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Evidence for a Common Molecular Mode of Action for Chemically Distinct Nonpeptide Antagonists at the Neurokinin-1 (Substance P) Receptor

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SUMMARY

The molecular mechanism of action of three chemically distinct nonpeptide antagonists, SR 140,333, FK 888, and RP 67,580, was compared with that of the previously characterized compound CP 96,345, using a series of chimeric constructs between their common target, the rat neurokinin (NK)-1 (substance P) receptor, and the homologous nonresponsive NK-3 (NKB) receptor. The ability of all four nonpeptide compounds to displace radiolabeled substance P from the NK-1 receptor and their ability to inhibit the peptide-induced increase in inositol phosphate turnover were critically dependent on structural elements located in an area from the middle of the second extracellular loop through transmembrane segments V and VI to the middle of the third extracellular loop of the NK-1 receptor. Dissection of the domain around the outer part of transmembrane segments V and VI into smaller segments demonstrated that the individual nonpeptide antagonists, in agreement with their distinct chemical structures, were dependent on different subepitopes within the common putative binding domain. Full NK-1-like susceptibility to SR 140,333, FK 888, and CP 96,345 could be transferred to the NK-3 receptor by exchange of transmembrane segments V and VI and adjacent parts with corresponding segments from the NK-1 receptor. For SR 140,333 and CP 96,345, almost the same effect could be achieved by transfer of two discontinuous segments around the top of transmembrane segments V and VI. RP 67,580 shared interaction sites with the other compounds around the top of transmembrane segment VI but appeared also to be dependent on transmembrane segment VII. It is concluded that four nonpeptide antagonists, despite overt chemical differences, appear to block NK-1 receptor function by interacting in distinct ways with a common site located spatially around the outer part of transmembrane segment VI.

More than 40 small and medium-size peptides have been described as important chemical messengers, hormones, and neuropeptides in mammals (1). Because peptides and peptide analogues in general are inefficient drugs, mainly due to their unfavorable bioavailability and stability, nonpeptide compounds are currently being developed in many peptide systems not only as drugs but also as pharmacological and physiological tools (2). Within the last few years a series of high affinity nonpeptide antagonists have been described for the NK-1 (SP) receptor (Fig. 1) (3–6). These nonpeptide compounds may become a new class of analgesic and anti-inflammatory drugs, because SP is believed to play an important role in afferent transmission of pain stimuli in the spinal cord and in the neurogenic contribution to the inflammatory process (7–9). Two of the nonpeptide compounds, CP 96,345 and RP 67,580, were developed from lead compounds discovered through screening of chemical files (4, 10). One, SR 140,333, was developed and optimized on the basis of a high affinity NK-2 receptor antagonist, SR 48,968, which originally was discovered by file screening (6, 11). The last one, FK 888, was developed from an octapeptide antagonist lead in which the minimally active tripeptide was identified and subsequently minimized into a greatly modified dipeptide (5). All of these compounds are orally active and all bind to the human NK-1 receptor with high selectivity and single-digit nanomolar affinity (3–6). However, as shown in Fig. 1, despite their common pharmacological capability the compounds are chemically very different, just as

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ABBREVIATIONS: NK, neurokinin; SP, substance P; ^{125}I-BH-SP, ^{125}I-Bolton Hunter-labeled substance P; ELE, eledoisin; ^{125}I-BH-ELE, ^{125}I-Bolton Hunter-labeled eledoisin; TM, transmembrane segment; PI, inositol phosphate; CHO, Chinese hamster ovary; CCK, cholecystokinin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CR, chimeric receptor.

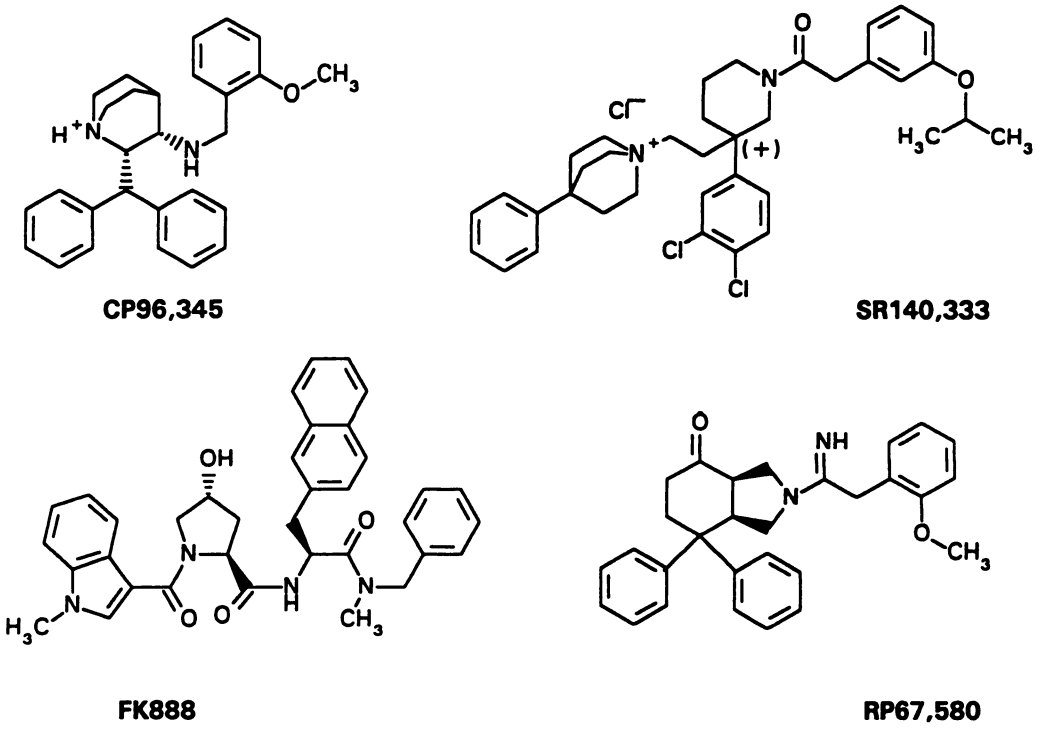


Fig. 1. Structures of the NK-1-specific nonpeptide antagonists CP 96,345, RP 67,580, and SR 140,333 (3, 4, 6). The lead compounds for the development of CP 96,345, RP 67,580, and SR 140,333 were identified by screening of chemical files (3, 4, 6). In contrast, the lead compound for the development of FK 888 was a tripeptide identified as the minimally active element in the structure of an octapeptide SP antagonist substituted with three D-tryptophans (14, 15).

none of them chemically resembles the natural peptide ligand SP (7).

