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Determination of the Amino Acid Residues in Substance P Conferring Selectivity and Specificity for the Rat Neurokinin Receptors

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SUMMARY

We have measured the affinity of various analogs and fragments of the tachykinin substance P for the cloned rat NK1, NK2, and NK3 receptors heterologously expressed in Chinese hamster ovary cells. The hydrophobic carboxyl-terminal pentapeptide sequence substance P-(7–11) binds with similar affinity (2–20 μM) to all three receptors. Our data suggest that addition of one to three amino-terminal residues to this sequence results in the optimization of its interaction within the binding pocket of the NK1 receptor. The addition of Pro-Gln-Gln to the carboxyl-terminal pentapeptide sequence increases affinity for the NK1 receptor, either by providing additional binding interactions or by modifying the conformation of the carboxyl-terminal sequence. This latter hypothesis is supported by the observation that physalaemin and phyllomedusin, which also contain a proline residue in the position analogous to the proline residue 4 of substance P, are also selective for NK1 receptors. Tachykinins that lack this proline have no higher affinity for NK1 than [pGlu] substance P-(6–11). Conversely, addition of Pro-Gln-Gln to the carboxyl-terminal pentapeptide sequence is unfavorable for NK2 and NK3 receptor binding. Preliminary data suggest that tachykinins with high affinity (K_d < 500 nM) for NK2 receptors contain an aspartate residue in the position analogous to residue 5 of substance P, suggesting that an ionic interaction with the receptor may contribute binding energy. Further experiments will be required to determine the structural determinants of the NK1, NK2, and NK3 receptors responsible for these binding properties.

Tachykinins are a family of peptides that share the carboxyl-terminal sequence -Phe-X-Gly-Leu-Met-NH_2. The mammalian tachykinins substance P, neurokinin A, and neurokinin B (see Fig. 1 for tachykinin sequences) have high affinity for the neurokinin receptor subtypes NK1, NK2, and NK3, respectively. Lee *et al.* (1) first described functionally distinct receptors for tachykinins in various tissue preparations. Subsequently, the pharmacology of these receptor subtypes was described by using selective radioligands, such as ^{125}I-BHSP and ^{125}I-BHE, to characterize binding sites in peripheral tissues and brain (2–6). Recently, more selective ligands were developed in order to study the distribution and selectivity of these receptors (for review, see Ref. 7). The limitations of this approach are that ligands are not completely selective and that tissue sources rarely contain a homogeneous receptor subtype population.

Recently, cDNA clones encoding functional rat NK1, NK2, and NK3 receptors have been described (8–11). The receptors are members of the superfamily of G protein-coupled receptors characterized by a seven-transmembrane domain motif. The NK1, NK2, and NK3 receptors have the expected selectivity for substance P, neurokinin A, and neurokinin B, respectively, when expressed in *Xenopus* oocytes or COS cells (8–11).

In the present investigation, we have characterized the pharmacology of agonist binding to the cloned receptors stably expressed in CHO cells. This strategy provides a homogeneous population of each of the three receptor subtypes and thus circumvents the ambiguities inherent in utilizing ligands with poor selectivity. The heterologously expressed receptors show clear subtype-specific differences in their interactions with tachykinin analogs. The results of this study suggest a molecular basis for the differential binding of peptide analogs to the three receptor subtypes.

Materials and Methods

All synthetic peptides were purchased from Peninsula Laboratories. ^{125}I-SP, ^{125}I-BHNA, and ^{125}I-BHE were from New England Nuclear Corp.

Cloning of rat NK1, NK2, and NK3 receptors. cDNAs for the three receptors were synthesized from RNA and amplified in two rounds of polymerase chain reaction, using nested primers based on the nucleotide sequences previously reported (8–11). All primers were constructed with an *Eco*RI restriction site at their 5′ ends. The NK1 receptor cDNA was synthesized from rat brain mRNA using a primer corresponding to nucleotides 1222–1245 and was amplified with sense primers −24 to −1 and −12 to 18 and antisense primers 1246–1269 and 1222–1245. The NK2 receptor cDNA was synthesized from rat stomach

ABBREVIATIONS: ^{125}I-BHSP, ^{125}I-Bolton Hunter-labeled substance P; ^{125}I-SP, ^{125}I-[Tyr^8]substance P; ^{125}I-BHNA, ^{125}I-Bolton Hunter-labeled neurokinin A; ^{125}I-BHE, ^{125}I-Bolton Hunter-labeled eledoisin; G protein, guanine nucleotide-binding protein; CHO, Chinese hamster ovary; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

