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# Differential Activation of Intracellular Effector by Two Isoforms of Human Neurokinin-1 Receptor

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**SUMMARY**

Two isoforms of the human neurokinin-1 receptor were cloned and characterized in heterologous expression systems of mammalian cell culture and *Xenopus* oocytes. The two isoforms differ only in the length of the encoded polypeptide. The peptide-binding properties of the long form of human neurokinin-1 receptor are consistent with those of the native neurokinin-1 receptor of mammalian tissues, where substance P is the most potent agonist. Peptide agonists elicit an oscillating current in *Xenopus* oocytes expressing the long form. In contrast, the short form of human neurokinin-1 receptor expressed in COS cells binds substance P with an apparent affinity at least 10-fold lower than that of the long form, and it elicits the electrophysiological response only weakly in *Xenopus* oocytes. These data suggest that the short form couples to a different effector system. Sequence analysis suggested that the two isoforms may arise from alternative pre-mRNA splicing. These results indicate that multiple forms of the human neurokinin-1 receptor exist and the differential activation of intracellular effector may be involved in generating the complex biological effects of substance P.

The neurotransmitter peptide SP plays an important role in pain transmission and neurogenic inflammatory diseases (1, 2). It binds preferentially to the NK1R to elicit its biological response (3, 4). The cDNA of rat NK1R has been cloned, and the deduced protein sequence indicates that it is a membrane receptor with seven putative transmembrane segments (5, 6). This predicted topological structure is characteristic of receptors that activate G proteins. Studies using mutagenesis and antipeptide antibodies with several G protein-coupled receptors have implicated the proximal regions of the second, third, and fourth intracellular segments in mediating the interactions of the receptors with G proteins (7–10).

Heterologous expression of the cloned rat NK1R in *Xenopus* oocytes has demonstrated that the activation of this receptor upon SP binding results in an oscillating current, presumably mediated by a G protein and the phosphatidylinositol pathway. However, NK1R from different tissues do not always conform to the same profile of agonist selectivity (3), suggesting the possibility of differential binding sites and/or second messenger pathways for the NK1R. In the present studies, we report the isolation of two cDNA clones that encode two isoforms of the human NK1R of different sizes, and the expressed receptors elicit electrophysiological response in oocytes with very different efficacies. The only difference between the two isoform sequences is the length of the carboxyl-terminal tail. These data suggest a role for the carboxyl-terminal region of NK1R in determining the specificity of G protein activation. The possibility of the activation of other effector systems by the short form is discussed.

## Materials and Methods

**Isolation of human NK1R cDNA.** Human mRNA was prepared from three human glioblastoma cell lines T98G, CCF-STTG1, and U87MG (American Type Culture Collection, Rockville, MD). PCR amplification was carried out using the GeneAmp kit (Perkin Elmer Cetus, Norwalk, CT). The PCR annealing temperature was 55° and cycle number was 30 unless specified. To obtain the cDNA encoding the central core region of human NK1R, first-strand cDNA was synthesized using oligo(dT) primer and amplified by rat primers based on the sequence of the second and seventh transmembrane segments (TGCATGGCTGCATTCAAT, TGCATGGCTGCCTTCAA, and ACAGTAGATGATGGGGTTGTACAT) in a primary PCR (annealing at 40°). The primary PCR product was further amplified in a secondary PCR under the same conditions. The secondary PCR product was amplified with rat primers (TGCATGGCTGCATTCAAT, TGCATGGCTGCCTTCAA, CAGGTA[G/C]ACCTGCTGGATGAACTT) in a tertiary PCR (annealing at 45°). A 600-bp fragment was purified from the tertiary PCR product and subcloned into pBluescript SK+ (Stratagene, La Jolla, CA). The DNA sequence was determined by the dideoxy chain termination method, from both directions. The nucleotide sequence of the cDNA is similar to the region of rat NK1R encoding amino acids 91–280.

**ABBREVIATIONS:** SP, substance P; BHSP, Bolton-Hunter-labeled substance P; G protein, guanine nucleotide-binding protein; NK1R, neurokinin-1 receptor; NKB, neurokinin B; PCR, polymerase chain reaction; SK, substance K; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; bp, base pairs.

To obtain the cDNA encoding the amino-terminal region of human NK1R, cDNA was synthesized using five antisense primers based on the human sequence (nucleotides 446–427, 646–626, 671–652, 766–746, and 821–802, as defined in Fig. 1A). The first-strand cDNA was amplified by rat primers based on the amino-terminal sequence of rat NK1R (ATGGATAACGTCCTTCCTAT and ATGGACAATGTGCTGCCCA) and one human primer (nucleotides 446–427), in a primary PCR. The primary PCR product was amplified with the same rat primers and one human primer (nucleotides 410–391) in a secondary PCR. The secondary PCR product was amplified with the same rat primers and one human primer (nucleotides 369–349) in a tertiary PCR. A 500-bp fragment was identified by hybridization analysis with a human oligonucleotide probe (nucleotides 333–313). This cDNA fragment encodes an amino acid sequence similar to the amino-terminal sequence of the rat NK1R, preceded by a 5′ untranslated sequence. It was apparent that the degenerate rat NK1R amino-terminal primers hybridized incidentally to the 5′ untranslated region of human NK1R mRNA under low stringency conditions.

