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The Unpredicted High Affinities of a Large Number of Naturally Occurring Tachykinins for Chimeric NK_1/NK_3 Receptors Suggest a Role for an Inhibitory Domain in Determining Receptor Specificity*

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Ye Tian, Lan-Hsin Wu, Dale L. Oxender, and Fu-Zon Chung‡

From the Department of Biotechnology, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Co., Ann Arbor, Michigan 48105-1047

Three chimeric receptors were constructed by exchanging exon sequences between human NK_1 and NK_3 receptor genes. The resulting chimeric receptors not only retained high affinities for their natural ligands substance P and neurokinin B but also exhibited surprisingly high affinities for other naturally occurring tachykinins including neurokinin A, neuropeptide K, neuropeptide γ, eledoisin, kassinin, physalaemin, and phyllomedusin. In contrast, these chimeric receptors displayed a wide range of variability in their affinities for non-naturally occurring ligands including selective agonists and antagonists of NK_1, NK_2, and NK_3 receptors. Since the only common feature among these naturally occurring neurokinin peptides is the conserved C-terminal sequences, our data suggest that these conserved sequences must play the major role in conferring high affinity binding to the chimeric receptors. To explain the apparently "improved" affinities of these naturally occurring ligands for the chimeric receptors as compared with their affinities for the parent NK_1 and NK3 receptors, we are proposing that certain inhibitory domains that are present in the NK_1 and/or NK_3 receptors are compromised in these chimeric receptors. Upon disruption of these inhibitory domains during the formation of chimeras, the naturally occurring ligands can interact more favorably with chimeric receptors through their conserved C-terminal sequences. Based on this hypothesis, the binding affinities of natural tachykinin ligands may be largely determined by their conserved C-terminal sequences, whereas receptor selectivities of these ligands are influenced more by the presence or absence of inhibitory domains rather than specific binding domains on their target receptors.

Substance P, neurokinin A (NKA),[1] and neurokinin B (NKB), the major mammalian neurokinin peptides, belong to the tachykinin family and share a common C-terminal sequence of -Phe-Xaa-Gly-Leu-Met-NH_2. There are at least three neurokinin receptor subtypes, NK_1, NK_2, and NK_3, that have been proposed to mediate the biological functions of these neurokinin peptides. Substance P has higher affinity to the NK_1 receptor than to the other two neurokinin receptor subtypes and is believed to act as an important neurotransmitter (1). The human NK_1 receptor is widely distributed in both central and peripheral nervous systems and has been proposed to be involved in many physiological and pathological conditions such as noxious stimuli, neurogenic inflammation, emesis, intestinal motility, vasodilation, smooth muscle contraction, salivary and airway secretion as well as immune response (1–3). The NK_2 receptor is widely distributed in the peripheral nervous system such as in the smooth muscles of the respiratory, gastrointestinal, and urinary tract. Among three tachykinins, NKA has the highest affinity to the NK_2 receptor. Activation of this receptor subtype results in facilitation of transmitter release, neuronal excitation, and stimulation of certain immune cells (3). NK_2 receptors have also been detected in the central nervous system. However, the exact function of this receptor in the central nervous system has yet to be delineated. Compared with the NK_1 and NK_2 receptors, much less is known about the biological function of the NK_3 receptor. NK_3 receptors are mainly distributed in the central nervous system (4), and NKB has the highest affinity to this receptor subtype. With the recent development of selective NK_3 antagonists (5, 6), it is expected that the biological function of the NK_3 receptor will be elucidated in the near future.

All three neurokinin receptor genes have been cloned (7–9). Based on the deduced protein sequences, neurokinin receptors belong to the G-protein-coupled receptor superfamily with the structural characteristics of seven transmembrane helices (TM). All three neurokinin receptors share a high degree of homology (10, 11). Careful examination of the gene structures of the neurokinin receptors has revealed that all three neurokinin receptors are encoded by a five-exon gene structure (7–9, 11). Furthermore, the exon/intron junction sites among all three neurokinin receptor genes are also fully conserved (9, 12). It is conceivable that all of the neurokinin receptor genes may have evolved from a common ancestor gene, and the protein sequences encoded by individual exons may serve as functional motifs for these receptors. If these assumptions are correct, then by shuffling exons among different receptor genes, it should be possible to create "new" tachykinin receptors that might have different pharmacological profiles than the existing tachykinin receptors. Based on this hypothesis, we constructed three chimeric receptors by shuffling exons between human NK_1 and NK_3 receptor genes (Fig. 1). The choice for using NK_1 and NK_3 receptors is based on the fact that these two tachykinin receptor subtypes have the most different pharmacological profiles among the three tachykinin receptors. In the present study, substance P, neurokinin A (NKA), NKB, as well as several nonmammalian tachykinin peptides (Fig. 2) were employed to examine their interactions with three chimeric recep-

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‡ To whom correspondence should be addressed: Dept. of Biotechnology, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Co., 2800 Plymouth Rd., Ann Arbor, MI 48105. Tel.: 313-998-5911; Fax: 313-998-5970; E-mail: chungf@aa.wl.com.

[1] The abbreviations used are: NKA, neurokinin A; NKB, neurokinin B; BHSP, Bolton-Hunter-labeled substance P; SP, substance P; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; TM, transmembrane.