		1 2 3 4 5 6 7 8 9 10 11
A.	Substance P	R-P-K-P-Q-Q-F-F-G-L-M-NH_2
	Neurokinin A	H-K-T-D-S-F-V-G-L-M-NH_2
	Neurokinin B	D-M-H-D-F-F-V-G-L-M-NH_2
B.	Physalaemin	pE-A-D-P-N-K-F-Y-G-L-M-NH_2
	Kassinin	D-V-P-K-S-D-Q-F-V-G-L-M-NH_2
	Phyllomedusin	pE-N-P-N-R-F-I-G-L-M-NH_2
	Eledoisin	pE-P-S-K-D-A-F-I-G-L-M-NH_2
C.	Senktide	Suc-D-F-MeF-G-L-M-NH_2
	Septide	pE-F-F-P-L-M-NH_2

Fig. 1. Sequence of mammalian (A), nonmammalian (B), and synthetic (C) tachykinins.

total RNA using the antisense primer 1206–1224 and was amplified using sense primers −92 to −73 and −70 to −51 and antisense primers 1206–1224 and 1178–1197. The NK3 receptor cDNA was synthesized from rat brain mRNA using the antisense primers 1357–1377 and 1378–1398 and was amplified using these two antisense primers and the sense primers −99 to −78 and −74 to −54. All three cDNAs were subcloned into pBluescript SK+ at the *Eco*RI site. The integrity of the cDNA clones was confirmed by sequencing.

Expression of NK1, NK2, and NK3 receptors in COS and CHO cells. Rat NK1, NK2, and NK3 receptor cDNAs were transfected into COS cells, using pCDM8 (NK1) or pcDNA/NEO (NK2 and NK3) expression vectors (Invitrogen), by electroporation. Cells were harvested 3 days after transfection for binding studies. Stable cell lines were selected from neomycin-resistant clones by ligand binding, after electroporation into CHO/DHFR$^-$/TK$^+$ cells, using pRC/CMV (NK1 and NK2) or pcDNA/NEO (NK3) expression vectors. Cell lines were selected and maintained in Iscove's modified Dulbecco's medium containing 25 mM HEPES, 2 mM L-glutamine, 100 units of penicillin/ml, 100 μg of streptomycin/ml (GIBCO), 10% fetal bovine serum (heat inactivated), 1/500 hypoxanthine-thymidine (American Type Culture Collection), and 1 mg/ml G418.

Binding to rat NK1, NK2, and NK3 receptors. Monolayer cultures (175-cm^2 flask) were washed twice with 20 ml of phosphate-buffered saline and then suspended by treatment at 37° for 5 min with cell dissociation solution (enzyme-free, catalog no. S-004H; Specialty Media, Inc.). The cells were washed and resuspended in assay buffer. CHO cells expressing NK1, NK2, or NK3 receptors (5×10^4, 2×10^5, or 2×10^5, respectively) were incubated with ^{125}I-SP, ^{125}I-BHNA, or ^{125}I-BHE (0.1 nM), respectively, in 0.5 ml of 50 mM Tris, pH 7.5, containing 150 mM NaCl, 5 mM $MnCl_2$, 0.2 mg/ml bovine serum albumin, and protease inhibitors. The protease inhibitor cocktail contained 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 20 μg/ml chymostatin for NK1 and NK2 assays and 40 μg/ml bacitracin, 4 μg/ml leupeptin, and 10 μM phosphoramidon for NK3 assays. At equilibrium (>45 min), the incubations were filtered over GF/C filters that had been presoaked in 0.5% polyethylenimine, using a 96-well Tomtek harvester. Nonspecific binding was determined using excess substance P (1 μM), neurokinin A (1 μM), or eledoisin (10 μM) for NK1, NK2, and NK3 assays, respectively. Nonspecific binding was <10% of total ligand binding to the cells and was subtracted to calculate the specific binding to receptors. Assays were performed in duplicate, and each titration was repeated twice. The variation in the data points between separate experiments was <10%.

Results

cDNA clones for the rat NK1, NK2, and NK3 receptors were obtained, using polymerase chain reaction amplification, from rat brain, rat stomach, and rat brain mRNA, respectively. The integrity of the cDNA encoding the rat NK1, NK2, and NK3 receptors was determined by DNA sequence analysis and by transient expression of the receptor proteins in COS cells. These rat NK1, NK2, and NK3 receptors display the appropriate specificity for substance P, neurokinin A, and neurokinin B, respectively (Fig. 2).