To obtain the cDNA encoding the carboxyl-terminal region of human NK1R, oligo(dT)-primed cDNA was ligated with *Eco*RI linker and then ligated into pBluescript SK+. The ligated plasmid DNA was amplified in a primary PCR with two human primers (nucleotides 714–734 and 746–766) and a vector-specific primer, T3 (Stratagene). The primary PCR product was amplified in a secondary PCR with one human primer (nucleotides 790–809) and the same vector-specific primer T3. The secondary PCR product was amplified in a tertiary PCR with one human primer (nucleotides 813–832) and vector-specific primer SK (Stratagene). DNA fragments of 800 bp and 400 bp were identified by hybridization analysis with a human oligonucleotide probe (nucleotides 828–845).

To obtain a full length cDNA, primers based on the sequence of the untranslated region were used. cDNA was synthesized from either human glioblastoma mRNA or human brain mRNA (Clontech, Palo Alto, CA) with the long form primer (nucleotides 1377–1357) or the short form primer (nucleotides 1028–1009, as defined in Fig. 1B). First-strand cDNA was amplified in a primary PCR with two human primers (nucleotides 1377–1357 and −107 to −88 for the long form or nucleotides 1028–1009 and −107 to −88 for the short form). The primary PCR product was amplified in a secondary PCR with two human primers (nucleotides 1356–1338 and −37 to −18 for the long form or nucleotides 999–982 and −37 to −18 for the short form). A 1300-bp DNA fragment (long form) and a 1100-bp DNA fragment (short form), respectively, were purified and subcloned into Bluescript SK+.

**Expression of the cloned human NK1R.** *In vitro* RNA transcript was synthesized from the cDNA in Bluescript SK+ by T7 RNA polymerase (Stratagene) and purified by Sephadex G-50 spin column. One or two nanograms of RNA were injected into each oocyte. *Xenopus* oocyte isolation, RNA microinjection, and two-electrode voltage-clamp were described previously (11). The peak current in response to various peptide agonists was measured at a holding potential of −80 mV.

To express the human NK1R transiently in COS, the cDNA was cloned into the expression vector pCDM9, which was derived from pCDM8 (Invitrogen, San Diego, CA) after insertion of the ampicillin resistance gene (nucleotides 1973–2964 from Bluescript SK+) into the *Sac*II site of pCDM8. Transfection of plasmid DNA into COS cells was achieved by electroporation.

The competition binding assay of human NK1R expressed in COS was modified from the whole-cell assay (6). Monolayer cell culture of COS was dissociated with a nonenzymatic solution (Specialty Media, Lavallette, NJ) and resuspended in an appropriate volume of the binding buffer (50 mM Tris, pH 7.5, 5 mM $MnCl_2$, 150 mM NaCl, 0.04 mg/ml bacitracin, 0.004 mg/ml leupeptin, 0.2 mg/ml bovine serum albumin, 0.01 mM phosphoramidon), such that 200 μl of the cell suspension would give rise to no more than 10,000 cpm of specific $^{125}$I-BHSP binding. Two hundred microliters of cells were added to a tube containing 20 μl of 2.5 nM $^{125}$I-BHSP (NEN, North Billercia, MA) (see also Ref. 12) and 20 μl of unlabeled peptide at various concentrations. The tubes were incubated at 4° for 1 hr with gentle shaking. The unbound radioactivity was separated from bound radioactivity on GF/C filters (Brandel, Gaithersburg, MD) that had been prewetted with a 0.1% (w/v) solution of 50% (v/v) polyethylenimine. The filters were washed with 3 ml of wash buffer (50 mM Tris, pH 7.5, 5 mM $MnCl_2$, 150 mM NaCl) three times, and the radioactivity was determined with a γ-counter. For equilibrium binding assays, a stock solution of $^{125}$I-SP was prepared from a 90-fold isotopic dilution of $^{125}$I-BHSP (42 nM) with unlabeled SP (42 nM). The reaction mixture contained 20 μl of buffer (for total binding) or 120 μM SP (for nonspecific binding), 200 μl of $^{125}$I-SP at various concentrations, and 200 μl of cell suspension.