Previously we have shown that the action of CP 96,345 is dependent upon three subdomains situated around the outer part of TM V and TM VI, through construction of a series of chimeric receptors between the NK-1 receptor and the NK-3 (NKB) receptor (12). We were even able to convey CP 96,345 susceptibility to the previously nonresponsive NK-3 receptor by moving a discontinuous complex of nonconserved residues from the NK-1 receptor to the NK-3 receptor (12). Recently, we have found that the NK-2-specific compound SR 48,968 also acts through an epitope located spatially around the outer part of TM VI on its target receptor (13). Exchange of 17 nonconserved residues around the top of TM VI and VII conveyed full susceptibility to the NK-2-specific nonpeptide compound to the NK-1 receptor without affecting its affinity and specificity for the natural peptide agonists (13). In the present investigation, we have studied the molecular mechanism of action of three additional nonpeptide antagonists that are all capable of binding with high affinity and specificity to the NK-1 receptor and thereby of disrupting its function. Surprisingly, we found that, despite their chemical differences, all four nonpeptide antagonists shown in Fig. 1 appear to act through epitopes that are located in the same general area of the NK-1 receptor, spatially situated around the top of TM VI.

Materials and Methods

Peptide and nonpeptide ligands. SP, NKB, and ELE were purchased from Peninsula (St. Helens, Merseyside, UK). The following nonpeptide compounds were used: CP 96,345 [(2*S*,3*S*)-(*cis*)-(2-diphenylmethyl)-*N*-[(2-methoxyphenyl)methyl]-1-azabicyclo[2.2.2]octan-3-amine], which was kindly provided by Drs. John A. Lowe III and R. Michael Snider, Pfizer Inc. (Groton, CT); SR 140,333 [(*S*)-1-[2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl]-4-phenyl-1-azabicyclo[2.2.2]octane chloride] (6); FK 888 [N^2-[(4*R*)-4-hydroxy-1-[(1-methyl-1*H*-indole-3-yl)-L-carbonyl-L-prolyl]]-*N*-methyl-*N* -(phenylmethyl)-3-(2-naphthyl)-L-alaninamide] (14, 15); and RP 67,580 [(3a*R*,7a*R*)-2-[1-imino-2-(2-methoxyphenyl)ethyl]-7,7-diphenyl-4-perhydroisoindol-4-one] (4).

Construction of chimeric receptors. The cDNAs encoding the rat NK-1 and NK-3 receptors (16, 17) were generously provided by Dr. S. Nakanishi, Institute for Immunology, Kyoto University (Kyoto, Japan). The chimeric receptors, NK1(NK3-TM7), NK1(NK3-TM5–7), and NK3(NK1-TM5–6), between the rat NK-1 and NK-3 receptors were constructed as described in detail previously, by use of either preexisting or introduced unique restriction sites located at equivalent positions in the receptor cDNAs (12). For construction of NK1(NK3-TM7), NK1(NK3-TM5–7), and NK3(NK1-TM5–6), the *ClaI* and *BglII* restriction sites located in the cDNAs at positions corresponding to the amino acid positions in the NK-1 receptor of Ile-182 and Glu-276, respectively, were used. The restriction sites were introduced by site-directed mutagenesis in the M13 system as described (12, 18, 19). Chimeric receptors NK1(NK3/183–196) (containing segment A), NK1(NK3/197–207) (containing segment B), NK1(NK3/262–270) (containing segment C), and NK1(NK3/271–276) (containing segment D) were all constructed by site-directed mutagenesis as described (12). The appropriate restriction endonuclease fragments encoding the chimeric/mutated constructs were cloned into the pTEJ8 expression vector (20). The structure of the recombinant genes was verified by restriction endonuclease mapping and by DNA sequence analysis. The wild-type NK-1 and NK-3 receptors were cloned into the pTEJ-8 eukaryotic expression vector as described (21).

Transfections and tissue culture. The expression plasmids containing the cDNAs encoding the wild-type NK-1 and NK-3 receptors and the chimeric receptors were transiently transfected into COS-7

cells by the calcium phosphate precipitation method, according to previously reported methods (20, 21).

Binding experiments. Monoiodinated ^{125}I-BH-SP and ^{125}I-BH-ELE were prepared and purified by high performance liquid chromatography as described in detail previously (17, 22). The transfected COS-7 cells were transferred to 12-well culture plates (0.1–1.0 × 10^5 cells/well) 1 day after transfection and 24 hr before the binding experiments were performed as described (21). The number of cells per well was determined by the expression efficiency of the individual plasmids, aiming at 5–10% binding of the added radioligand in the competition binding experiments. Binding experiments were performed for 3 hr at 4° with 50 pM ^{125}I-BH-SP or ^{125}I-BH-ELE plus variable amounts of unlabeled peptide or nonpeptide compound in 0.5 ml of 50 mM Tris·HCl buffer, pH 7.4, containing 150 mM NaCl, 5 mM $MnCl_2$, and 0.1% (w/v) bovine serum albumin (Sigma) and supplemented with protease inhibitors, 100 μg/ml bacitracin, and 10 μg/ml chymostatin (both from Sigma). All determinations were performed in triplicate and the nonspecific binding was determined as the binding in the presence of either 1 μM SP or ELE. The specific binding constituted >80% of the total binding. The binding data were analyzed and IC_{50} values were determined by computerized nonlinear regression analysis using InPlot (GraphPad Software, San Diego, CA). K_d and B_{max} values for binding of radiolabeled SP and ELE to the different receptors were assessed from competition binding experiments with 10–12 different concentrations of the corresponding unlabeled peptide, using the equations $K_d = IC_{50} - L$ and $B_{max} = B_0 \cdot IC_{50}/L$, where L is the concentration of free radioligand and B_0 is specifically bound radioligand (23). K_i values were calculated using the equation $K_i = IC_{50}/(1 + L/K_d)$ (24).