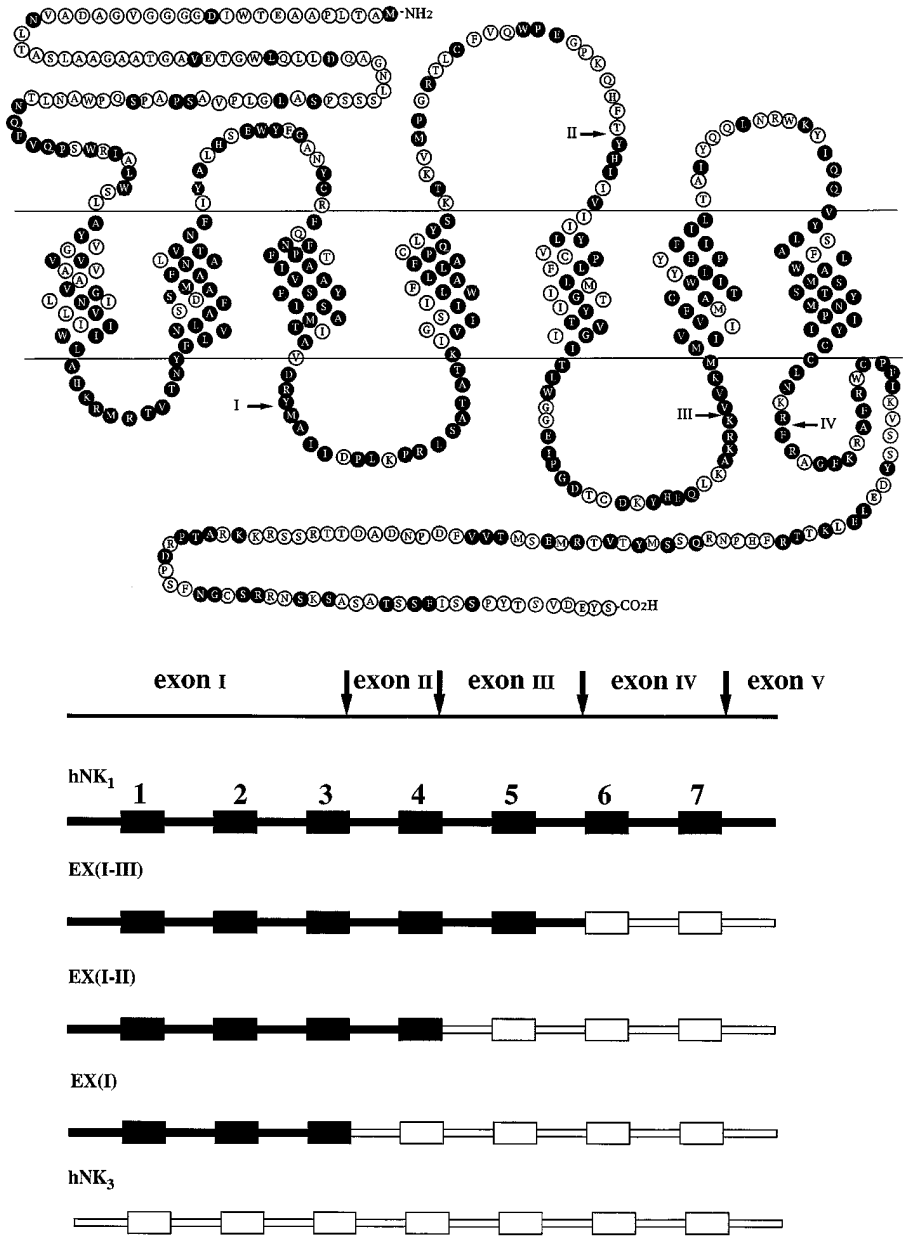

FIG. 1. **Proposed structure of the human NK_3 receptor and schematic representations of the human NK_1, NK_3, and three NK_1/NK_3 chimeric receptors.** *Upper panel,* the proposed structure of the human NK_3 receptor. *Solid circles with white letters* indicate amino acid residues conserved between the human NK_1 and NK_3 receptors. *Open circles with black letters* indicate amino acid residues specific for the human NK_3 receptor. Junctions between adjacent exons are indicated by *arrows* and are numbered in order. *Lower panel,* schematic representations of the human NK_1, NK_3, and three chimeric receptors. Seven transmembrane helices are presented as rectangular blocks with numbers corresponding to the TMI to TMVII. *Filled blocks* and *bars* represent human NK_1 sequences, and *open blocks* and *bars* represent NK_3 sequences. *Arrows* indicate junctions between adjacent exons.

tors constructed by exon shuffling. Furthermore, the binding characteristics of some highly selective peptide agonists and non-peptide antagonists that belong to each of three tachykinin receptor categories were also examined. Based on findings from these studies, a hypothesis involving inhibitory domains has been proposed to explain the unique binding properties observed in these chimeric receptors.

EXPERIMENTAL PROCEDURES

Materials—^{125}I-Bolton-Hunter-labeled substance P (^{125}I-BHSP), ^{125}I-NKA, ^{125}I-eledoisin, and [^{125}I-MePhe^7]NKB were purchased from DuPont NEN. Substance P, octa-substance P (SP 4–11), hexa-substance P (SP 6–11), penta-substance P (SP 7–11), NKA, NKB, eledoisin, neuropeptide K, neuropeptide-γ, kassinin, physalaemin, phyllomedusin, [Sar^9,Met(O$_2$)^{11}]SP, SPOMe, septide, GR64349, senktide, [Pro^7]NKB, and [MePhe^7]NKB were purchased from Peninsula Laboratories, Inc. (Belmont, CA). SR140,333, SR142,801 and SR48,968 were kindly provided by Dr. X. Emonds-Alt of Sanofi Recherché (France). [Ala$^{\beta 8}$]NKA_{4-10}, L-703,606, and L-659877 were purchased from RBI (Natick, MA). *myo*-[^{3}H]Inositol was purchased from Amersham Corp.

Cloning of the Human NK1 and NK3 Receptor Genes and Construction of NK1/NK3 Chimeric Receptors—Individual exons of human NK_1 or NK_3 receptor genes were amplified directly from the human genomic DNA (purchased from Clontech) using polymerase chain reaction. Oligonucleotides, 24–30-mers in length, corresponding to 5′ or 3′ ends of individual exons, were used as primers in the amplification reaction. Full-length human NK_1 or NK_3 receptor genes were assembled by connecting individual exons in a recombinant polymerase chain reaction using primers containing overlapping sequences of adjacent exons (13). Chimeric receptors, EX(I), EX(I–II), and EX(I–III), were constructed by shuffling exons between human NK_1 and NK_3 receptors. As depicted in Fig. 1, the N-terminal protein sequences of the constructed EX(I), EX(I–II), and EX(I–III) chimeric receptors are encoded by exon (1), exon (1–2), and exon (1–3) of the human NK_1 receptor gene, respectively. The remaining C-terminal sequences of these chimeric receptors were derived from corresponding exons of the human NK_3 receptor gene (Fig. 1). The authenticity of entire gene sequences was confirmed by dideoxy sequencing. The full-length human NK_1, NK_3 receptor, or recombinant chimeric receptor DNA sequences were inserted into an expression vector pRC/CMV. The recombinant plasmids were transfected into CHO cells by electroporation, and permanent cell lines were selected in the presence of G418 as described previously (14).

Receptor Binding Assay—CHO cells expressing either human NK_1, NK_3 receptors, or various chimeras were cultured in Ham's F-12 nutrient mixture supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Cells were seeded to 96-well Wallac (Gaithersburg, MD) rigid cross-talk corrected cell culture plate 1 day before the experiment.

Mammalian Tachykinins

SP	Arg-Pro-Lys-Pro-Gln-Gln-**Phe**-Phe-**Gly** -**Leu**-Met-**NH2**
NKA	His-Lys-Thr-Asp-Ser-**Phe**-Val-**Gly** -**Leu**-Met-**NH2**
NKB	Asp-Met-His-Asp-Phe-**Phe**-Val-**Gly** -**Leu**-Met-**NH2**
Neuropeptide K	Asp-Ala-Asp-Ser-Ser- Ile-Glu-Lys-Gln-Val-Ala-Leu-Leu-Lys-Ala-Leu-Tyr-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-**Phe**-Val-**Gly** -**Leu**-Met-**NH2**
Neuropeptide γ	Asp-Ala-Gly-His-Gly-Gln-Ile-Ser-His-Lys-Arg-His-Lys-Thr-Asp-Ser-**Phe**-Val-**Gly** -**Leu**-Met-**NH2**

Non-mammalian Tachykinins

Eledoisin	pGlu-Pro-Ser-Lys-Asp-Ala-**Phe**-Ile-**Gly** -**Leu**-Met-**NH2**
Kassinin	Asp-Val Pro-Lys-Ser-Asp-Gln-**Phe**-Val-**Gly** -**Leu**-Met-**NH2**
Physalaemin	pGlu-Ala-Asp-Pro-Asn-Lys-**Phe**-Tyr-**Gly** -**Leu**-Met-**NH2**
Phyllomedusin	pGlu-Asn-Pro-Asn-Arg-**Phe**-Ile-**Gly** -**Leu**-Met-**NH2**