**Other chemicals.** The rat NK1R cDNA was cloned as described previously (6). All peptides were obtained from Peninsula Laboratories (Belmont, CA). SP and SK stock solutions were made in 1 mg/ml bovine serum albumin, 50% methanol, 0.1 M acetic acid. NKB stock solution was made in dimethyl sulfoxide.

## Results

**Isolation of two isoforms of human NK1R cDNA and the structures of the deduced proteins.** The cDNA of human NK1R was isolated in four steps. (i) A cDNA clone encoding the transmembrane core region of the human NK1R was isolated from human glioblastoma mRNA by homologous PCR amplification using degenerate primers based on the rat NK1R sequence. (ii) A cDNA clone encoding the amino-terminal and 5′ untranslated regions of the human NK1R was isolated by PCR using antisense human NK1R primers and sense degenerate primers based on the rat NK1R sequence. (iii) cDNA clones encoding the carboxyl-terminal and 3′ untranslated regions of human NK1R were isolated from a cDNA library by PCR using sense human NK1R primers and a vector-specific primer. An 800-bp and a 400-bp cDNA fragment were obtained. Both clones encode the carboxyl-terminal region of the human NK1R, except that the 800-bp clone contains a stop codon after amino acid 407, whereas the 400-bp clone contains a stop codon after amino acid 311. The 3′ untranslated sequences of these two clones are also different. It is concluded that two isoforms of the human NK1R are present, one short form (311 amino acids) and one long form (407 amino acids). (iv) To confirm the authenticity of the two isoforms of human NK1R, two full length cDNAs were obtained by PCR using human primers based on the 5′ and 3′ untranslated regions. The complete sequences of the full length cDNAs for both isoforms are shown in Figure 1. Full length cDNA clones were also obtained from human brain mRNA with identical sequence (Fig. 2).

The amino acid sequences of the long and short forms of the human NK1R were deduced from their cDNA sequences (Fig. 1). The protein size of the long form is the same as that of the rat NK1R, with 22 amino acid substitutions between the two species. The short form contains a very short carboxyl-terminal sequence, extending only seven amino acid residues after the end of the seventh transmembrane segment; otherwise, the amino acid sequence is identical to that of the long form.

**Pharmacological characterization of human NK1R in *Xenopus* oocytes.** *In vitro* RNA transcripts from the cDNAs encoding either of the two forms of the human NK1R were injected into *Xenopus* oocytes for functional expression. Under voltage-clamp conditions, bath application of SP, SK, or NKB elicited an oscillating inward current (Fig. 3), whereas uninjected oocytes did not respond to any of the agonists. The

A

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                                                                  -122 GA AAAAGCCTTC CACCCTCCTG TCTGGCTTTA -91

GAAGGACCCT GAGCCCCAGG CGCCACGACA GGACTCTGCT GCAGAGGGGG GTTGTGTACA GATAGTAGGG CTTTACCGCC TAGCTTCGAA -1

ATG GAT AAC GTC CTC CCG GTG GAC TCA GAC CTC TCC CCA AAC ATC TCC ACT AAC ACC TCG GAA CCC AAT CAG TTC GTG CAA 81
MET Asp Asn Val Leu Pro Val Asp Ser Asp Leu Ser Pro Asn Ile Ser Thr Asn Thr Ser Glu Pro Asn Gln Phe Val Gln 27
                          M                       F                                   S

CCA GCC TGG CAA ATT GTC CTT TGG GCA GCT GCC TAC ACG GTC ATT GTG GTG ACC TCT GTG GTG GGC AAC GTG GTA GTG ATG 162
Pro Ala Trp Gln Ile Val Leu Trp Ala Ala Ala Tyr Thr Val Ile Val Val Thr Ser Val Val Gly Asn Val Val Val MET 54
     T                                                                                                   I

TGG ATC ATC TTA GCC CAC AAA AGA ATG AGG ACA GTG ACG AAC TAT TTT CTG GTG AAC CTG GCC TTC GCG GAG GCC TCC ATG 243
Trp Ile Ile Leu Ala His Lys Arg MET Arg Thr Val Thr Asn Tyr Phe Leu Val Asn Leu Ala Phe Ala Glu Ala Ser MET 81
                                                                                                        C

GCT GCA TTC AAT ACA GTG GTG AAC TTC ACC TAT GCT GTC CAC AAC GAA TGG TAC TAC GGC CTG TTC TAC TGC AAG TTC CAC 324
Ala Ala Phe Asn Thr Val Val Asn Phe Thr Tyr Ala Val His Asn Glu Trp Tyr Tyr Gly Leu Phe Tyr Cys Lys Phe His 108
                                                    V

AAC TTC TTC CCC ATC GCC GCT GTC TTC GCC AGT ATC TAC TCC ATG ACG GCT GTG GCC TTT GAT AGG TAC ATG GCC ATC ATA 405
Asn Phe Phe Pro Ile Ala Ala Val Phe Ala Ser Ile Tyr Ser MET Thr Ala Val Ala Phe Asp Arg Tyr MET Ala Ile Ile 135
                          L