PI turnover. CHO cell clones stably expressing the wild-type NK-1 receptor and chimeric receptor NK1(NK3-TM5–7) were established as described (18). Cells were seeded in 12-well culture plates at a density of 5 × 10^5 cells/well and were incubated with *myo*-[^{3}H]inositol (2.5 μCi/well) with PT6–271; Amersham, Little Chalfont, UK) for 24 hr in inositol-free RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.1 mg/ml gentamicin. The cells were washed twice in assay buffer (10 mM HEPES, pH 7.4, containing 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1% bovine serum albumin, and 10 mM glucose) and preincubated for 20 min at 37° in 0.5 ml of assay buffer containing 10 mM LiCl, after which antagonists were added, followed 5 min later by SP. After incubation for 20 min at 37°, cells were extracted on ice for 30 min with 0.5 ml of ice-cold 10% trifluoroacetic acid. The supernatants were neutralized according to the method of Sharpes and McCarl (25). For separation of generated [^{3}H]inositol phosphates from [^{3}H]inositol (26), the neutralized supernatants were diluted with 2.0 ml of water and incubated for 30 min with 1.0 ml of a 50% (w/v) slurry of Bio-Rad AG 1-X8 resin (100–200 mesh, formate form; Bio-Rad Laboratories, Hercules, CA). The resin was washed three times with 3 ml of 5 mM *myo*-inositol before the inositol phosphates were eluted from the resin with 1.0 ml of 1.0 M ammonium formate in 0.1 M formic acid. A 0.8-ml fraction of the eluate was counted in a liquid scintillation β counter.

Results

Localization of General Domains in the NK-1 Receptor Essential for the Action of Nonpeptide Antagonists

The chemical structures of the four nonpeptide antagonists investigated in the present study are shown in Fig. 1. They all bound to the NK-1 receptor with high affinities, as assessed by their ability to displace radiolabeled SP from COS-7 cells transiently expressing the wild-type rat receptor (Fig. 2, *upper*). In this rat system, SR 140,333 and RP 67,580 showed K_i values similar to that of the natural peptide ligand SP (approximately 1 nM), whereas CP 96,345 and FK 888 bound with lower affinities (K_i = 8.1 and 82 nM, respectively) (Table 1). It should be noted, however, that both CP 96,345 and FK 888 bind with considerably higher affinities to the human NK-1 receptor (5, 27, 28). Only FK 888 and SR 140,333 bound to the NK-3 receptor with a detectable affinity, albeit 2–3 orders of magnitude less than their affinity for the NK-1 receptor (Table 1).

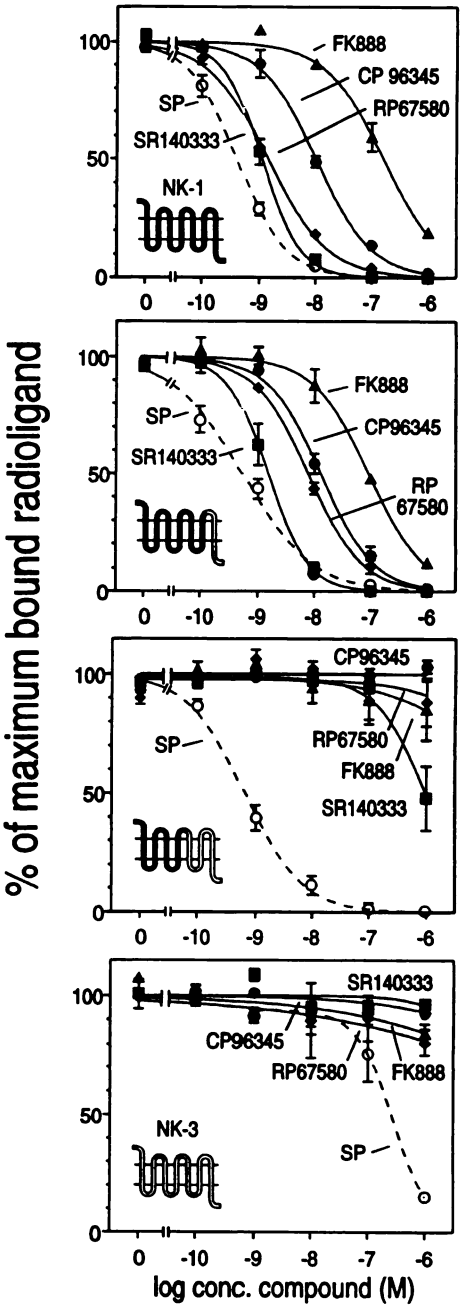


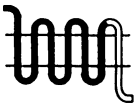
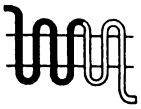
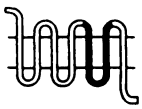
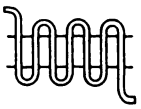
Fig. 2. Identification of a general domain essential for nonpeptide antagonist binding to the NK-1 receptor, by use of NK-1/NK-3 chimeric constructs. Competition binding of the peptide agonist SP (○) and the nonpeptide antagonists CP 96,345 (●), RP 67,580 (♦), FK 888 (▲), and SR 140,333 (■) with ^{125}I-BH-SP to (*from the top*) the wild-type NK-1 receptor, CR NK1(NK3-TM7), and CR NK1(NK3-TM5–7) and with ^{125}I-BH-ELE to the wild-type NK-3 receptor. Data are expressed as percentage of maximum bound radioligand (mean ± standard error, three to five experiments). A simplified structure of the receptor constructs is shown in each diagram. *Filled areas*, segments derived from the NK-1 receptor; *open areas*, segments derived from the NK-3 receptor.