FIG. 2. **Sequence of naturally occurring tachykinins.**

On the day of each experiment, cells were washed twice with phosphate-buffered saline (PBS), and appropriate agonists or antagonists were added and incubated in 0.2 nM ^{125}I-labeled ligand in PBS containing 0.4 mg/ml bovine serum albumin, 0.08 mg/ml bacitracin, 0.004 mg/ml chymostatin, 0.004 mg/ml leupeptin, 1 μM thiorphan, 25 μM phosphoramidon, and 2 mM $MnCl_2$. The cells were incubated for 1 h at room temperature, and the reactions were terminated by two washes with ice-cold PBS. Fifty μl of 2% SDS followed by 175 μl of Ready Gel (Beckman) were added to each well. Plates were vortexed, and the radioactivity was quantified in a Wallac 1450 microbeta scintillation counter. Nonspecific binding was determined in the presence of 1 μM unlabeled corresponding ligand. Receptor binding data were analyzed with nonlinear curve fitting using KaleidaGraph software package (PCS Inc, Reading, PA). IC_{50} values were determined using a modified Hill equation, % inhibition = {cpm(L) − cpm(1 μM cold ligand)}/{cpm (0) − cpm(1 μM cold ligand)} = $L^n/(IC_{50}^n + L_n)$, where cold ligand represents unlabeled ligand, L represents the concentration of unlabeled ligand, *n*, the Hill coefficient, and IC_{50}, the concentration of unlabeled ligand that causes 50% inhibition of the total specific binding of 0.2 nM radiolabeled ligand.

Measurement of Phosphatidylinositol Turnover—Agonist-induced phosphatidylinositol turnover was estimated by measuring inositol phosphate's accumulation in CHO cells expressing selective receptors as described previously (15). Briefly, cells (10,000/well) were seeded in 96-well cell culture plates 24 h before changing medium to Eagle's minimal essential medium/F-12 (with Earle's salt, with glutamine; Life Technologies, Inc.) containing 10 μCi/ml [^{3}H]inositol. After overnight incubation with [^{3}H]inositol, medium was removed, and cells were washed twice with assay buffer (minimal essential medium with 10 mM LiCl, 20 mM HEPES, and 1 mg/ml bovine serum albumin). Cells were then incubated with various concentrations of agonists for 1 h. Reactions were stopped by two washes with ice-cold PBS followed by the addition of 0.1 ml of ice-cold 5% trichloroacetic acid to each well. The trichloroacetic acid extract was applied to a cation exchange column containing AG 1-X8 resin (Bio-Rad) and washed three times with 5 mM myo-inositol. Inositol phosphate was eluted with 1 M ammonium formate, 0.1 M formic acid. Radioactivity was determined by liquid scintillation counting. Data were analyzed with nonlinear curve fitting using KaleidaGraph software package (PCS Inc, Reading, PA). EC_{50} values were determined using equation % maximal effect = {cpm (L) − cpm (0)}/{cpm(1 μM substance P) − cpm (0)} = L/(EC_{50} + L), where 0 represents the background count in the absence of agonist, L represents agonist concentration, and EC_{50} the concentration of agonist that causes half of the maximal effect.

TABLE I

K_d and B_{max} values of ^{125}I-BHSP binding to human NK_1, chimeric EX(I–III), EX(I–II), and EX(I) receptors and K_d and B_{max} values of [^{125}I-MePhe^7]NKB binding to human NK_3 receptors

Binding isotherms were determined as described under "Experimental Procedures." K_d and B_{max} values were determined by Scatchard analysis. The results are representative of at least three independent experiments.

Receptors	K_d	B_{max}
	nM	*receptors/cells*
Human NK_1	0.47 ± 0.01	9.1 ×10^5
EX(I–III)	1.22 ± 0.03	7.7 ×10^5
EX(I–II)	0.16 ± 0.01	1.6 ×10^5
EX(I)	0.36 ± 0.03	1.8 ×10^5
Human NK_3	5.4 ± 0.4	1.03×10^6

RESULTS

Binding Characteristics of the Human NK_1 and NK_3 Receptors—Human NK_1 and NK_3 receptors were expressed in CHO cells at high levels as indicated by their B_{max} values listed in Table I. Substance P and [MePhe^7]NKB exhibited high affinity to human NK_1 and NK_3 receptors (Table I), respectively, as have been previously reported (3, 14, 16, 17). Chimeric receptors, *i.e.* EX(I–III), EX(I–II) and EX(I), were also expressed at high levels in CHO cells. All three chimeric receptors exhibited high affinities for substance P with K_d values in nano- or subnanomolar range (Table I). Subsequently, ^{125}I-BHSP was used as the primary radioligand to examine binding affinities of other tachykinin peptides to these chimeric receptors. The high affinities of substance P to EX(I–III), EX(I–II), and EX(I) chimeric receptors also suggest that the structural integrity has been largely maintained in these chimeric receptors constructed by exon shuffling.

Binding of Naturally Occurring Tachykinin Peptides to NK1/NK3 Chimeric Receptors—In addition to substance P, NKA and NKB also demonstrated high potencies in displacing ^{125}I-BHSP binding to EX(I–III), EX(I–II), and EX(I) chimeric receptors (Fig. 3). In contrast, NKB and NKA were relatively weak in displacing ^{125}I-BHSP binding to human NK_1 receptors, whereas substance P and NKB were relatively weak in displacing [MePhe^7]NKB binding to human NK_3 receptors (Fig. 3). Since all three chimeric receptors contain sequences encoded by exon 1 of the NK_1 receptor gene as well as exon IV–V of the NK_3 receptor gene, the high affinity binding of substance P and NKB to these chimeras could be attributed to the conservation of these sequences in the chimeras. However, the high potencies of NKA in displacing ^{125}I-BHSP binding to these chimeric receptors were unexpected. Since these chimeric receptors were constructed using only human NK_1 and NK_3 receptor sequences, the high affinities of NKA to these receptors suggested that some residues conserved among NK_1, NK_2, and NK_3 receptors are probably involved. NKA shares a highly conserved C-terminal pentapeptide sequence with substance P and NKB (Fig. 2), and it is possible that the C-terminal sequence of NKA plays a major role in determining its binding affinities to these chimeric receptors. To further study this possibility, several other nonmammalian tachykinin peptides with variable N-terminal sequences were examined for their affinities to EX(I–III), EX(I–II), and EX(I) chimeric receptors. As shown in Table II, all naturally occurring tachykinins examined displayed high affinities to these chimeric receptors. Physalaemin and phyllomedusin, which have high affinities for the human NK_1 receptor and low affinities to the human NK_3 receptor, displayed high affinities for all three chimeric receptors with IC_{50} values in the nano- or subnanomolar range (Table II). Eledoisin, kassinin, neuropeptide K, and neuropeptide γ, which have relatively low affinities for either human