CAT CCC CTC CAG CCC CGG CTG TCA GCC ACA GCC ACC AAA GTG GTC ATC TGT GTC ATC TGG GTC CTG GCT CTC CTG CTG GCC 486
His Pro Leu Gln Pro Arg Leu Ser Ala Thr Ala Thr Lys Val Val Ile Cys Val Ile Trp Val Leu Ala Leu Leu Leu Ala 162
                                                  F

TTC CCC CAG GGC TAC TAC TCA ACC ACA GAG ACC ATG CCC AGC AGA GTC GTG TGC ATG ATC GAA TGG CCA GAG CAT CCG AAC 567
Phe Pro Gln Gly Tyr Tyr Ser Thr Thr Glu Thr MET Pro Ser Arg Val Val Cys MET Ile Glu Trp Pro Glu His Pro Asn 189

AAG ATT TAT GAG AAA GTG TAC CAC ATC TGT GTG ACT GTG CTG ATC TAC TTC CTC CCC CTG CTG GTG ATT GGC TAT GCA TAC 648
Lys Ile Tyr Glu Lys Val Tyr His Ile Cys Val Thr Val Leu Ile Tyr Phe Leu Pro Leu Leu Val Ile Gly Tyr Ala Tyr 216
 R   T                A

ACC GTA GTG GGA ATC ACA CTA TGG GCC AGT GAG ATC CCC GGG GAC TCC TCT GAC CGC TAC CAC GAG CAA GTC TCT GCC AAG 729
Thr Val Val Gly Ile Thr Leu Trp Ala Ser Glu Ile Pro Gly Asp Ser Ser Asp Arg Tyr His Glu Gln Val Ser Ala Lys 243

CGC AAG GTG GTC AAA ATG ATG ATT GTC GTG GTG TGC ACC TTC GCC ATC TGC TGG CTG CCC TTC CAC ATC TTC TTC CTC CTG 810
Arg Lys Val Val Lys MET MET Ile Val Val Val Cys Thr Phe Ala Ile Cys Trp Leu Pro Phe His Ile Phe Phe Leu Leu 270
                                                                                    V

CCC TAC ATC AAC CCA GAT CTC TAC CTG AAG AAG TTT ATC CAG CAG GTC TAC CTG GCC ATC ATG TGG CTG GCC ATG AGC TCC 891
Pro Tyr Ile Asn Pro Asp Leu Tyr Leu Lys Lys Phe Ile Gln Gln Val Tyr Leu Ala Ile MET Trp Leu Ala MET Ser Ser 297
                                                  S

ACC ATG TAC AAC CCC ATC ATC TAC TGC TGC CTC AAT GAC AGG TTC CGT CTG GGC TTC AAG CAT GCC TTC CGG TGC TGC CCC 972
Thr MET Tyr Asn Pro Ile Ile Tyr Cys Cys Leu Asn Asp Arg Phe Arg Leu Gly Phe Lys His Ala Phe Arg Cys Cys Pro 324

TTC ATC AGC GCC GGC GAC TAT GAG GGG CTG GAA ATG AAA TCC ACC CGG TAT CTC CAG ACC CAG GGC AGT GTG TAC AAA GTC 1053
Phe Ile Ser Ala Gly Asp Tyr Glu Gly Leu Glu MET Lys Ser Thr Arg Tyr Leu Gln Thr Gln Gly Ser Val Tyr Lys Val 351
                                                                                    S

AGC CGC CTG GAG ACC ACC ATC TCC ACA GTG GTG GGG GCC CAC GAG GAG GAG CCA GAG GAC GGC CCC AAG GCC ACA CCC TCG 1134
Ser Arg Leu Glu Thr Thr Ile Ser Thr Val Val Gly Ala His Glu Glu Glu Pro Glu Asp Gly Pro Lys Ala Thr Pro Ser 378
                                                                  E

TCC CTG GAC CTG ACC TCC AAC TGC TCT TCA CGA AGT GAC TCC AAG ACC ATG ACA GAG AGC TTC AGC TTC TCC TCC AAT GTG 1215
Ser Leu Asp Leu Thr Ser Asn Cys Ser Ser Arg Ser Asp Ser Lys Thr MET Thr Glu Ser Phe Ser Phe Ser Ser Asn Val 405
                          G                       N                                   S           Y           M