By using chimeric constructs between the NK-1 and NK-3 receptors, we previously localized structural elements of importance for the action of CP 96,345 to a region around the top of

TABLE 1

Binding affinities of the peptide ligands SP and NKB and the nonpeptide antagonists CP 96,345, RP 67,580, FK 888, and SR 140,333 for the wild-type NK-1 and NK-3 receptors and chimeric constructs between these

Simplified structures of the receptor constructs are shown; *filled areas*, segments derived from the NK-1 receptor; *open areas*, segments derived from the NK-3 receptor. The data are expressed as K_i values (mean ± standard error, three to five experiments). Hillslopes for the competitions curves are given in parentheses (mean ± standard error, n = 3–5) ^{125}I-BH-SP was used as radioligand for the NK-1 receptor, CR NK1(NK3-TM7), and CR NK1(NK3-TM5-7) and ^{125}I-BH-ELE was used as radioligand for the wild-type NK-3 receptor and CR NK3(NK1-TM5-6). B_{max} values for binding of ^{125}I-BH-SP to the receptor constructs were as follows (mean ± standard error, three or four experiments): wild-type NK-1, 90 ± 30 fmol/10^6 cells; CR NK1(NK3-TM7), 110 ± 30 fmol/10^6 cells; CR NK1(NK3-TM5-7), 23 ± 8 fmol/10^6 cells. K_d and B_{max} values for binding of ^{125}I-BH-ELE to the NK-3 receptor were 4.7 ± 1.1 nM and 51 ± 3 fmol/10^6 cells and for binding to CR NK3(NK1-TM5-6) were 4.7 ± 1.6 nM and 9 ± 6 fmol/10^6 cells, respectively.

	K_i				
	Wild-type NK-1	CR NK1(NK3-TM7)	CR NK1(NK3-TM5-7)	CR NK3(NK1-TM5-6)	Wild-type NK-3
					
	nM				
SP	0.27 ± 0.04 (0.98 ± 0.09)	0.61 ± 0.19 (0.76 ± 0.06)	0.72 ± 0.09 (0.90 ± 0.15)	63 ± 3 (1.2 ± 0.2)	300 ± 80 (0.87 ± 0.08)
NKB	425 ± 150[a] —	8.2 ± 2.5[a] (1.1 ± 0.1)	7.9 ± 0.8[a] (1.0 ± 0.2)	1.7 ± 0.3 (0.8 ± 0.05)	1.8 ± 0.2 (1.0 ± 0.1)
RP 67,580	1.21 ± 0.17 (0.83 ± 0.06)	6.3 ± 0.7 (0.92 ± 0.07)	>10,000 —	310 ± 70 (1.06 ± 0.03)	>10,000 —
FK 888	82 ± 15 (0.92 ± 0.09)	70 ± 4 (0.86 ± 0.14)	>10,000 —	11 ± 3 (0.92 ± 0.17)	6,700 ± 600 0.89 ± 0.14
SR 140,333	1.0 ± 0.2 (1.37 ± 0.09)	1.3 ± 0.4 (1.30 ± 0.16)	1,400 ± 800 (0.98 ± 0.06)	1.2 ± 0.8 (0.93 ± 0.16)	7,500 ± 2,500 (0.77 ± 0.12)
CP 96,345[b]	8.1 ± 1.2 (0.85 ± 0.09)	11 ± 3 (0.95 ± 0.06)	>10,000 —	15.2 ± 1.6 (0.83 ± 0.06)	>10,000 —

[a] Data obtained by stable expression in CHO cells (18).
[b] The data for CP 96,345 have been published previously (12) and are shown for comparison.

TM V and VI (12). In the present paper these chimeric constructs were used to characterize three new compounds; however, for direct comparison the binding data for CP 96,345 have been included in figures and tables. As observed for CP 96,345, exchange of TM VII and a part of the third extracellular loop of the NK-1 receptor with the corresponding sequence from the nonresponsive NK-3 receptor [yielding CR NK1(NK3-TM7)], did not impair binding of SR 140,333 and FK 888 (Fig. 2; Table 1). However, a small 5-fold decrease in the affinity for RP 67,580 was observed in this chimeric construct, compared with the wild-type NK-1 receptor (Fig. 2; Table 1), indicating that RP 67,580 may have points of interaction with nonconserved residues in TM VII.

The binding of all four nonpeptide antagonists was profoundly impaired when also TM V and TM VI plus half of extracellular loop 2 from the NK-3 receptor were introduced into the NK-1 receptor, yielding CR NK1(NK3-TM5–7) (Fig. 2; Table 1). Like CP 96,345, both RP 67,580 and FK 888 lost their ability to inhibit binding of radiolabeled SP to this chimeric receptor construct (Fig. 2; Table 1). Only SR 140,333 still had some effect on radiolabeled SP binding, albeit with an approximately 1000-fold decrease in affinity (Table 1). As described previously (12), the overall structure of the NK-1 receptor was not affected by the chimeric exchanges, as the control peptide, ELE, which binds equally well to both wild-type receptors, and the endogenous NK-1 ligand SP both bound with high unchanged affinity to this chimeric receptor (Table 1 and refill). Furthermore, no affinity change between chimeric receptor NK1(NK3-TM7) and NK1(NK3-TM5–7) was observed for the NK-3-selective endogenous ligand NKB (Table 1).

Separation of Agonist and Antagonist Functions

In CHO cells stably expressing the wild-type NK-1 receptor, SP potently stimulated PI turnover with an EC_{50} value of 2.0 ± 0.5 nM (mean ± standard error, two experiments) (Fig. 3, *upper*), which is in agreement with previously published results (29). In cells expressing the chimeric receptor in which the three carboxyl-terminal TM's had been exchanged with the corresponding segment of the NK-3 receptor [CR NK1(NK3-TM5–7)], SP stimulated PI turnover to a similar extent and with a similar dose-response curve (EC_{50} = 4.7 ± 0.4 nM, mean ± standard error, two experiments), in agreement with our binding data (Fig. 3, *upper*). All of the nonpeptide compounds acted as antagonists at the wild-type NK-1 receptor, as they dose-dependently inhibited the SP-induced increase in intracellular PI turnover (Fig. 3, *lower left*). However, FK 888, RP 67,580, and CP 96,345 lost all of their antagonistic effect on SP-induced PI turnover after the exchange of TM V through TM VII and the connecting segments with the corresponding NK-3 sequence [yielding CR NK1(NK3-TM5–7)] (Fig. 3). Only SR 140,333 retained some ability to inhibit the effect of SP in this construct but, again, with a dose-response curve that had been shifted >1000-fold to the right, in agreement with the binding results (Fig. 3, *lower right*).

