetrodotoxin, pH 7.4; and intracellular (electrode) solution, 120 mM KCl, 2 mM MgCl_2, 0.4 mM GTP, 1.5 mM ATP, 10 mM HEPES, and 1 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, pH 6.8.

**Competition Binding Assays.** Chinese hamster ovary (CHO) cells stably expressing the rNK1R and hNK1R were generously provided by Dr. James Krause (Neurogen Corp., Branford, CT). Competition binding was conducted with Bolton-Hunter [125I]Lys^3^-SP (Perkin Elmer-NEN, Boston, MA) at a concentration of 50 pM and increasing amounts, 1 pM to 1 μM, of unlabeled peptide. All peptides were prepared, and cells were harvested in Tris-buffered saline (50 mM Tris, 120 mM NaCl, pH 7.4) supplemented with bacitracin (0.2 mg/mL), chymostatin (20 μg/mL), leupeptin (20 μg/mL), and bovine serum albumin (0.1%). The cells were incubated in a Millipore Multiscreen 96-well CV filtration plate (Millipore Corporation, Billerica, MA) with radiolabeled SP (50,000 dpm/well) and unlabeled peptide for 2 h at 4°C. The total volume per well was 200 μL, and the cell count in each well was 100,000 cells. Cells were collected by filtration and washed to remove unbound ligand. The filters were collected, and the radiation was counted using a Packard Cobra II series auto-gamma counter (Packard, Meriden, CT). Nonspecific binding (typically 200 dpm) was determined in the presence of 1 μM unlabeled peptide. Specific binding was calculated by subtracting nonspecific binding from the total counts. Data points were normalized by dividing the specifically bound radiolabeled SP counts in the presence of unlabeled peptide by the specifically bound radiolabeled SP counts in the absence of unlabeled peptide that produced the least competition. Maximal specific binding under these conditions is typically <5000 dpm.

**GTP·S Binding.** GTP·S was measured by an antibody capture scintillation proximity assay (DeLapp, 2004). Membranes were prepared from NK1 receptor-expressing CHO cells by homogenization and centrifugation. Samples were taken for protein concentration measurement. Membranes containing 20 μg of protein, various concentrations of tachykinin peptides, and 200 pM [35S]GTP·S were added to a total of 200 μL of assay buffer (100 mM NaCl, 2 mM MgCl_2, 5 mM HEPES, 100 nM GDP, pH 7.4) and incubated at room temperature for 1 h. Membranes were solubilized with 0.35% NP-40 for 30 min. Anti-G_2 antibody (a kind gift from Dr. Thomas Geys, Pennington Biomedical Research Center, Baton Rouge, LA) was added at a dilution of 1:250 and incubated overnight at 4°C with shaking. The next morning, 50 μL of scintillation proximity assay polyvinyl toluene antibody binding beads (Amersham Biosciences, Piscataway, NJ) were added, and the tubes were incubated for 5 h at room temperature. The beads were pelleted, resuspended in 150 μL of assay buffer, and transferred to scintillation tubes for counting in an LS-6500 scintillation counter (Beckman Coulter, Fullerton, CA).

**Fluorescence Ca^{2+} Assays.** Stably transfected CHO cells were grown to 80 to 90% confluence in Falcon 24-well plates at 37°C and 5% CO_2 for 3 to 4 days prior to use. The cells were rinsed three times with a solution containing 115 mM NaCl, 2.3 mM CaCl_2, 1 mM MgCl_2, 5 mM KCl, 10 mM HEPES, and 10 mM glucose, pH 7.4. The cells were then incubated with this solution containing the organic anion transport inhibitor probenecid (2.5 mM) supplemented with 1 μM Fluo 3-AM, a visible light-excited Ca^{2+} indicator (Molecular Probes, Eugene, OR), and 0.1% Pluronic F-127 for 30 min at 37°C. The wells were rinsed again three times just before measurement of cytosolic Ca^{2+} levels and placed in a FLUOstar Galaxy fluorescent plate reader (BMG Lab Technologies, Durham, NC) at 37°C. The fluorescence was measured in an area of ~2.0 mm^2 in the center of each well. The number of CHO cells tested within this area was ~2000. The fluorescence emission at 520 nm following excitation at 485 nm was measured and used as an index of intracellular Ca^{2+} levels.