Clonal CHO cell lines were prepared and characterized. The rat NK1/CHO cell line expresses 23,000 receptors/cell and has a K_d for ^{125}I-SP of 0.1 nM. The rat NK2/CHO cell line expresses 15,000 receptors/cell and has a K_d for ^{125}I-BHNA of 1 nM. The rat NK3/CHO cell line expresses 2400 receptors/cell and has a K_d for ^{125}I-BHE of 0.2 nM.

We tested the ability of several substance P fragments and tachykinin analogs to inhibit radioligand binding to NK1, NK2, and NK3 receptors (Figs. 3 and 4). These data are summarized in Table 1. Carboxyl-terminal fragments of substance P as small as [*p*Glu]substance P-(6–11) have high affinity for the NK1 receptor (Fig. 3). In contrast, substance P and carboxyl-terminal fragments six to nine residues in length are 10–100-fold less potent at the NK2 and NK3 receptors. Substance P-(7–11) and shorter carboxyl-terminal fragments show reduced affinities at the NK1 receptor and, in fact, have similar affinities for the NK1, NK2, and NK3 receptors (Fig. 3; Table 1).

Substance P free acid and the amino-terminal fragments substance P-(1–9) and substance P-(1–4) have poor affinity for all three receptor subtypes (Table 1). The methyl ester of substance P has 10-fold and 5-fold lower affinity than substance P at NK1 and NK2 receptors, respectively. It is less active than substance P at the NK3 receptor also, but its precise affinity for the NK3 receptor could not be determined.

The nonmammalian tachykinins eledoisin and kassinin have similar affinities for the NK1, NK2, and NK3 receptors (Fig. 4; Table 1). In contrast, physalaemin and phyllomedusin have higher affinity for NK1 than NK2 or NK3 receptors.

Attempts have been made in several laboratories to prepare synthetic tachykinins that are receptor subtype selective (12–15). Our data confirm that [Sar^9, Met(O$_2$)^{11}] substance P (12) and septide (13) are selective for NK1 receptors and that [Nle^{10}] neurokinin A-(4–10) (14) and senktide (15) are selective for NK2 and NK3 receptors, respectively (Table 1).

Discussion

Several groups have characterized the binding of ^{125}I-BHSP and ^{125}I-BHE to rat cortex membranes (2, 3, 16). These two

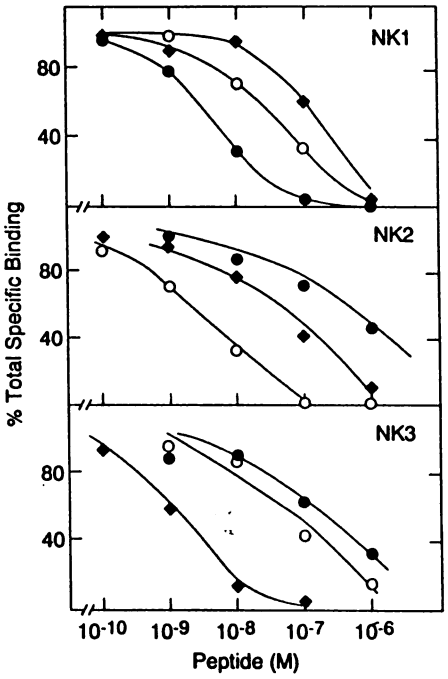

Fig. 2. Inhibition of ligand binding to NK1, NK2, and NK3 receptors, transiently expressed in COS cells, by substance P (●), neurokinin A (○), and neurokinin B (♦). Ligands (0.2 nM) were incubated with 0.4–2 × 10^5 COS cells in the presence or absence of competing peptides, as described in Materials and Methods. The cells express 1–2 × 10^4 receptors/cell. Data points are the average of two separate experiments.

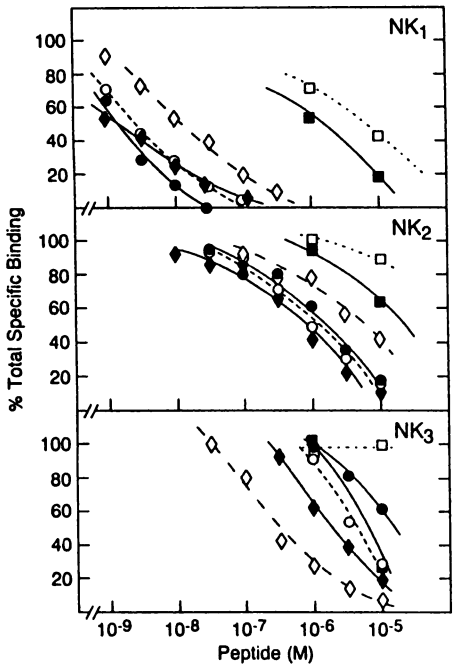

Fig. 3. Inhibition of binding to NK1, NK2, and NK3 receptors, expressed in CHO cells, by substance P (●), substance P-(3–11) (○), substance P-(4–11) (♦), [*p*Glu]substance P-(6–11) (◇), substance P-(7–11) (■), and substance P free acid (□). Ligands were incubated with CHO cells as described in Materials and Methods. Data points are the average of two separate experiments.