Localization of Subdomains Important for Nonpeptide Antagonist Action

The region around the top of TM V and VI of the NK-1 receptor was dissected into four subdomains, designated A, B, C, and D (see Fig. 5), which were exchanged with the corresponding segments of the NK-3 receptor. These constructs showed that, although the compounds are dependent on a

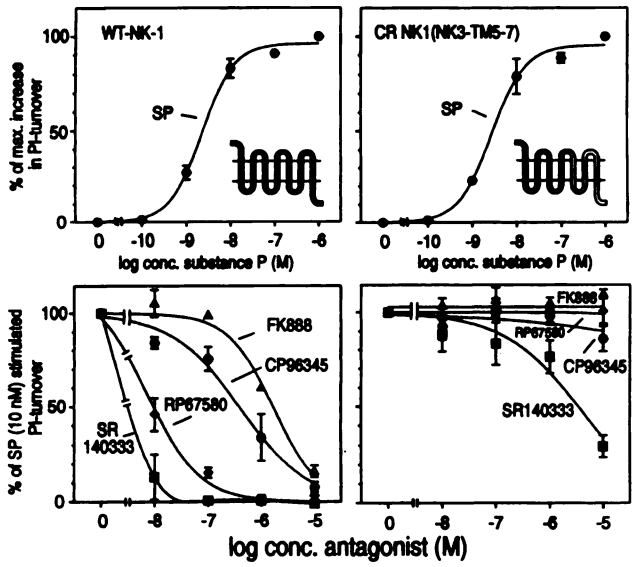


Fig. 3. Separation of agonist and antagonist action through construction of chimeric receptor NK1(NK3-TM5–7). *Upper*, stimulation of PI turnover by SP in CHO cells stably expressing the wild-type NK-1 receptor (*left*) and chimeric receptor NK1(NK3-TM5–7) (*right*). Data are expressed as percentage of maximum increase in the presence of 1 μM SP (mean ± standard error, two experiments). The average increase above basal PI turnover was 7 ± 1-fold for the NK-1 receptor and 18 ± 3-fold for CR NK1(NK3-TM5–7) in the clones studied. *Lower*, inhibition of SP-induced PI turnover (10 nM) by CP 96,345 (●), RP 67,580 (♦), FK 888 (▲), and SR 140,333 (■) in CHO cells stably expressing the wild-type NK-1 receptor (*left*) and CR NK1(NK3-TM5–7) (*right*). A simplified structure of the receptor constructs is shown in each diagram. *Filled areas*, segments derived from the NK-1 receptor; *open areas*, segments derived from the NK-3 receptor.

common general domain of the receptor, they appear to interact in distinct ways with the different subdomains.

RP 67,580 and FK 888. In contrast to what we found for CP 96,345 (12), the action of both RP 67,580 and FK 888 on the NK-1 receptor was severely affected by the introduction of the three nonconserved residues in domain C from the NK-3 receptor (Fig. 4; Table 2). Conversely, domain B from the NK-3 receptor, which decreased the affinity of CP 96,345 10-fold, did not affect RP 67,580 and FK 888 affinity. Furthermore, both of these latter compounds were only marginally affected by the introduction of the A or D epitopes, which decreased the affinity for CP 96,345 almost 200-fold and 40-fold, respectively (12) (Fig. 4; Table 2).

SR 140,333. The most obvious effect with SR 140,333 was observed with domain D. However, even this was only a 7-fold shift in apparent affinity (Fig. 4; Table 2). Domains B and C had no effect, and with domain A only a 2–3-fold decrease in affinity for SR 140,333 was detected (Fig. 4; Table 2). Thus, by these exchanges of subdomains we were unable to locate in more detail compelling structural reasons for the affinity decrease of >1000-fold for SR 140,333 between CR NK1(NK3-TM7) and CR NK1(NK3-TM5–7) (Fig. 2). A possible explanation would be that nonconserved residues located in different subdomains act together in a synergistic fashion to facilitate the action of SR 140,333.

Genetic Transfer of the Putative Binding Site for Nonpeptide Antagonists from the NK-1 Receptor to the NK-3 Receptor

Up to this point we have attempted to localize structural elements that are essential for the action of the nonpeptide