After loading the cells, concentrations ranging from 1 pM to 1 μM of each peptide were applied to the cells to acquire the data for the concentration-response relationships. A single concentration of peptide was applied to each well. The order in which the various concentrations were applied was varied from plate to plate. Typically, the same concentration was applied to a column of four wells on each plate, and six concentrations were tested per plate. Ionomycin (10 μM) was applied at the end of each experiment to normalize the agonist-induced responses between wells. Data are expressed as a percentage of the maximal response (E_max) for each agonist.

To examine the concentration dependence of desensitization, cells were incubated with agonist at concentrations from 0.1 nM to 1 μM for 5 min at 37°C and then washed three times over the next 30 s. As shown in previous studies (Bennett et al., 2002), this is sufficient to remove the agonist with little desensitization occurring. Next, a constant concentration of SP (100 nM) was applied, and the change in the fluorescence in response to SP was recorded. Ionomycin (10 μM) was applied after the second application to normalize the agonist-induced responses between experiments. The amplitude of the response to 100 nM SP indicates the degree of desensitization of the receptor. These data are expressed as the percentage by which the SP response was decreased as a function of preincubating agonist concentration.

**Statistics and Data Analysis.** Data are expressed as mean ± S.E.M. n values range from 6 to 12 for each data point. GraphPad Prism software (GraphPad Software Inc., San Diego, CA) was used to fit concentration-response curves to the Hill equation and to make statistical comparisons of the curves. Except for the GTP·S binding curves, none of the Hill coefficients were significantly different from 1; therefore, the coefficient was set to 1 for the curve fits and comparisons of those curves. Figures were composed in SigmaPlot.

**Results**

**Concentration-Response Curve for Inhibition of the M-Type Potassium Current.** The amino acid sequences of the ranatachykkin peptides and SP are shown in Fig. 1. As these peptides were originally isolated from bullfrog (Kangawa et al., 1993), their actions on neurons isolated from bullfrog sympathetic ganglia have been examined. These peptides express the bullfrog SP receptor, which exhibits a 69% overall amino acid sequence identity with the rat and human NK1 receptors, with the amino acid residues in regions of the receptor known to be important for SP binding being even more highly conserved (Simmons et al., 1997). When applied to these neurons, SP results in a concentration-dependent inhibition of a time- and voltage-sensitive K^+ current termed I_M (Simmons et al., 1994). The concentration-response curves for the RTK peptides to inhibit I_M (Fig. 2) show that they all inhibit I_M to the same maximal extent;
however, the EC$_{50}$s vary. The rank order of potency for inhibiting $I_m$ is RTKA > SP > RTKB = RTKC, with EC$_{50}$ values of 1.3, 3.8, 12.6, and 13 nM, respectively. A similar rank order of potency has been observed for increases in intracellular Ca$^{2+}$ in CHO cells stably expressing the bullfrog SP receptor (Perrine et al., 2000).

In addition to eliciting cell signaling responses such as inhibition of $I_m$ and increases in intracellular Ca$^{2+}$, binding of substance P also leads to receptor desensitization in bullfrog sympathetic neurons (Jan and Jan, 1982; Simmons et al., 1990), in heterologous expression systems (Kwatra et al., 1993), and in vivo (Moohchala and Sawynok, 1984). To examine desensitization of the bullfrog SP receptor, CHO cells expressing the receptor were incubated in various concentrations of SP or the RTKs for 5 min. The degree of desensitization produced was measured as the decrease in the amplitude of a subsequent Ca$^{2+}$ response induced by a fixed concentration of SP (100 nM). Both SP and RTKA were able to produce 100% desensitization of the SP response, but RTKB and RTKC produced only 50 to 60% desensitization at concentrations at which maximal inhibition of $I_m$ and increases of Ca$^{2+}$ were observed (Fig. 3). Thus, although all of the peptides could produce equal maximal effects for inhibition of $I_m$ and elevation of intracellular Ca$^{2+}$, they differed in their ability to produce receptor desensitization.