CTC TCC TAG GCCACAGGGC CTTTGGCAGG TGCAGCCCCC ACTGCCTTTG ACCTGCCTCC CTTCATGCAT GGAAATTCCC TTCATCTGGA 1304
Leu Ser
     A

ACCATCAGAA ACACCCTCAC ACTGGGACTT GCAAAAAGGG TCAGTATGGG TTAGGGAAAA CATTCCATCC TTGAGTCAAA AAATCTCAAT 1394

TCTTCCCTAT CTTTGCCACC CTCATGCTGT GTGACTCAAA CCAAATCACT GAACTTTGCT GAGCCTGTAA AATAAAAGGT CGGACCAGCT 1484

TTTCCCAAAA GCCCATTCAT TCCATTCTGG AAGTGACTTT GGCTGCATGC GAGTGCTCAT TTCAGGAT 1552
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B

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892                                                                 933
ACC ATG TAC AAC CCC ATC ATC TAC TGC TGC CTC AAT GAC AGG TGA GGATCCCAAC CCCATGAGCT CTCCAGGGGC CACAAGACCA 976
Thr MET Tyr Asn Pro Ile Ile Tyr Cys Cys Leu Asn Asp Arg
                                                    311

TCTACATACA CAGTGGCCAA GCGGCATCCT AAATGAGTAA ACCCAGCTGT GAGACAAGAG GGACAAGTGG GGACTGCAGC TAACTTATCA 1066

TCACACAACT CAGCCTGGCT GATTATCACC ATCCAGGAAT GGGAGCCCGG AGTAGACTGA TTTTCTTTTT TTCTTTTCCA 1146
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**Fig. 1.** Nucleotide sequence and amino acid sequence of the long form of human NK1R (A) and the short form of human NK1R (B). The putative transmembrane segments are *underlined*. The rat NK1R amino acids, in single-letter code, are included below the equivalent position where divergence is found. In B, only the carboxyl-terminal region (starting from nucleotide 892) and 3′ untranslated region are shown. The upstream sequence (from −122 to 933) of the short form is identical to the long form of the human NK1R.


**Fig. 2.** Agarose gel electrophoresis of the full length cDNA for the short and long forms of human NK1R derived from human brain mRNA. *Lane 1*, PCR products of the short form of the human NK1R. *Lane 2*, PCR products of the long form of the human NK1R. *Lane M*, DNA size markers, four of which are labeled, by their nucleotide lengths, on the *side*.

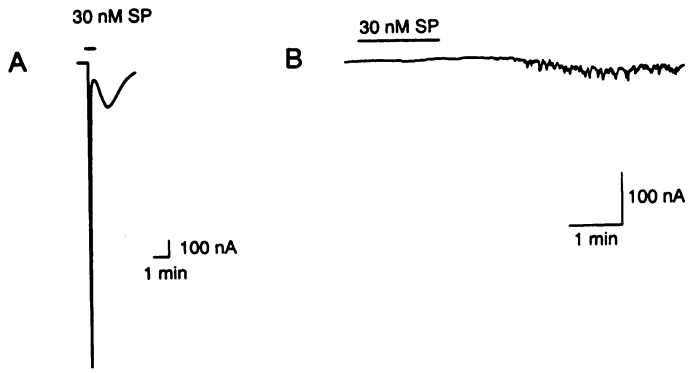


**Fig. 3.** Chloride current response elicited by SP from *Xenopus* oocytes expressing the human NK1R. A, Response of an oocyte injected with 1 ng of RNA of the long form human NK1R. B, Response of an oocyte injected with 2 ng of RNA of the short form human NK1R. *Horizontal bar above the current traces*, duration of SP application.

oscillating current could be completely inhibited by intracellular injection of 50 nl of 15 mM EGTA before the physiological measurement. The current response elicited by 30 nM SP from oocytes injected with 1 or 2 ng of the long form RNA was large (in the range of 500–4000 nA, depending on *Xenopus laevis* donor). However, the current response elicited by 30 nM SP from oocytes injected with the short form RNA was about 100-fold smaller than that of the long form. Oocytes injected with the short form RNA usually gave rise to responses of <100 nA, with a slow onset (Fig. 3B). In some experiments in which the maximal response to SP of the long form was <1000 nA, no significant response was observed from oocytes injected with the short form.

The dose-response curves for the long form of the human NK1R are shown in Fig. 4A. The potency order for the three peptide agonists is consistent with the pharmacological definition of the NK1R. SK and NKB were approximately equipotent at the human NK1R, whereas SK was more potent than NKB at the rat NK1R (Fig. 4; Table 1).

**Pharmacological characterization of human NK1R in COS cells.** To measure ligand binding affinity, the human NK1R cDNA was transfected into COS cells. The equilibrium dissociation constants of peptide agonists for the long form were determined by measuring the displacement of $^{125}$I-BHSP. As shown in Fig. 5, the order of ligand binding affinity (SP > SK > NKB) correlated with the order of efficacy as measured in oocytes. Like the $EC_{50}$ values of receptor activation in oocytes, the binding affinities of SK and NKB at the long form of the human NK1R were also closer to each other than at the rat NK1R (Table 1).