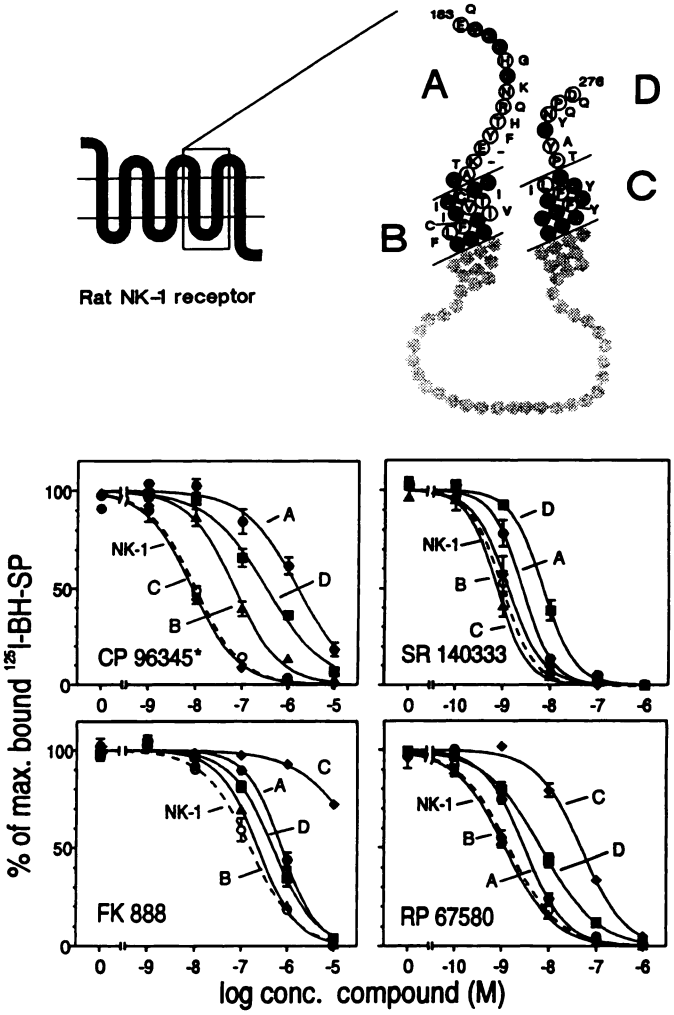


Fig. 4. Identification of subdomains in the NK-1 receptor involved in binding of four different nonpeptide antagonists. *Top*, structure of the NK-1 receptor in the region around TM V and VI. *Segments A, B, C,* and *D* were exchanged with the corresponding segments from the NK-3 receptor. *Black circles*, amino acid residues conserved between the NK-1 and NK-3 receptors; *white circles*, residues specific for the NK-1 receptor. The corresponding amino acid residues of the NK-3 receptor are indicated. *Bottom*, competition binding of CP 96,345 (*upper left*), SR 140,333 (*upper right*), FK 888 (*lower left*), and RP 67,580 (*lower right*) with ^{125}I-BH-SP to the wild-type NK-1 receptor (○), NK1(NK3/183–196) (segment A) (●), NK1(NK3/197–207) (segment B) (▲), NK1(NK3/262–270) (segment C) (♦), and NK1(NK3/271–276) (segment D) (■) is shown. Data are expressed as percentage of maximum bound radioligand (mean ± standard error, three to five experiments). *, The data for CP 96,345 have been published previously and are shown for comparison.

compounds by destroying binding through introduction of sequences from a receptor that did not bind the compounds. The effect of these chimeric substitutions on nonpeptide binding could theoretically be caused by some indirect effects on the general structure of the receptor, i.e., changes that were not reflected in altered binding of the peptide ligands or in impaired functional coupling. In an attempt to build up the binding ability for the nonpeptide compounds instead of destroying it, we generated a series of reciprocal chimeric constructs, as illustrated in Fig. 5. Transfer of segments A, B, D, and A plus D and TM V and VI (yielding CR 5–6) from the NK-1 receptor to the NK-3 receptor all resulted in chimeric receptors that bound radiolabeled ELE with similar high affinity, indicating

TABLE 2

Binding affinities of the peptide ligand SP and the nonpeptide antagonists CP 96,345, RP 67,580, FK 888, and SR 140,333 for the wild-type NK-1 receptor and for chimeric constructs in which segments of the NK-1 receptor around the top of TM V and VI were exchanged with corresponding segments from the NK-3 receptor

A, NK1(NK3/183-196); B, NK1(NK3/197-207); C, NK1(NK3/262-270); D, NK1(NK3/271-276). The start of segment A corresponds to the junction between the two receptor fragments in CR NK1(NK3-TM5-7) and the end of segment D to the junction in CR NK1(NK3-TM7). The data are expressed as K_i values (mean ± standard error, three to five experiments) Hillslopes for the competition curves are given in parentheses (mean ± standard error, 3–5 experiments). B_{max} values for binding of ^{125}I-BH-SP to the receptor constructs were as follows (mean ± standard error, three or four experiments): wild-type NK-1, 90 ± 30 fmol/10^6 cells; A, 38 ± 13 fmol/10^6 cells; B, 210 ± 30 fmol/10^5 cells; C, 330 ± 170 fmol/10^6 cells; D, 21 ± 2 fmol/10^5 cells.

	K_i				
	Wild-type NK-1	CR NK1(NK3/183-196) (A)	CR NK1(NK3/197-207) (B)	CR NK1(NK3/262-270) (C)	CR NK1(NK3/271-276) (D)
			nM		
SP	0.27 ± 0.04	0.5 ± 0.2	0.15 ± 0.03	0.25 ± 0.06	0.18 ± 0.05
	(0.98 ± 0.09)	(0.92 ± 0.03)	(1.12 ± 0.08)	(1.06 ± 0.11)	(0.85 ± 0.08)
RP 67,580	1.21 ± 0.17	2.8 ± 0.3	0.98 ± 0.17	40 ± 4	6 ± 1
	(0.83 ± 0.06)	(0.92 ± 0.07)	(0.84 ± 0.05)	(0.94 ± 0.05)	(0.74 ± 0.09)
FK 888	82 ± 15	700 ± 100	174 ± 19	>10,000	430 ± 90
	(0.92 ± 0.09)	(1.1 ± 0.1)	(1.01 ± 0.03)	—	(0.96 ± 0.05)
SR 140,333	1.0 ± 0.2	2.7 ± 0.5	0.60 ± 0.09	1.2 ± 0.3	7 ± 1
	(1.37 ± 0.09)	(1.4 ± 0.2)	(1.3 ± 0.2)	(1.28 ± 0.05)	(1.32 ± 0.12)
CP 96,345[a]	8.1 ± 1.2	1,520 ± 330	58 ± 9	7.5 ± 0.8	340 ± 50
	(0.85 ± 0.09)	(0.90 ± 0.04)	(0.88 ± 0.05)	(1.03 ± 0.06)	(0.72 ± 0.01)

[a] These data have been published previously (12) and are shown for comparison.