To determine whether the differential actions of tachykinin peptides were an exclusive feature of the bullfrog receptor, they would also be observed at mammalian receptors, a series of further experiments has been conducted with respect to the actions of SP and RTKC at mammalian NK$_1$ receptors. Receptor binding, receptor-G protein coupling, Ca$^{2+}$ signaling, and desensitization have all been measured, and the results have been quantified in terms of concentration-response relationships.

**Binding and Functional Assays of Substance P and Ranatachykinin C at the Rat NK$_1$ Receptor.** Competition binding studies of SP and RTKC for $^{125}$I-labeled SP binding to the rat receptor revealed an IC$_{50}$ for SP of 0.4 nM (pEC$_{50}$ = 9.39 ± 0.14) and for RTKC of 32.6 nM (pEC$_{50}$ = 7.49 ± 0.09) (Fig. 4). To assess receptor-G protein coupling, GTPyS binding stimulated by various concentrations of SP and RTKC was measured by antibody capture scintillation proximity assay (Fig. 5). The EC$_{50}$ for SP was 0.5 nM (pEC$_{50}$ = 9.30 ± 0.19), close to the IC$_{50}$ observed in the binding studies. For RTKC, the EC$_{50}$ for stimulating GTPyS binding was 2.5 nM (pEC$_{50}$ = 8.60 ± 0.07), 13-fold lower than the IC$_{50}$ for binding. The concentration-response curves for GTPyS binding are the only ones for which the slope was found to be statistically different from 1; for both of the GTPyS binding curves, the slope was close to 0.5.

The concentration response curves for SP and RTKC to produce Ca$^{2+}$ elevations in rat NK$_1$ receptor-expressing CHO cells (Fig. 6) gave EC$_{50}$s for SP of 2.6 nM (pEC$_{50}$ = 8.59 ± 0.05) and for RTKC of 7.3 nM (pEC$_{50}$ = 8.14 ± 0.14). For SP, this value is 6.5 times greater than the IC$_{50}$ for binding. For RTKC, this value is 4.5-fold less than the IC$_{50}$ for binding. In contrast, the desensitization of the response of the rat NK$_1$ receptor to SP (Fig. 7) showed an EC$_{50}$ for SP of 0.9 nM (pEC$_{50}$ = 9.05 ± 0.06), giving a potency relative to binding of 2.3, whereas the EC$_{50}$ for RTKC to produce desensitization was 50.6 nM (pEC$_{50}$ = 7.30 ± 0.08), giving a potency relative to binding of 1.6.

**Binding and Functional Assays of Substance P and Ranatachykinin C at the Human NK$_1$ Receptor.** To confirm the generalizability of these effects to the NK$_1$ receptor.
of other mammalian species, further experiments were conducted with CHO cells expressing the human NK1 receptor. In a similar series of experiments, the relative abilities of SP and RTKC to bind to the receptor, to produce Ca\textsuperscript{2+}/H\textsubscript{11001} elevations, and to desensitize the human NK1 receptor have been measured (Fig. 8). The IC\textsubscript{50\textsuperscript{8}} for binding were 3 nM (pEC\textsubscript{50} = 8.52 ± 0.13) for SP and 21 nM (pEC\textsubscript{50} = 7.67 ± 0.15) for RTKC. The EC\textsubscript{50} for SP to produce Ca\textsuperscript{2+} elevations was 3.3-fold greater, 10.0 nM (pEC\textsubscript{50} = 8.00 ± 0.05), whereas for RTKC, it was 1.4-fold less, 15.3 nM (pEC\textsubscript{50} = 7.82 ± 0.08). In contrast to this, the EC\textsubscript{50} for SP to produce desensitization of the response of the human NK1 receptor was 2.5 nM (pEC\textsubscript{50} = 8.58 ± 0.07), less than the IC\textsubscript{50} for binding, whereas for RTKC, the EC\textsubscript{50} was 29.2 nM (pEC\textsubscript{50} = 9.30 ± 0.05), almost 2-fold greater than the IC\textsubscript{50} for binding.