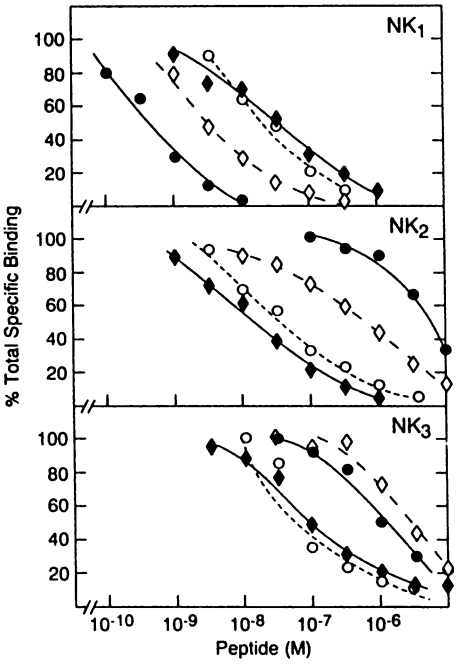

Fig. 4. Inhibition of binding to NK1, NK2, and NK3 receptors, expressed in CHO cells, by physalaemin (●), eledoisin (○), kassinin (♦), and phyllomedusin (◇). Ligands were incubated with CHO cells as described in Materials and Methods. Data points are the average of two separate experiments.

binding sites display different profiles of tachykinin specificity, suggesting that these radioligands bind preferentially to different receptor sites. The data previously obtained with ^{125}I-BHSP in cortex membranes (2, 16) correspond closely to the data

TABLE 1

Inhibition of ligand binding to NK1, NK2, and NK3 receptors by tachykinins

Values are the average derived from two separate experiments. Duplicates did not differ by >2-fold.

	IC_{50}		
	NK1	NK2	NK3
	nM		
Substance P	1.6	2,200	18,000
Neurokinin A	16	2.8	1,300
Neurokinin B	71	25	3.5
Substance P-(3–11)	2.8	1,000	4,500
Substance P-(4–11)	1.8	1,000	2,000
[*p*Glu]Substance P-(6–11)	13	6,300	350
Substance P-(7–11)	>1,000	>1,000	>1,000
Substance P-(8–11)	≫10,000	≫10,000	≫10,000
Substance P free acid	6,300	≫10,000	≫10,000
Substance P methyl ester	13	10,000	≫10,000
Substance P-(1–9)	≫10,000	≫10,000	≫10,000
Substance P-(1–4)	≫10,000	≫10,000	≫10,000
Physalaemin	0.5	5,000	1,000
Kassinin	35	14	80
Phyllomedusin	3	560	2,500
Eledoisin	20	32	56
[Sar^9,Met(O$_2$)^{11}]Substance P	1	>10,000	≫10,000
[Nle^{10}]Neurokinin A-(4–10)	13,000	180	≫10,000
Senktide	≫10,000	≫10,000	18
Septide	100	>10,000	≫10,000

reported here for the NK1 receptor, confirming that ^{125}I-BHSP is selective for the NK1 receptor.

Neurokinin B has the highest affinity of the mammalian tachykinins for the ^{125}I-BHE binding site in rat cortex membranes (2, 3). In contrast, the ^{125}I-BHE binding site in membranes prepared from peripheral tissues, such as rat duodenum and mouse urinary bladder, has higher affinity for neurokinin A (5). These two binding sites were thought to represent NK3 and NK2 receptors, respectively. Although more selective but lower affinity agonist ligands for these two sites have been described recently and used to localize NK2 and NK3 receptors selectively in the central nervous system and periphery (13, 14), the receptor pharmacology has largely been determined using the nonselective ligand ^{125}I-BHE (2, 3, 5). Because the endogenous membrane preparations do not contain homogeneous receptor populations, the data reported here for the NK2 and NK3 receptors differ qualitatively and quantitatively from the previous reports.