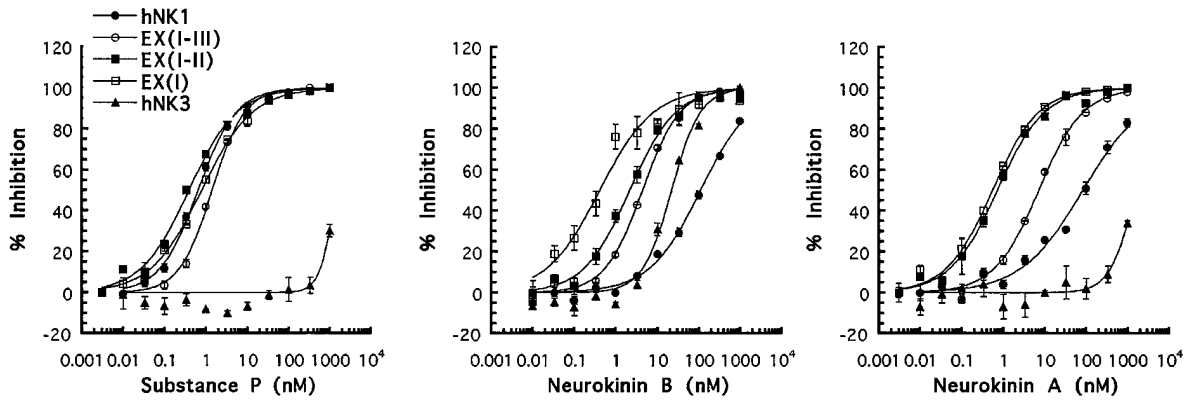

FIG. 3. **Competition binding isotherms of substance P, neurokinin A, and neurokinin B to human NK_1, NK_3, and three chimeric receptors.** ^{125}I-BHSP (0.2 nM) was used to label human NK_1 and chimeric receptors, and [^{125}I–MePhe^7]NKB was used to label human NK_3 receptors as described under "Experimental Procedures." Data were presented as a percent inhibition of specific binding of 0.2 nM ^{125}I-BHSP or [^{125}I-MePhe^7]NKB. The results were representative of three to five independent experiments. Each point represents the mean of triplicate experiments and *vertical bar* the standard error of the mean.

TABLE II

IC_{50} values of some natural tachykinin peptides on ^{125}I-BHSP binding to human NK_1, chimeric EX(I–III), EX(I–II), and EX(I) receptors and on [^{125}I-MePhe^7]NKB binding to human NK_3 receptors

Competition binding curves were determined as described under "Experimental Procedures." IC_{50} values were calculated as the concentrations that caused 50% inhibition of the specific binding of 0.2 nM ^{125}I-BHSP to human NK_1 and three chimeric receptors or 0.2 nM [^{125}I-MePhe^7]NKB to human NK_3 receptors. The results are representative of at least three independent experiments.

	IC_{50} (nM)				
	$hNKI_1$	EX(I–III)	EX(I–II)	EX(I)	hNK_3
Substance P	0.80 ± 0.01	1.40 ± 0.09	0.52 ± 0.06	0.79 ± 0.03	>1000
NKA	84.3 ± 11.1	7.2 ± 0.5	6.5 ± 1.1	2.3 ± 0.4	>1000
NKB	111 ± 10	4.4 ± 0.2	2.2 ± 0.3	0.59 ± 0.04	17.9 ± 2.0
Eledoisin	25.0 ± 4.1	3.82 ± 0.38	2.67 ± 0.20	0.16 ± 0.01	486 ± 74
Kassinin	170 ± 85	9.63 ± 0.60	4.71 ± 0.51	0.103 ± 0.007	150 ± 52
NP-α	25.2 ± 3.0	3.36 ± 0.33	1.80 0.14	0.84 ± 0.07	NB
NP-K	331 ± 35	9.38 ± 1.26	3.53 ± 0.33	1.48 ± 0.11	NB
Physalaemin	0.75 ± 0.06	0.96 ± 0.07	0.218 ± 0.009	0.16 ± 0.01	>1000
Phyllomedusin	3.62 ± 0.50	1.77 ± 0.50	0.84 ± 0.04	0.30 ± 0.04	>1000

NK_1 or NK_3 receptors (11, 18), also demonstrated high affinities for these chimeric receptors (IC_{50} values in the nano- or subnanomolar range, Table II). Similar results were obtained using either ^{125}I-NKA or ^{125}I-eledoisin as radioligands (data not shown). Since all eight naturally occurring tachykinin peptides demonstrated similar high affinities for EX(I–III), EX(I–II), and EX(I) chimeric receptors but contain very different N-terminal sequences, it seems to suggest that the C-terminal common sequences largely determine the binding affinities of these tachykinin peptides to the chimeras.

To confirm the high affinities of these peptides for chimeric receptors, direct binding assay using radiolabeled NKA and eledoisin was carried out. K_d and B_{max} values of ^{125}I-NKA and ^{125}I-eledoisin binding to all three chimeric receptors are listed in Table III. Data derived from direct binding experiments are consistent with data derived from the competition experiments using ^{125}I-BHSP (Table II).

Agonist-induced Phosphatidylinositol Turnover—Since human NK_1 and NK_3 receptors expressed in CHO cells have been shown to be functionally coupled to the phospholipase C signal transduction pathway (19, 20), the potencies of substance P, NKA, and NKB to induce phosphatidylinositol turnover in CHO cells expressing EX(I–III), EX(I–II), and EX(I) chimeric receptors as well as NK_1 and NK_3 receptors were examined. As has been reported, substance P was very potent (EC_{50}, 0.66 ± 0.11 nM) in stimulation of phosphatidylinositol turnover in CHO cells expressing human NK_1 receptors (Fig. 4). NKA and NKB were also potent in stimulating human NK_1 receptors (EC_{50}, 4.4 ± 0.6 and 7.8 ± 1.0 nM, respectively), although not as potent as substance P. On the other hand, NKB was more potent (EC_{50}, 3.8 ± 0.5 nM) than substance P or NKA (EC_{50}, 219 ± 42 and 87 ± 12 nM, respectively) in stimulating phosphatidylinositol turnover in cells expressing human NK_3 receptors (Fig. 4), which was also consistent with the binding data and the notion that NKB is the preferred ligand for this receptor subtype. Consistent with the binding data, substance P, NKA, and NKB demonstrated high potencies in stimulating phosphatidylinositol turnover in cells expressing EX(I–III) chimeric receptors with EC_{50} values of 8.8 ± 0.8, 32.0 ± 7.1, and 33.6 ± 9.1 nM, respectively (Fig. 4), and in cells expressing EX(I–II) receptors (EC_{50}, 0.24 ± 0.04, 2.0 ± 0.4, and 7.2 ± 1.6 nM, respectively). The EX(I) chimeric receptor is also functionally coupled to the phospholipase C signal transduction pathway. However, due to the unusual high background in unstimulated cells, stimulation of CHO cells expressing this chimeric receptor resulted in less than 2-fold increase in phosphatidylinositol turnover (data not shown). Data from the func-

TABLE III

K_d and B_{max} values of ^{125}I-NKA and ^{125}I-eledoisin binding to chimeric EX(I–III), EX(I–II), and EX(I) receptors

K_d and B_{max} values of ^{125}I-NKA and ^{125}I-eledoisin binding to each individual chimeric receptor were determined as described under "Experimental Procedures." The results are representative of at least three independent experiments.