To compare the relative abilities of these two peptides to produce signal activation and desensitization, the desensitization/activation ratio has been calculated as the ratio of the EC\textsubscript{50} of the concentration-response curve for the Ca\textsuperscript{2+} response to the EC\textsubscript{50} of the concentration-response curve for desensitization. These values for both the rat and human NK\textsubscript{1} receptors are presented in Table 1. In both cases, SP produces desensitization at lower concentrations than it produces a Ca\textsuperscript{2+} response, whereas RTKC produces Ca\textsuperscript{2+} responses at lower concentrations than it produces desensitization.

**Statistical Analysis (Analysis of Variance and F Test).** Statistical comparisons have been made to compare the concentration-response curves of the human NK\textsubscript{1} receptor. The concentration-response curve for SP to produce a Ca\textsuperscript{2+} response is significantly different from the SP concentration-desensitization curve \(p < 0.001, F = 136 (1,322)\). The curves for RTKC to produce signal activation and desensitization are significantly different \(p = 0.009, F = 7.045 (1,210)\). There is not a significant difference between the SP and RTKC activation curves, but there is a significant difference between the SP and RTKC desensitization curves \(p < 0.0001, F = 148.6 (1,298)\).

**Discussion**

In classic models of drug action, the primary event is binding of a ligand (L) to its receptor (R). The binding of an agonistic ligand to a membrane receptor leads to an intracellular effect, E, such that \(L + R \rightleftharpoons LR \rightarrow E\). The concentration dependence of the effect hinges on the binding affinity of L for R. The magnitude of the effect depends on the intrinsic efficacy of L. There are numerous examples showing that activation of a single receptor can lead to activation of multiple intracellular signaling events (Perez and Karnik, 2005), such that

\[
L_1 + R \rightleftharpoons L_1R \leftarrow E_1 \rightarrow E_2
\]

In traditional receptor theory, the relative degree of activation of \(E_1\) or \(E_2\) is the same for every agonist. For example,
an agonist with half the efficacy of a reference agonist for \( E_1 \) should also have half the efficacy of the reference agonist for \( E_2 \). More recently, however, evidence from a variety of systems suggests that some ligands, acting at a single receptor, may preferentially activate \( E_1 \), whereas other ligands, acting at the same receptor in the same system, may preferentially activate \( E_2 \), as follows:

\[
L_1 + R \rightleftharpoons L_1R \quad \rightarrow \quad \text{E}_1 \\
L_2 + R \rightleftharpoons L_2R \quad \rightarrow \quad \text{E}_2
\]

The possibilities for agonist-specific functional selectivity seem particularly conceivable with respect to peptide ligands of G protein coupled receptors because these ligands are relatively large molecules that span and interact with multiple extracellular and transmembrane domains of the receptor. There are some interesting biological implications for ligand selectivity of peptide ligands. In an organism containing both activating and desensitizing ligands, functional selectivity could provide a way for multiple ligands to transmit specific information through a single receptor. SP would activate but produce less desensitization. RTKC would activate and rapidly desensitize the receptor. In this manner, the SP-mediated signals would provide a strong, short-lived signal, whereas at similar concentrations, the RTKC signal would be less intense and longer lived.

TABLE 1
Desensitization/activation ratios for SP and RTKC at rat and human NK\(_1\) receptors

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Rat NK(<em>1) Receptor (EC(</em>{50}))</th>
<th>Human NK(<em>1) Receptor (EC(</em>{50}))</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Desensitization/Activation</td>
<td>Desensitization/Activation</td>
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<tr>
<td>Signal</td>
<td>Ratio</td>
<td>Signal</td>
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<tr>
<td>activation</td>
<td>nM</td>
<td>activation</td>
</tr>
<tr>
<td>SP</td>
<td>2.6</td>
<td>0.35</td>
</tr>
<tr>
<td>RTKC</td>
<td>7.3</td>
<td>6.93</td>
</tr>
</tbody>
</table>