As expected from previous studies (2, 3, 16), only the carboxyl-terminal fragments of substance P that are five or more amino acid residues in length have measurable affinity for NK1, NK2, and NK3 receptors. The hydrophobic carboxyl-terminal pentapeptide substance P-(7–11) has similar affinity for all three receptors. The addition of a [*p*Glu] residue to this "core" sequence increases the affinity for the rat NK1 receptor 100-fold but only increases the affinity 4-fold and 17-fold for NK2 and NK3 receptors, respectively. Substance P-(5–11) is equipotent to [*p*Glu]substance P-(6–11) at the rat NK1 receptor (data not shown), whereas substance P-(4–11) and substance P-(3–11) are equipotent to substance P.

These data suggest that the carboxyl-terminal hydrophobic "core" sequence of substance P, F-F-G-L-M-NH_2, interacts with all three receptors in a similar fashion, with an affinity of 2–20 μM. The specificity for the NK1 receptor is conferred by addition of one to three additional amino-terminal residues to this core peptide. The addition of these amino-terminal residues is likely to provide additional binding interactions with the

receptor and to modify the conformation of the "core" sequence, to optimize its interaction within the binding pocket of the NK1 receptor. This latter hypothesis is supported by the observation that the nonmammalian tachykinins physalaemin and phyllomedusin, which have completely different amino-terminal sequences from substance P, are also selective for NK1 receptors. These peptides all have a proline residue in the position analogous to residue 4 of substance P, whereas tachykinins lacking this proline (i.e., eledoisin, kassinin, neurokinin A, and neurokinin B) have no higher affinity for the NK1 receptor than does [*p*Glu]substance P-(6–11). Proline in this position could potentially modify the conformation of the carboxyl-terminal sequence, to increase its affinity for the NK1 receptor.

In contrast to the NK1 receptor, addition of the three amino-terminal residues Pro-Gln-Gln to the carboxyl-terminal pentapeptide sequence of substance P produces only a 24-fold and <2-fold increase in affinity for NK2 or NK3 receptors, respectively. In fact, all of those tachykinins containing proline in the position analogous to residue 4 of substance P have greatly reduced affinity for NK2 and NK3 receptors, compared with the tachykinins lacking this residue, suggesting that the peptide conformation preferred for the NK1 receptor is unfavorable for NK2 and NK3 receptor binding.

All of the tachykinins that have high affinity (K_d < 500 nM) for the NK2 receptor (i.e., neurokinin A, neurokinin B, kassinin, and eledoisin) contain an aspartate at the position corresponding to residue 5 of substance P, suggesting that an ionic interaction with the receptor may contribute binding energy. Of these four peptides, only neurokinin A has a K_d of >0.1 μM for the NK3 receptor, suggesting that this interaction may be important for NK3 binding as well. As was previously observed in rat cortex membranes (2), modification of Lys^4 of eledoisin with the Bolton-Hunter reagent increases its affinity for NK3 receptors and its selectivity for this receptor versus NK1 receptors.

After this manuscript was submitted, Ingi *et al.* (17) reported similar experiments using membranes from transiently transfected COS cells. Their conclusions agree with ours, but several quantitative differences in the affinities observed for the tachykinin analogs should be noted. Substance P, neurokinin A, and neurokinin B have higher affinity for their respective receptors in COS cell membranes than we have observed in intact CHO cells. The percentage of the receptor population in the high affinity/G protein-coupled state is generally lower in intact cells than in membranes, and we have data suggesting that the observed affinity of substance P in rat NK1/CHO cell membranes is 3-fold higher than in intact cells. In addition, Ingi *et al.* have observed that substance P and neurokinin A have higher affinity for NK3 receptors expressed in COS cell membranes than we have observed in intact CHO cells. We have also observed this in intact COS cells (Fig. 1) and, therefore, the discrepancy may reflect a difference in cell type. In addition to these differences, our studies were performed at a receptor concentration that bound <5% of the available ligand, whereas those of Ingi *et al.* were done under conditions where up to 30% of the ligand was bound. The quantitative differences observed in these studies are most likely due to these differences in experimental design.

This pharmacological characterization of the individually expressed NK1, NK2, and NK3 receptors provides insights into the structural properties of the tachykinin peptides required for selectivity for NK1 receptors versus NK2 and NK3 receptors. Although we have obtained preliminary evidence suggesting that ionic interactions may be important for NK2 and NK3 receptor binding, analysis of fragments and analogs of neurokinin A and neurokinin B will be required to identify directly the structural determinants that confer selectivity between the NK2 and NK3 receptors. Further experiments using genetic analysis of the receptor proteins will be required to determine the structural elements within the receptors that are responsible for conferring these differential binding properties.

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