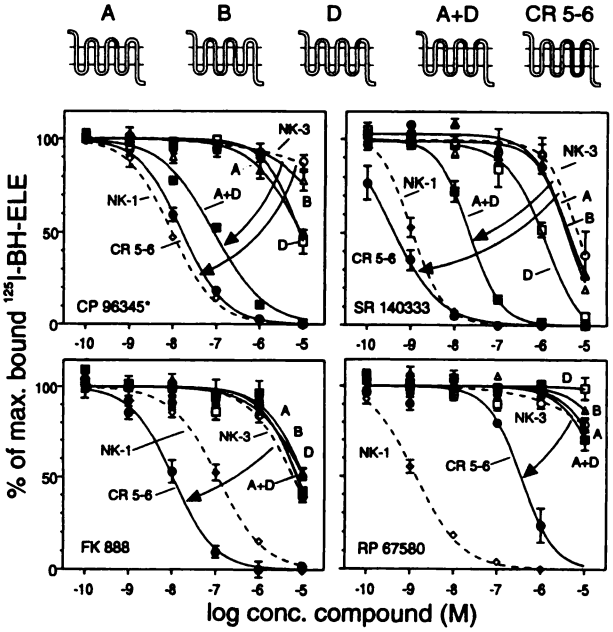


Fig. 5. Genetic transfer of nonpeptide NK-1 antagonist binding to the nonresponsive NK-3 receptor. Simplified structures of the NK-3 receptor substituted with NK-1 receptor segments (*solid areas*) are shown *above the panels*. Competition binding of CP 96,345 (*upper left*), SR 140,333 (*upper right*), FK 888 (*lower left*), and RP 67,580 (*lower left*) with ^{125}I-BH-SP to the wild-type NK-1 receptor (◇) and with ^{125}I-BH-ELE to the wild-type NK-3 receptor (○) and chimeric receptors NK3(NK1/223–233) (segment A) (▲), NK3(NK1/234–245) (segment B) (△), NK3(NK1/309–314) (segment D) (□), NK3(NK1/223–233,309–314) (segments A plus D) (■), and NK3(NK1-TM5–6) (●) is shown. Data are expressed as percentage of maximum bound radioligand (mean ± standard error, three to five experiments). K_d and B_{max} values for binding of ^{125}I-BH-ELE to the mutated receptors transiently expressed in COS-7 cells were as follows (mean ± standard error, three or four experiments): NK3(NK1/223–233) (segment A), K_d = 0.9 ± 0.2, B_{max} = 430 ± 70 fmol/10^5 cells; NK3(NK1/234–245) (segment B), K_d = 2.4 ± 0.8 nM, B_{max} = 100 ± 40 fmol/10^5 cells; NK3(NK1/309–314) (segment D), K_d = 1.7 ± 0.8 nM, B_{max} = 200 ± 80 fmol/10^5 cells; NK3(NK1/223–233,309–314), K_d = 0.78 ± 0.01 nM, B_{max} = 61 ± 6 fmol/10^5 cells; NK3(NK1-TM5–6), K_d = 4.7 ± 1.6 nM, B_{max} = 9 ± 6 fmol/10^5 cells. *, The data for CP 96,345 have been published previously and are shown for comparison.

that the overall structure of these constructs was conserved (see legend to Fig. 5). Unfortunately, transfer of segment C was accompanied by a poor expression level, and the construct showed only very low affinity binding of the control peptide ELE (K_d > 30–50 nM). This likely reflects a structural incompatibility of the NK-3 construct containing the three nonconserved residues from the NK-1 receptor in subdomain C. Thus, the characterization of the binding domain for RP 67,580 and FK 888, which both were especially dependent on domain C in the NK-1 constructs (Fig. 4), cannot be as detailed in the present paper as is that for CP 96,345 and SR 140,333. Previously, we found that the combined transfer of the discontinuous segments A and D from the NK-3 receptor to the NK-1 receptor conveyed to the NK-3 receptor binding affinity for CP 96,345 that was only <6-fold below the affinity of the compound for the wild-type NK-1 receptor. Complete NK-1-like affinity was obtained by the transfer of the whole TM V and VI (CR 5–6) to the NK-3 receptor (12).

SR 140,333. Also, in these constructs SR 140,333 behaved surprisingly similarly to CP 96,345 (Fig. 5). Transfer of domains A plus D increased the affinity 340-fold (K_i = 22 ± 3.5 nM, mean ± standard error, three experiments) and transfer of the whole TM V and VI (CR 5–6) conveyed full NK-1-like affinity for SR 140,333 (Fig. 5; Table 1). Transfer of segment A or B alone had only a marginal influence on SR 140,333 binding. However, transfer of domain D increased the affinity almost 10-fold (Fig. 5). Thus, for both SR 140,333 and CP 96,345 the segments around TM V and VI of the NK-1 receptor appear to contain not only essential but also sufficient structural information for the action of these compounds on the NK-1 receptor. It is interesting to note that the 'synergistic effect' of the combined transfer of domains A and D, i.e., the increase in binding affinity for A plus D, is larger than the sum of increments for A and D (Fig. 5).

FK 888. The combined transfer of TM V and VI (CR 5–6) to the NK-3 receptor also improved the affinity for FK 888 by almost 3 orders of magnitude (Fig. 5). Surprisingly, the affinity of FK 888 for CR 5–6 was approximately 10-fold higher than the affinity for the wild-type rat NK-1 receptor (Fig. 4, *bottom*,

lower left). However, recently we have found that a single substitution in the rat NK-1 receptor, Leu[116] to valine, is a major structural reason for the decreased affinity of FK 888 for the rat NK-1 receptor, compared with the human receptor (30). Importantly, the rat NK-3 receptor, like the homologous human NK-1 receptor, already has a valine residue at this position, which could explain the particularly high affinity of FK 888 for the chimeric construct CR 5–6.

The ability of FK 888 to displace radiolabeled ELE from the NK-3 receptor was not improved by the transfer of segments A, B, D, or A plus D from the NK-1 receptor (Fig. 5), in agreement with the fact that only substitution of subdomain C in the NK-1 receptor had a major effect on FK 888 binding (Fig. 4).

RP 67,580. The binding affinity of RP 67,580 increased from undetectable levels to a K_i value of 310 nM for CR 5–6 (Fig. 5; Table 1). However, the affinity for CR 5–6 was still approximately 100-fold less than the affinity of the compound for the wild-type NK-1 receptor. Thus, structural elements around TM V and VI, conceivably especially in subdomain C (Fig. 4), are important but not sufficient for the action of RP 67,580. It should be noted, however, that RP 67,580 was the only compound that was affected when TM VII from the NK-3 receptor was built into the NK-1 receptor (Fig. 2, *second panel*). Thus, RP 67,580 may have points of interaction in the NK-1 receptor, conceivably in TM VII, that are not included in the segment that was transferred to the NK-3 receptor mutant shown in Fig. 5. As expected, based on the results shown in Fig. 4, introduction of segment A, B, D, or A plus D into the NK-3 receptor did not improve its affinity for RP 67,580.

Discussion

Despite their differences in mode of discovery and development and their obvious differences in chemical structure, the functions of all four nonpeptide antagonists shown in Fig. 1 appear to be critically dependent on structural elements spatially located around the top of TM VI of the NK-1 receptor. However, in accordance with their distinct chemical structures, we found that the different compounds deviate in their dependency on different subdomains within this common domain. As reported previously, there is no evidence indicating that the peptide agonist SP has any major points of interaction shared with the nonpeptide antagonists in this domain of the receptor (12).