Receptor	^{125}I-NKA		^{125}I-Eledoisin	
	K_d, nM	B_{max} receptors/cell	K_d, nM	B_{max} receptors/cell
EX(I–III)	1.62 ± 0.06	11.8×10^5	2.69 ± 0.29	31.2×10^5
EX(I–II)	0.52 ± 0.05	2.8×10^5	0.96 ± 0.09	6.2×10^5
EX(I)	0.35 ± 0.04	1.3×10^5	0.40 ± 0.04	5.0×10^5

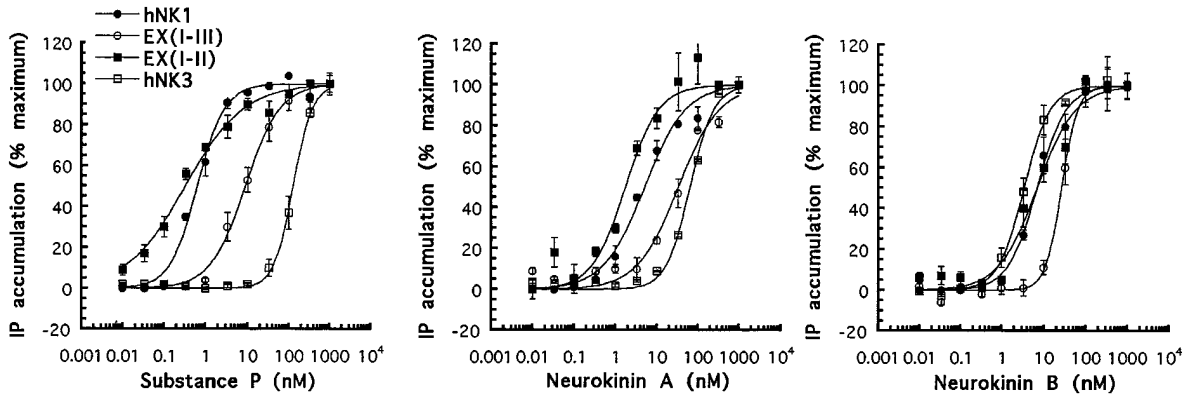

FIG. 4. **Agonist-induced inositol phosphate accumulation in CHO cells expressing human NK_1, NK_3, or chimeric receptors.** Substance P, neurokinin A-, and neurokinin B-induced phosphatidylinositol turnover were measured in cells expressing human NK_1, NK_3, or chimeric EX(I–III), EX(I–II), and EX(I) receptors as described under "Experimental Procedures." Data are expressed as a percentage of the maximal stimulation by 1 μM substance P. Each point represents the mean of triplicate experiments and *vertical bar* the standard error of the mean.

tional assays suggest that all three chimeric receptors not only can interact with tachykinin ligands but also are capable of coupling to the phospholipase C signal transduction pathway.

Binding of [MePhe^7]NKB to Chimeric Receptors—[MePhe^7]NKB is a potent and highly selective agonist for the NK_3 receptor and has been widely used in radiolabeling this receptor subtype (16, 21). Competition binding assays using [^{125}I-MePhe^7]NKB as the tracer to study ligand-receptor interaction with EX(I–III), EX(I–II), and EX(I) chimeric receptors show poor labeling (data not shown). Direct comparison of [^{125}I-MePhe^7]NKB binding to the wild-type human NK_3 and to EX(I–II) receptors was carried out to examine the binding characteristics of [^{125}I-MePhe^7]NKB for these receptors. [^{125}I-MePhe^7]NKB displayed high affinity binding to human NK_3 receptors with K_d of 2.8 ± 0.2 nM (Fig. 5). Functionally, [MePhe^7]NKB was potent in stimulating phosphatidylinositol turnover in cells expressing the human NK_3 receptor. On the other hand, the natural ligand NKB displayed relatively low potency in displacing [^{125}I-MePhe^7]NKB binding to the human NK_3 receptor (IC_{50}, 23.0 ± 2.4 nM; Fig. 5) and in stimulating phosphatidylinositol turnover in cells expressing the human NK_3 receptor (Fig. 4; EC_{50} of 18.5 ± 1.9 nM). [MePhe^7]NKB, therefore, displayed an order of magnitude higher affinity for the human NK_3 receptor than that found in NKB. Examination of the binding of [MePhe^7]NKB and NKB to EX(I–II) chimeric receptors revealed that the relative potencies of two compounds are completely reversed in the chimeric receptor. For instance, NKB displaced ^{125}I-BHSP binding to EX(I–II) receptors with an IC_{50} value of 6.3 ± 0.8 nM, whereas [MePhe^7]NKB has an IC_{50} value of 165 ± 22 nM. Similarly, NKB displayed a potency of 7.2 ± 1.6 nM (EC_{50}) in stimulating phosphatidylinositol turnover in cells expressing EX(I–II) receptors, and in the same experiment [MePhe^7]NKB had an EC_{50} of 71.7 ± 25 nM. Based on these studies, ^{125}I-eledoisin instead of [^{125}I-MePhe^7]NKB was used as the radioligand to characterize the binding of NK_3 ligands to chimeric receptors.

Receptor Binding Characteristics of Selective Agonists and Antagonists—Although ^{125}I-BHSP, ^{125}I-NKA, and ^{125}I-eledoisin have been widely used as radioligands to characterize NK_1, NK2, and NK_3 receptors, respectively, it has not been possible to characterize the same receptor using all three radioligands as a result of their differences in affinities for different receptor subtypes. Since all three radioligands displayed high affinities to these chimeric receptors, it was possible to characterize a chimeric receptor using any of the three radioligands. In the following experiments, the binding characteristics of some highly selective agonists and antagonists to each subtype of tachykinin receptors were studied using the best of choice of radioligand for these chimeric receptors.

Three highly selective NK_1 agonists, [Sar^9,Met(O$_2$)^{11}]SP, SPOMe, and septide, were characterized for their affinities for chimeric receptors using ^{125}I-BHSP. The inhibition of ^{125}I-BHSP binding to chimeric receptors by these agonists indicated very different interaction patterns (Table IV). The potency of [Sar^9,Met(O$_2$)^{11}]SP for inhibition of ^{125}I-BHSP binding remained relatively high for EX(I–III) and EX(I–II) but was very low for EX(I) chimeric receptors (Table IV), suggesting that the N-terminal NK_1 receptor sequence, especially sequences that include TMIV, may play an important role in the interaction of this highly selective NK_1 agonist with the human NK_1 receptor. SPOMe, a carboxyl-terminal-modified substance P derivative, showed little affinity for all three chimeric receptors (Table IV), indicating that the interaction sites for this selective substance P derivative are quite different from that for substance P. It seems to suggest that some nonconserved amino acids in the C terminus (TMVI and TMVII) of the human NK_1 receptor may contribute to the specific interaction of SPOMe with the human NK_1 receptor, since replacement of this sequence with its counterpart of the human NK_3 receptor resulted in a total loss of binding for SPOMe. Due to its high potency in functional assays and low potency in displacing radiolabeled SP, septide appears to interact with NK_1 receptors differently from that of substance P (22). As shown in Table IV, sequential addition of sequences encoded by exon 2 and exon 3 of the NK_1 receptor (as in EX(I–II) and EX(I–III)) actually reduced instead of increased their abilities to interact with septide, indicating a complex interaction profile for this NK_1 agonist. SR140,333 and L703,606 are highly selective non-peptide NK_1 antagonists. Both antagonists demonstrated no binding to all three chimeric receptors (Table IV), suggesting that the major interaction sites for these selective non-peptide NK_1 antagonists may not be present in these chimeric receptors. Direct binding assay using ^{125}I-L703,606 or [^{3}H]SR140,333 also confirmed the lack of binding for these two NK_1 antagonists to chimeric EX(I–III), EX(I–II), and EX(I) receptors (data not shown).