SP is an established neuropeptide neurotransmitter in mammals; however, to date the presence of RTKC in mammalian nervous system has not been reported. Another possibility for peptide functional selectivity could arise from metabolism of larger peptides. One major difference between SP and RTKC is the lack of the N-terminal Arg residue in RTKC. SP(2–11) has not been reported as a metabolite of SP, but it has been shown that SP is metabolized in the spinal cord to SP(1–7) (Sakurada et al., 1985) and that accumulation of this fragment is correlated with desensitization to SP-induced behaviors (Igwe et al., 1990). At the same time, C-terminal fragments of SP activate the receptor in a manner similar to SP but produce much less desensitization (Tschope et al., 1995; Vigna, 2001).

These differences in desensitization to different ligands could be interpreted as indicating the presence of two different NK\(_1\) receptor subtypes. A number of studies have shown two binding sites associated with the NK\(_1\) receptor, leading to suggestions that these sites either represent two populations of receptors coupled to different G proteins (Holst et al., 2001) or represent two different molecular forms of the receptor (Alves et al., 2006). The major binding site on the NK\(_1\) receptor has been termed the NK-1M site (Sagan and Lavielle, 2001). The less abundant binding site, termed NK-1m, is equivalent to the septide-sensitive binding site (Sagan et al., 1997), has a subnanomolar affinity for SP, and is coupled to PLC via G\(_i\). There are several features of the NK-1m site that are inconsistent with the data presented here. In the presence of two binding sites, the competition binding curves for SP should show two binding sites. The competition binding curves show no evidence for two binding sites and are best fit by a single-site competition model. Ligands selective for the NK-1m site, like septide, NKA, and NKB, are only able to displace SP from the NK-1M site at concentrations in the micromolar range. RTKC can displace SP in the nM range. Differences in desensitization and internalization of...
on the order of 100 nM (unpublished data).

modulate NK1 receptor function with agents that specifically provided helpful comments on the manuscript.

port. Dr. Shane A. Perrine made major contributions to the data and

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gene responses and desensitization and show that the ability of a tachykinin receptor agonist to produce signal activation and its ability to produce desensitization are not necessarily correlated. Previous stud-

eries have shown that SP analogs differ in their abilities to activate the phospholipase C or adenylate cyclase second messenger pathways (Sagan et al., 1996; Sachon et al., 2002). These findings have several implications in terms of drug-

receptor actions. First, the data suggest that it may be pos-

tible to design ligands to preferentially produce signal activa-

tion or desensitization. The ability to differentially activate one or another signaling pathway could provide novel or improved therapies. For example, a ligand that produces desensitization without activation may present a different therapeutic profile than a receptor antagonist. An-

other possibility is that one receptor-activated pathway may be more strongly correlated with therapeutic efficacy or side effects, properties that could be exploited in drug develop-

ment.

NK receptors are involved in pain, asthma, inflammation, major depression, and stress-induced anxiety. The ability to modulate NK receptor function with agents that specifically produce receptor desensitization could provide a novel and useful alternative therapeutic approach to these disorders.

This phenomenon of ligand-dependent selectivity with re-

spect to signaling through G protein coupled receptors has been described by a variety of terms including functional selectivity, ligand-directed trafficking, and conformation-specific agonism, among others (Simmons, 2005). Functional selectivity has been well described among amine receptors including serotonergic (Roth and Chuang, 1987; Berg et al., 1998), dopaminergic (Lewis et al., 1998; Gay et al., 2004), and

adrenergic (Jim et al., 1985) receptors. Ligand functional selectivity has also been demonstrated for several other peptide receptors (Hunyady et al., 1994; Whistler et al., 1999; Zhang et al., 1999; Kapusta et al., 2005; McLaughlin et al., 2005; Nickolls et al., 2005).

The ability to develop drugs that could consistently and re-

produbly activate a receptor without producing desensitiza-

tion could lead to considerable improvements in our ability to treat a variety of diseases. Conversely, the discovery that it was possible to produce desensitization of a G protein-coupled re-

ceptor in the absence of signal activation could be the basis for the development of a novel mechanism of drug action.

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