Under the aforementioned conditions of the competition assay with 0.2 nM $^{125}$I-BHSP, no significant level of $^{125}$I-BHSP binding to the short form of the human NK1R was detected in COS cells, indicating a decreased binding affinity for SP. Saturation binding was thus carried out to estimate the binding affinity. As shown in Fig. 6, the $K_d$ value of SP binding to the short form was shown to be >10 nM, whereas the $K_d$ of SP binding to the long form was 1.1 ± 0.3 nM. An exact $K_d$ value for the short form could not be determined, due to the limits of the accessible $^{125}$I-BHSP concentrations.

## Discussion

The present work describes the cloning and expression of two isoforms of the human NK1R. The long form of the human NK1R, when expressed in *Xenopus* oocytes and mammalian cells, shows similar relative potencies for the endogenous agonists as does the rat NK1R, although the absolute binding affinities are higher for the human NK1R (Table 1). Furthermore, the binding affinities and activation efficacies of SK and NKB are closer to each other for the long form of the human NK1R. Comparison of the deduced amino acid sequences for the rat NK1R and the long form of the human NK1R revealed 22 substitutions. The observed differences in agonist binding affinity must, therefore, be attributable to this sequence divergence. Further studies utilizing mutagenesis approaches should reveal the structural basis of peptide-receptor interactions.

The discovery of two isoforms of the human NK1R cDNA may explain some of the complex phenomena that have been

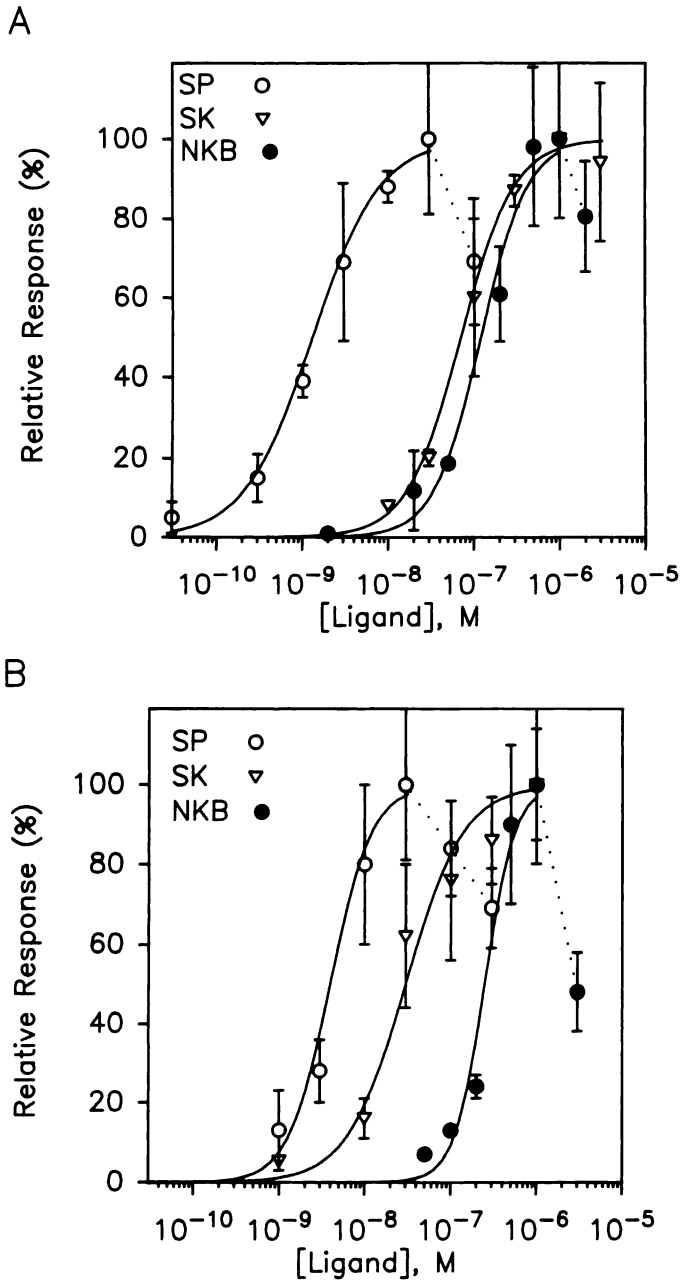


**Fig. 4.** Dose-response curves of the agonist-elicited chloride current response in oocytes injected with NK1R RNA. A, Long form of human NK1R. B, Rat NK1R. For each dose-response curve, the current response was normalized to the maximal response of that peptide. Each *point* represents the average response from at least three oocytes. Each oocyte was exposed to agonist only once. *Error bars*, standard error.