The binding affinities of selective NK_3 agonists ([MePhe^7]NKB, NKB, senktide, and Pro^7-NKB) and selective antagonists (PD157, 672, and SR142, 801) to chimeric receptors were also studied using ^{125}I-eledoisin. Although all of the NK_3 agonists examined exhibited high affinities for EX(I) chimeric receptors, [MePhe^7]NKB, senktide, and Pro^7-NKB demonstrated lower affinity to the other two chimeric receptors (Table IV). In contrast, NKB and eledoisin, the two natural

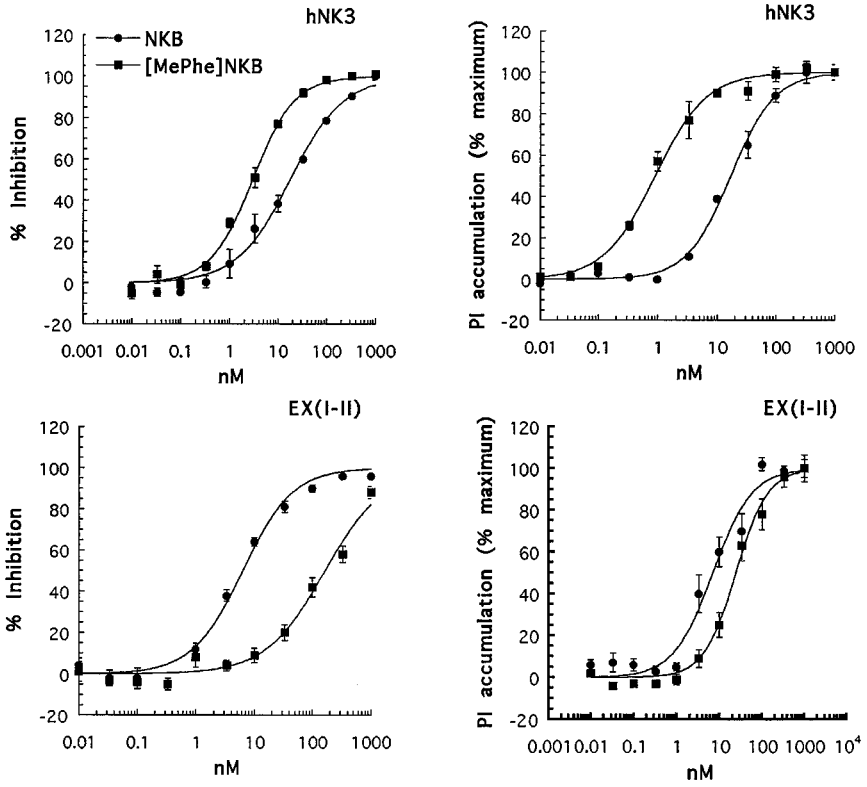

FIG. 5. **Comparison between NKB and [MePhe^7]NKB in their binding affinities and functional potencies for chimeric receptors expressed in CHO cells.** The competition binding isotherms of NKB or [MePhe^7]NKB to human NK_3 or EX(I–II) chimeric receptors were determined as described under "Experimental Procedures." Data were presented as a percentage inhibition of the specific binding of 0.2 nM [^{125}I-MePhe^7]NKB to human NK_3 receptors or 0.2 nM ^{125}I-BHSP to EX(I–II) receptor by NKB or [MePhe^7]NKB. The agonist potencies were determined by measuring the NKB or [MePhe^7]NKB-induced phosphatidylinositol turnover in CHO cells expressing either human NK_3 or EX(I–II) receptors. Results were representative of three independent experiments. Each point represents the mean of triplicate experiments and *vertical bar* the standard error of the mean.

TABLE IV

IC_{50} values of selective tachykinin agonists and antagonists on ^{125}I-BHSP, ^{125}I-NKA, or ^{125}I-eledoisin binding to human NK_1, NK_3, and chimeric EX(I–III), EX(I–II), and EX(I) receptors

Competition binding curves determined as described under "Experimental Procedures." 0.2 nM of ^{125}I-BHSP, ^{125}I-NKA, or ^{125}I-eledoisin were used as the radioligand selection for NK_1, NK_2, or NK_3 ligands, respectively. IC_{50} values were calculated as the concentrations that caused 50% inhibition of the specific binding of radiolabeled compounds. The results are representative of at least two independent experiments. NB, no binding.

	IC_{50} (nM)				
	hNK_1	EX(I–III)	EX(I–II)	EX(I)	hNK_3
BHSP					
Sar,Met-SP	0.16 ± 0.02	2.04 ± 0.13	6.47 ± 1.10	199.7 ± 18.4	
SPOMe	37.5 ± 3.7	NB	401 ± 84	NB	
Septide	120 ± 10	850.8 ± 81.2	561 ± 103	166.2 ± 10.5	
SR140,333	1.03 ± 0.10	NB	>1000	NB	NB
L-703,606	5.0 ± 0.6	NB	NB	NB	NB
NKA					
NKA	84.3 ± 11.1	1.80 ± 0.10	0.72 0.06	0.62 ± 0.04	>1000
Ala$^{\beta 8}$NKA		>1000	466 ± 24	55.1 ± 5.2	
GR64349		173 ± 88	150 ± 33	17.4 ± 2.6	
SR48968	>1000	492 ± 70	358 ± 34	>100	764 ± 95
L-659877		>1000	>1000	>>1000	
ELD					
MePhe^7-NKB	NB	47.2 ± 3.4	41.2 ± 3.8	1.46 ± 0.14	2.98 ± 0.23
Senktide	NB	109.1 ± 46.5	176 ± 111	2.59 ± 0.51	29.3 ± 5.6
Eledoisin	25.0 ± 4.1	2.89 ± 0.29	1.16 ± 0.09	0.60 ± 0.04	486 ± 74
NKB	109 ± 14	1.62 ± 0.19	0.48 ± 0.07	0.41 ± 0.08	23.0 ± 2.4
Pro^7-NKB	NB	>1000	675 ± 400	73.6 ± 20	335 ± 74
PD157,672		NB	>>1000	>1000	12.1 ± 0.83
SR142,801	NB	945 ± 144	121.3 ± 34.8	12.1 ± 2.6	2.04 ± 0.18

occurring tachykinin peptides, exhibited high potencies in displacing ^{125}I-eledoisin binding to all three chimeric receptors (Table IV), which is consistent with the previous results obtained using ^{125}I-BHSP as the radioligands (Table II). The discrepancy in binding affinities between natural and non-natural tachykinin peptides may be explain by the fact that unlike NKB and eledoisin, [MePhe^7]NKB, senktide, and Pro^7-NKB all have modified C-terminal pentapeptide sequences. Although they have high affinities to the wild-type human NK_3 receptor and demonstrated high selectivity, their interaction sites with the NK_3 receptor may be different from that of the natural tachykinin peptides. Additional interaction sites for these modified tachykinin agonists may have been lost in EX (I–II) and EX(I–III) chimeric receptors. PD157,672, a highly selective and potent antagonist, also failed to bind to any chimeric receptors. Since antagonist may not necessarily share the same interaction sites with agonist and PD157,672 is a relatively small molecule as compared with that of the natural ligands, the binding site(s) of PD157,672 could be located in a more restricted region that may have been lost in these chimeric receptors. Another highly selective and potent NK3 antagonist SR142,801(23), on the other hand, retained high af-

TABLE V
IC_{50} values of substance P and its C-terminal fragments on ^{125}I-BHSP binding to human NK_1, chimeric EX(I–III), EX(I–II), and EX(I) receptors

Competition binding curves were determined as described under "Experimental Procedures." IC_{50} values were calculated as the concentrations that caused 50% inhibition of the specific binding of 0.2 nM ^{125}I-BHSP to human NK_1 and three chimeric receptors. The results are representative of at least three independent experiments. NB, no binding.