Direct or indirect effect of mutations on binding? From the results of the present study it is not possible to determine with certainty whether the identified domain and subdomains of the NK-1 receptor are directly or indirectly involved in the actual binding of the nonpeptide antagonists. It is interesting to note that a quantitatively rather similar effect in response to point mutations (i.e., a 10–30-fold decrease in binding affinity) has been interpreted in some cases as an indirect effect (27, 28, 30) and in another as a direct effect on the binding of CP 96,345 (31). Importantly, the residue identified in the latter paper, His[197], is located in the middle of the general domain identified in the present study to be of major importance for the action of all four nonpeptide antagonists (Fig. 4). By systematic substitutions we have found that a series of residues, all located on one side of the helix in TM VI and presumably facing His[197] at the top of TM V, are particularly important for the action of the nonpeptide antagonists.[1] This suggests that we probably are dealing with at least part of the actual binding site for the nonpeptide antagonists.

General mechanism of action for nonpeptide antagonists? Recently, we found that a high affinity nonpeptide antagonist specific for the tachykinin NK-2 receptor also interacts with its target receptor within the same general area around TM VI. By exchanging domains around the top of TM VI and VII between, in this case, the NK-2 and NK-1 receptors, we were able to switch the specificity for the nonpeptide compounds without affecting the specificity and affinity for the endogenous peptide ligands SP and NKA (13). Thus, the action of an NK-2 antagonist, SR 48,968, is also critically dependent on residues located spatially around the top of TM VI, not in the NK-1 receptor but in its target, the NK-2 receptor (13).

In the CCK system several nonpeptide antagonists have been developed, and recently the first points of interaction between the benzodiazepine-based CCK antagonists and the CCK-B receptor were identified. Kopin and co-workers (31) showed that the species selectivity for L365,260 and L364,718 was dependent on the nature of a single aliphatic residue located in the outer part of TM VI, corresponding to residue 264 in region C of the NK-1 receptor (Fig. 5). Importantly, also, in the case of the CCK receptor the binding profile for peptide agonist was independent of the aliphatic character of this residue. Thus, CCK nonpeptide antagonists, at least of the diazepam class, also appear to have important points of interaction at the top of TM VI of their target receptor, independently of the points of interaction with the natural peptide ligand (31).

All of the studies discussed above used either species differences or differences among subtypes of receptors as the basis for chimeric genetic exchanges (12, 13, 31). Through this approach one is initially able only to identify points of interaction with residues that are not conserved among the 'parent' receptors. However, Strader and co-workers (32) took an alternative methodological approach; they performed a discontinuous 'alanine scan' of all of the polar residues that could be involved in hydrogen bonding and that were located in the outer parts of the TM of the NK-1 receptor. In this way they identified a conserved histidyl residue located at the top of TM V (His[197] in subdomain B in Fig. 5) as being important in the binding of CP 96,345 (32). By combining a series of different substitutions at this point with a series of analogs of CP 96,345, they arrived at the conclusion that there is an amino-aromatic interaction between the imidazole side chain and the diphenyl group of the nonpeptide antagonist. Interestingly, not even this residue of the CP 96,345 binding site, which is conserved among all tachykinin receptors, is involved in the binding of the natural peptide agonist (32). Mutational analysis of the peptide binding sites on the NK-1 receptor has identified mainly residues located in the amino-terminal extracellular segment, in the first extracellular loops, and around the top of the first TM (18, 33). Thus, we have, as yet, no structural information that points to an actual overlap between the peptide agonist binding site and nonpeptide antagonist binding sites. All available information indicates that at least a major part of the interaction of nonpeptide antagonists and their target receptors occurs with residues located spatially around the top of TM VI, spanning in some cases to the top of TM V and in some cases

[1] U. Gether, S. Zofmann, and T. W. Schwartz, unpublished observations.

to TM VII. None of the identified points of interaction have been demonstrated to be involved in the binding of the natural peptide agonist.

The majority of nonpeptide antagonists that have been studied in the NK-1, NK-2, and CCK-B systems were discovered initially through file screening (2, 7). It is remarkable that apparently all compounds that have been selected through these empirical procedures appear to share a general mode of interaction with their target receptors. Apparently, the lead compounds that are selected in the screening process have a preference for binding to this particular part of the receptor and thereby inhibit the binding of the peptide agonist. This could be due to the fact that the major site at which G proteins allosterically influence the affinity for agonist binding is found just below the common binding site for the nonpeptide antagonists in TM VI (34, 35). Thus, the molecular mechanism of action for the nonpeptide antagonists could be related to an interference with the overall proper function of the receptor, perhaps by keeping the receptor in a conformation disfavoring agonist binding and receptor activation. However, even more remarkable is the fact that one of the compounds, FK 888, was developed through a more or less rational procedure on the basis of a peptide lead structure, a tripeptide identified as the minimal active part of an octapeptide antagonist (5). Nevertheless, FK 888, which is a dipeptide, is dependent on nonconserved residues located in the same part of the receptor as that important for all of the empirically discovered compounds. It is probably noteworthy that the lead compound for FK 888 was an antagonist substituted with three D-tryptophan residues (5). The prominent stereochemical modification of the backbone around the aromatic residues may force the side chains into interactions with parts of the receptor with which the peptide agonist normally does not engage. During the additional peptide modification the medicinal chemical work may inadvertently have been directed towards optimizing interactions with such epitopes located just outside the peptide agonist site. It will be interesting to characterize the interaction points between small nonpeptide or peptoid antagonists that have been developed on the basis of other peptide leads (8). Such compounds could very well have different modes of action.

Development of agonist-independent antagonists for G protein-coupled receptors. An ultimate consequence of the observation that the nonpeptide antagonists, at least to a major degree, interact differently with the receptor than do the natural ligands would be that 'nonpeptide' antagonists could be developed for all G protein-coupled receptors, independently of the chemical nature of their natural ligands. Thus, through file screening it should be possible to find lead compounds that through a similar mechanism can disturb the function of, for example, prostaglandin receptors, the thrombin receptor, glycoprotein hormone receptors, any new subtype of monoamine receptors, and even perhaps certain odorant receptors. It is even possible that some of the well established antagonists for monoamine receptors, for example those that chemically are very different from the agonist, may in fact function in a similar way as the nonpeptide antagonists for the peptide receptors. That is, such compounds may disturb the general function of the receptor molecule instead of just preventing agonist binding by occupying parts of the agonist binding site. In these cases, the characterization of the molecular mechanism of action is more complicated because the agonists do have major interaction points in the same area (36, 37).

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