TABLE 1

**Comparison of $K_d$ values for peptide binding and $EC_{50}$ values for receptor activation for the long form of the human NK1R and the rat NK1R**

| | $K_d$[a] | | $EC_{50}$[b] | |
|---|---|---|---|---|
| | Rat NK1R | Human NK1R | Rat NK1R | Human NK1R |
| | nM | | nM | |
| SP | 3.2 ± 0.7 (5) | 0.7 ± 0.2 (4) | 4 ± 2 (7) | 1 ± 2 (4) |
| SK | 55 ± 26 (3) | 25 ± 6 (4) | 30 ± 8 (3) | 79 ± 12 (3) |
| NKB | 179 ± 11 (3) | 57 ± 9 (4) | 250 ± 38 (4) | 100 ± 40 (4) |

[a] The $K_d$ values were determined from the competition binding assays in COS cells. The $K_d$ values for the rat NK1R determined here are consistent with previously reported values (6). For SP, $K_d$ was calculated according the equation $K_{d(SP)} = IC_{50} - [^{125}I\text{-BHSP}]$. For SK and NKB, $K_d$ was calculated according to $K_d = IC_{50}/(1 + [^{125}I\text{-BHSP}]/K_{d(SP)})$. Mean ± standard error values are shown, and the numbers of independent determinations are indicated in parentheses.

[b] $EC_{50}$ values were determined from the electrophysiological assay in oocytes. The data in Fig. 4 were fitted to the logistic equation $y = 100/(1 + (EC_{50}/L)^a)$, in which $y$ is the relative response, $L$ is the peptide concentration, and $a$ is the slope factor. Mean ± standard error values are shown. The numbers of independent experiments are indicated in parentheses.

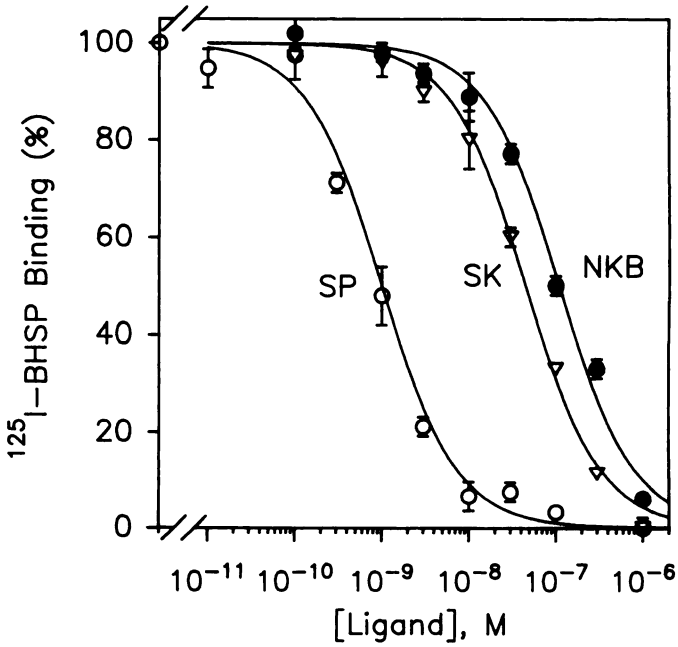


**Fig. 5.** Inhibition of $^{125}$I-BHSP binding to human NK1R by unlabeled peptides. The *solid lines* were calculated according to the equation $[cpm(L) - cpm(1\ \mu M\ SP)]/[cpm(0) - cpm(1\ \mu M\ SP)] = IC_{50}/(L + IC_{50})$, in which cpm($L$) and cpm(0) represent bound $^{125}$I-BHSP in the presence and absence of unlabeled peptides, respectively; and L is the peptide concentration. Each *point* represents the average of triplicates. *Error bars*, standard error.

observed by pharmacological and physiological analyses of the neurokinin receptor system. Heterologous expression of three subtypes of neurokinin receptors (termed NK1, NK2, and NK3) has indicated that the electrophysiological response is presumably mediated by the phosphatidylinositol pathway and calcium (6, 13, 14). Although intracellular $Ca^{2+}$ increases have been demonstrated in neurons in response to SP (15), other effectors have also been reported. For example, the inhibition of the M-current by SP in sympathetic neurons and smooth muscle cells does not require increased $Ca^{2+}$ levels (16). Other intracellular messengers may mediate the desensitization of the M-current inhibition, which occurs on a delayed time scale (17). Kinase-independent modulation of $Ca^{2+}$ and $K^+$ channels by SP also suggests direct G protein action at these channels (18).