	IC_{50} (nM)			
	hNK_1	EX(I–III)	EX(I–II)	EX(I)
Substance P	1.29 ± 0.09	1.36 ± 0.17	0.62 ± 0.02	0.91 ± 0.16
Sub P (4-11)	15.0 ± 4.4	2.93 ± 0.19	1.40 ± 0.13	0.73 ± 0.03
Sub P (6-11)	427 ± 79	10.7 ± 2.1	2.82 ± 0.18	1.15 ± 0.15
Sub P (7-11)	NB	NB	1043 ± 257	104 ± 23

finity to the EX(I) chimeric receptor and gradually decreased its affinities to EX(I–II) and EX(I–III) chimeric receptors (Table IV), confirming the previous observation that this antagonist may have more scattered interaction sites than that of PD157,672 (5).

Selective NK_2 agonists Ala$^{\beta 8}$-NKA and GR64349 displayed similar binding profiles as that of selective NK_3 agonist in displacing ^{125}I-NKA binding to all three chimeric receptors. Both Ala$^{\beta 8}$-NKA and GR64349 exhibited relatively high affinities to EX(I) receptors and gradually decreased their affinities to EX(I–II) and EX(I–III) receptors, suggesting that the sequences encoded by exon 2 and exon 3 of the NK_3 receptor gene may play important roles in the interaction of these agonists with the chimeras. Non-peptide NK_2 antagonist L-659877 failed to displace ^{125}I-NKA binding to all three chimeric receptors, whereas SR48,968 displayed only modest affinities (Table IV) to all three chimeric receptors as compared with its affinity to the human NK_2 receptor (14), suggesting that the high affinity binding sites for L-659877 and SR48,968 may not be present in these chimeric receptors.

Binding of Carboxyl-terminal Fragments of Substance P to Chimeric Receptors—The data in Table II and Table IV seem to suggest that the unmodified C-terminal pentapeptide sequences are essential for high affinity binding of various tachykinin ligands to the chimeric receptors. To further evaluate the importance of the conserved sequence, three C-terminal fragments of substance P were examined for their affinities for the chimeric receptors. As shown in Table V, SP 7–11 showed no binding to NK_1 or EX(I–III) receptors but displayed modest affinities for EX(I) and EX(II) receptors. SP 6–11 displayed low affinity for NK_1 receptor, but its affinities for the chimeric receptors are in the nanomolar range. The affinities of SP 4–11 for the chimeric receptors are similar to that found for substance P. These data confirm the importance of the conserved C-terminal pentapeptide sequence in determining binding affinities of tachykinin peptides for the chimeric receptors. The difference in binding affinity between SP 7–11 and SP 6–11 for the chimeric receptors, however, suggests that in order for the conserved C-terminal sequence of substance P to be fully active, addition of at least one extra amino acid residue at its N-terminal end is required.

DISCUSSION

In this study, three chimeric receptors were constructed based on the highly conserved structural arrangement of mammalian neurokinin receptor genes. An unexpected finding was that of nine naturally occurring tachykinin peptides examined, *i.e.* substance P, NKA, NKB, eledoisin, kassinin, neuropeptide K, neuropeptide γ, physalaemin, and phyllomedusin, all demonstrated high affinity binding to these chimeric receptors with K_d values in the nano- or subnanomolar range. Most of these natural tachykinin peptides displayed higher affinities for the chimeric receptors than for their parent human NK_1 or NK_3 receptors. For chimeras constructed between rat NK_1 and NK_3 receptors, Gether *et al.* (24) have also observed a similar increase in affinities of NKA and eledoisin (24).

Based on the "message and address" hypothesis (25, 26), it has been proposed that the highly conserved C-terminal sequence -Phe-Xaa-Gly-Leu-Met-NH_2 of all neurokinin peptides may serve as the "message," which can relay the signal to the receptor and result in receptor-mediated intracellular signal transduction. On the other hand, the N-terminal highly variable sequences in each neurokinin peptide may serve as the "address," with which the selectivity of individual neurokinin peptides is determined (25, 26). Based on this hypothesis, extensive receptor mutagenesis studies have been conducted in search of specific receptor sequences that may interact directly and specifically with the address portion of naturally occurring neurokinin peptides (27). However, most of the receptor sequences identified to be important for its natural ligand binding are sequences conserved among different receptor subtypes (28, 29), indicating that these receptor sequences may be interacting with the message instead of the address portion of the neurokinin peptides.

To explain our data with the message and address theory, we would have to assume that several new high affinity binding sites specific for various address sequences were created simultaneously in these chimeric receptors. It seems very unlikely that these high affinity binding sites can be created randomly by combining two receptor sequences that contain only low affinity binding sites for these ligands. An alternative interpretation of our data from an evolutionary point of view seems attractive. Based on the highly conserved gene structure of neurokinin receptors, it may be hypothesized that these receptors have evolved from the same ancestral gene. The primordial neurokinin receptor binds to the primordial peptide by interacting with its C-terminal pentapeptide sequence. During the course of evolution, different neurokinin receptor subtypes as well as different neurokinin peptides may have diverged from their common ancestor; however, the common binding sites for the primordial peptide to different subtypes of neurokinin receptors might have been preserved during the course of evolution. Selectivity for individual receptor subtypes for their preferred peptide ligand may have evolved not by creating additional binding sites for each specific ligand but by acquiring steric hindrances (most likely located in the extracellular loops) that will allow only the preferred ligands to interact with the common binding site. These steric hindrances or inhibitory domains could result from the collective effect of many or just a few amino acid residues that can form spatial obstacles to prevent other peptides from reaching the common interaction site. Based on this hypothesis, the high affinity binding of these naturally occurring tachykinin peptides to the chimeric receptors can be simply explained by the disruption of the inhibitory domains in these chimeras, which then allows other natural ligands containing the conserved C-terminal sequence to interact favorably with the common binding site. This hypothesis may also explain the difficulties in trying to find selective natural ligand interaction sites in each receptor subtype. Mutagenesis results indicate that amino acid residues critical for neurokinin binding to NK_1 or NK_2 receptors are mostly conserved among the three neurokinin receptors and are located in the transmembrane domains (28, 29). Consistent with our hypothesis, the existence of distinct negative determinants that restrict the ligand-receptor interaction has also been reported in receptors for the luteinizing hormone and follicle-stimulating hormone (30). In the case of neurokinin receptors, construc-

tion of chimeric receptors by exon shuffling may have reversed the evolutionary process by removing these inhibitory domains. As a result, not only substance P and NKB but also six other tachykinin peptides all displayed high and similar affinities to these chimeric receptors. According to this hypothesis, it may be further postulated that the inhibitory domains for substance P and NKA would be located in the N-terminal region encoded by the exon 1 of the human NK_3 receptor gene. This conclusion is supported by the fact that replacement of these sequences with their corresponding sequences of the human NK_1 receptor resulted in an increase in binding affinities of substance P to these chimeric receptors. Using the same argument, the inhibitory domains for NKB may also be postulated to be located in the C-terminal region encoded by exon 4–5 of the human NK_1 receptor gene. Since exon 5 only encodes the C-terminal sequence that is believed to be located intracellularly (Fig. 1), the inhibitory domains for NKB can be further confined to the region encoded by exon 4 of the human NK_1 receptor gene. For other naturally occurring tachykinin peptides, various inhibitory domains located in the N-terminal region of NK_1 and/or C-terminal region of NK_3 receptor(s) may be involved in preventing these peptides from interacting optimally with the common binding site.

The results from studying the binding characteristics of several non-natural selective agonists and antagonists in these chimeric receptors are also quite informative. All of the selective agonists with modified C terminus have lower affinities for these chimeric receptors than that of their natural counterparts, indicating the importance of having the intact conserved C terminus to be fully recognized by the common binding site (Table IV). With the exception of SR48,968 and SR142,801, all antagonists examined show little or no binding to these chimeric receptors (Table IV). The lack of binding of antagonists may indicate that these inhibitory domains are likely the favorable targets for antagonist binding. This observation may also have important pharmaceutical implications in the future design and development of new tachykinin antagonists. Binding studies involving substance P fragments also confirmed the importance of the conserved C-terminal sequence in determining binding affinities to the chimeric receptors (Table V). The N-terminal sequence of substance P, although it may not be directly involved in receptor binding, is probably very important in maintaining the proper conformation of the C-terminal peptide as is evident by the poor binding of SP 7–11 to the chimeric receptors (Table V).

In summary, functional chimeric receptors were constructed by shuffling exons between the human NK1 and NK3 receptor genes. High affinity binding of nine naturally occurring tachykinin peptides to these chimeric receptors suggests that there may be a common binding pocket for all neurokinin peptides. The following hypothesis was proposed to explain our data. The binding affinities of natural ligands are largely determined by the interaction of their C-terminal conserved sequences with a common binding site in these receptors, whereas the selectivities of natural ligands are mostly influenced by the presence or absence of inhibitory domains that serve as negative determinants to prevent ligand-receptor interaction.

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REFERENCES

1. Regoli, D., Boudon, A., and Fauchere, J. L. (1994) *Pharmacol. Rev.* **46,** 551–599
2. Mantyh, P. W. (1991) *Ann. N. Y. Acad. Sci.* **632,** 263–271
3. Patacchini, R., and Maggi, C. A. (1995) *Arch. Int. Pharmacodyn. Ther.* **329,** 161–184
4. Bannon, M. J., Brownschidle, L. A., Tian, Y., Whitty, C. J., Poosch, M. S., Dsa, C., and Moody, C. A. (1995) *Brain Res.* **695,** 19–24
5. Chung, F.-Z., Wu, L.-H., Tian, Y., Vartanian, M. A., Lee, H., Bikker, J., Humblet, C., Pritchard, M. C., Raphy, J., Sumanchauhan, N., Horwell, D. C., Lalwani, N. D., and Oxender, D. L. (1995) *Mol. Pharmacol.* **48,** 711–716
6. Ourydonat, F., Carayon, P., Thurneyssen, O., Pailhon, V., Emondsalt, X., Soubrie, P., and Lefur, G. (1995) *J. Pharmacol. Exp. Ther.* **274,** 148–154
7. Takeda, Y., Chou, K. B., Takeda, J., Sachais, B. S., and Krause, J. E. (1991) *Biochem. Biophys. Res. Commun.* **179,** 1232–1240
8. Gerard, N. P., Eddy, R. L. J., Shows, T. B., and Gerard, C. (1990) *J. Biol. Chem.* **265,** 20455–20462
9. Takahashi, K., Tanaka, A., Hara, M., and Nakanishi, S. (1992) *Eur. J. Biochem.* **204,** 1025–1033
10. Krause, J. E., Blount, P., and Sachais, B. S. (1994) in *The Receptors* (Bylund, D. B., ed), pp. 165–218, Humana Press, Omaha, NE
11. Buell, G., Schulz, M. F., Arkinstall, S. J., Maury, K., Missotten, M., Adami, N., Talabot, F., and Kawashima, E. (1992) *FEBS Lett.* **299,** 90–95
12. Graham, A., Hopkins, B., Powell, S. J., Danks, P., and Briggs, I. (1991) *Biochem. Biophys. Res. Commun.* **177,** 8–16
13. Wu, L.-H., Vartanian, M. A., Oxender, D. L., and Chung, F.-Z. (1994) *Biochem. Biophys. Res. Commun.* **198,** 961–966
14. Chung, F.-Z., Wu, L.-H., Vartanian, M. A., Watling, K. J., Guard, S., Woodruff, G. N., and Oxender, D. L. (1994) *Biochem. Biophys. Res. Commun.* **198,** 967–972
15. Mauzy, C. A., Hwang, O., Egloff, A. M., Wu, L.-H., and Chung, F.-Z. (1992) *Biochem. Biophys. Res. Commun.* **186,** 277–284
16. Sumanchauhan, N., Grimson, P., Guard, S., Madden, Z., Chung, F.-Z., Watling, K., Pinnock, R., and Woodruff, G. (1994) *Eur. J. Pharmacol. Mol. Pharmacol. Sect.* **269,** 65–72
17. Mussap, C. J., Geraghty, D. P., and Burcher, E. (1993) *J. Neurochem.* **60,** 1987–2009
18. Bergstrom, L., Torrens, Y., Saffroy, M., Beaujouan, J. C., Lavielle, S., Chassaing, G., Morgat, J. L., Glowinski, J., and Marquet, A. (1987) *J. Neurochem.* **48,** 125–133
19. Heuillet, E., Menager, J., Fardin, V., Flamand, O., Bock, M., Garret, C., Crespo, A., Fallourd, A. M., and Doble, A. (1993) *J. Neurochem.* **60,** 868–876
20. Mantyh, P. W., Pinnock, R. D., Downes, C. P., Goedert, M., and Hunt, S. P. (1984) *Nature* **309,** 795–797
21. Sadowski, S., Huang, R. R., Fong, T. M., Marko, O., and Cascieri, M. A. (1993) *Neuropeptides* **24,** 317–319
22. Pradier, L., Menager, J., Le Guern, J., Bock, M. D., Heuillet, E., Fardin, V., Garret, C., Doble, A., and Mayaux, J. F. (1994) *Mol. Pharmacol.* **45,** 287–293
23. Patacchini, R., Bartho, L., Holzer, P., and Maggi, C. A. (1995) *Eur. J. Pharmacol.* **278,** 17–25
24. Gether, U., Johansen, T. E., and Schwartz, T. W. (1993a) *J. Biol. Chem.* **268,** 7893–7898
25. Cascieri, M. A., Huang, R. R., Fong, T. M., Cheung, A. H., Sadowski, S., Ber, E., and Strader, C. D. (1992) *Mol. Pharmacol.* **41,** 1096–1099
26. Yokota, Y., Akazawa, C., Ohkubo, H., and Nakanishi, S. (1992) *EMBO J.* **11,** 3585–3591
27. Fong, T. M., Huang, R. R. C., Yu, H., Swain, C. J., Underwood, D., Cascieri, M. A., and Strader, C. D. (1995) *Can. J. Physiol. Pharmacol.* **73,** 860–865
28. Huang, R.-R. C., Yu, H., Strader, C. D., and Fong, T. M. (1994) *Biochemistry* **33,** 3007–3013
29. Huang, R.-R. C., Vicario, P. P., Strader, C. D., and Fong, T. M. (1995) *Biochemistry* **34,** 10048–10055
30. Moyle, W. R., Campbell, R. K., Myers, R. V., Bernard, M. P., Han, Y., and Wang, X. (1994) *Nature* **368,** 251–255

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The Unpredicted High Affinities of a Large Number of Naturally Occurring Tachykinins for Chimeric NK_1/NK_3 Receptors Suggest a Role for an Inhibitory Domain in Determining Receptor Specificity

Ye Tian, Lan-Hsin Wu, Dale L. Oxender and Fu-Zon Chung

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