The observation that the short form of the human NK1R is less responsive to SP than the long form (Fig. 3) could reflect either a lower intrinsic binding affinity or a preference of the short form for a different G protein. The former appears less likely, because it has been shown that the binding sites of several G protein-coupled receptors involve the transmembrane domain but not the intracellular carboxyl-terminal tail (9). The latter interpretation appears more likely, because the binding affinity of agonists can be increased by the presence of a G

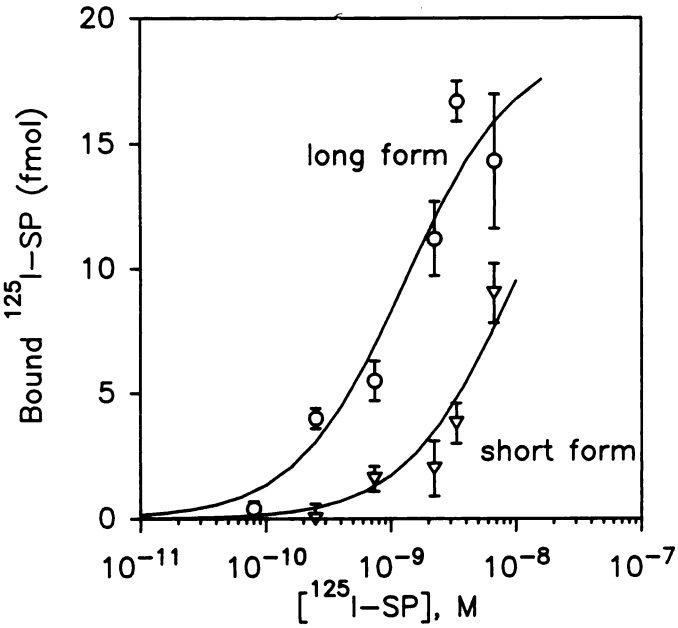


**Fig. 6.** Equilibrium binding of $^{125}$I-SP to the short form and the long form of the human NK1R. Nonspecific binding was determined by including 5.7 μM unlabeled SP at each concentration of $^{125}$I-SP and was subtracted from total binding. The *solid line* was calculated according the equation $B = B_{max} \cdot L/(L + K_d)$, in which $L$ represents $^{125}$I-SP concentration and $B$ represents bound $^{125}$I-SP. Each *point* represents the average of triplicates. *Error bars*, standard error.

protein that interacts with the receptor (19). According to this interpretation, the short form of the human NK1R couples to a G protein that is not present in COS cells, resulting in the apparent low binding affinity for SP in these cells (Fig. 6). The short form can elicit the electrophysiological response in *Xenopus* oocytes, but the magnitude of the response is much smaller and is undetectable in some oocytes. An analogous situation has been described for the muscarinic M2 receptor expressed in human embryonic kidney cells and *Xenopus* oocytes. At low concentrations of carbamylcholine, the M2 receptor activates $G_i$ and inhibits adenylyl cyclase, as has been observed *in vivo*. At high agonist concentrations, however, these heterologously expressed receptors stimulate phosphatidylinositol turnover (20). The magnitude of the chloride current response mediated by the M2 receptor expressed in oocytes is small and variable among oocytes (7). Thus, the apparent low affinity of the agonist-receptor interaction reflects the weak coupling of the receptor with an inappropriate G protein. Such a situation can result from the overexpression of receptors in these heterologous systems (21). Until an appropriate effector can be identified for the short form of human NK1R, the possibility of physiological irrelevance cannot be ruled out.

It is not known at the present time how the two isoforms of the human NK1R are generated *in vivo*. Analysis of the cDNA sequences indicates that the sequences of the 5′ untranslated region and the coding region are identical between both isoforms, whereas the 3′ untranslated sequences are different, with no obvious relationship. It is likely that the two isoforms arise from alternative pre-mRNA splicing. It has been shown that the gene structures for the human NK2R and the rat NK1R are quite similar (22, 23). In both the human NK2R gene and the rat NK1R gene, the junction between exon 4 and exon 5 is at the same position as the stop codon of the short form of human NK1R. In the genomic sequences of both the human NK2R and the rat NK1R, the three nucleotides following the end of exon 4 are TGA, which also comprise the stop codon in the short form of human NK1R. In the cDNA of the short form of the human NK1R, the nucleotide sequence around the stop codon (CAGGTGAG) conforms to the consensus 5′ splice site in eukaryotic pre-mRNAs (24). Therefore, it is likely that the short form of the human NK1R is generated by omitting the splicing of the intron between exon 4 and exon 5 during the process of pre-mRNA splicing, provided that the human NK1R gene structure is very similar to that of the human NK2R and the rat NK1R. Isolation of the human NK1R gene will allow direct assessment of the splicing junctions. Because both isoforms of the human NK1R can be readily obtained from either glioblastoma mRNA or brain mRNA, it seems that such an alternative splicing pattern does not represent an abnormal physiological condition. The alternative splicing would generate a greater information-processing capacity in neuronal pathways involving neurokinins.

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