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The Species Selectivity of Chemically Distinct Tachykinin Nonpeptide Antagonists Is Dependent on Common Divergent Residues of the Rat and Human Neurokinin-1 Receptors

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SUMMARY

During evolution mutations have occurred in peptide receptors that are neutral with respect to binding of the natural peptide ligand but frequently affect the binding of nonpeptide antagonists. By systematically introducing the nonconserved residues from the human neurokinin (NK)-1 receptor into the corresponding rat receptor we have attempted to localize the structural elements that are responsible for 15–76-fold higher affinity of three tachykinin nonpeptide antagonists for the human receptor, compared with the corresponding rat receptor. Surprisingly, exchange of the four divergent residues located around the previously located apparent binding site for CP 96,345 and FK 888 at the top of transmembrane segment (TM) V and VI, either alone or as a group, did not affect the binding of these nonpeptide compounds. However, substitution of Ser^{290} in TM VII of the rat receptor with isoleucine present in the human receptor increased the affinity for FK 888 20-fold and that for CP 96345 6-fold, corresponding to an affinity that was only about 4-fold less than the affinity for the human NK-1 receptor. Full human-like affinity for FK 888 and CP 96,345 could be conveyed to the rat receptor by the combined substitution of Ser^{290} in TM VII to isoleucine and Leu^{116} in TM III to valine. The NK-2 receptor-selective compound SR 48,968 was found to bind with low affinity to the human NK-1 receptor but with 15-fold even lower affinity to the rat receptor. Substitution of residue 290, which is situated within the previously located binding site for this compound, could completely account for this difference. These data demonstrate that the species selectivities of the nonpeptide antagonists CP 96345, FK 888, and SR 48,968, independently of clear differences in their chemical structures and modes of discovery, have a similar structural basis, being dependent on two divergent residues that apparently are not involved in peptide agonist binding.

In many peptide systems nonpeptide compounds are currently being developed not only as drugs but also as pharmacological and physiological tools (1). Within the past few years a series of highly selective and potent nonpeptide antagonists have been described for the NK-1 (substance P) and NK-2 (NKA) receptors (Fig. 1) (2, 3). These nonpeptide compounds have already demonstrated the importance of substance P in both nociceptive and inflammatory responses, and it is believed that nonpeptide substance P antagonists may represent a novel class of both analgesic and anti-inflammatory drugs (2–4).

Nonpeptide antagonists are most commonly developed from lead compounds discovered through screening of large chemical files (1). These include the quinuclidine derivative CP 96,345 and the perhydroisoindole derivative RP 67,580, which both are highly selective NK-1 receptor antagonists (5, 6). Another NK-1 receptor nonpeptide antagonist, SR 140,333, was developed and optimized on the basis of the high affinity NK-2 receptor antagonist SR 48,968, which in fact was also discovered originally by file screening (7, 8). However, the nonpeptide NK-1-selective compound 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 (9). Thus, the chemical structure and mode of discovery of nonpeptide tachykinin antagonists are very different in spite of their common pharmacological capability, just as none of the structures resemble the naturally occurring peptide agonists.

In contrast to the natural peptide ligands, nonpeptide antagonists often show substantial selectivity among different species variants of their target receptor (1). In the tachykinin system, CP 96,345 is known to be much less potent at the rat and mouse NK-1 receptor than at the NK-1 receptor in most other species, including humans (10, 11). Similarly, FK 888 has been described to be much more potent at the guinea pig than

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ABBREVIATIONS: NK, neurokinin; BH, Bolton Hunter; bp, base pair(s); TM, transmembrane segment.

at the rat NK-1 receptor (9), whereas the reverse selectivity has been reported for RP 67,580 (6). However, only 22 residues of 407 amino acid residues differ between, for example, the rat and human NK-1 receptors (12, 13). Apparently, during evolution mutations have occurred that, obviously due to evolutionary pressure, do not affect the binding site of the naturally occurring peptide agonist but profoundly affect the nonpeptide antagonist binding site. This is in agreement with our recent data using chimeric constructs between the NK-1 receptor and the homologous NK-2 and NK-3 (NKB) receptors, which indicated the existence of distinct binding domains for peptide agonists and nonpeptide antagonists on the NK-1 and NK-2 receptors (14, 15). Thus, delineation of structural elements that determine the species selectivity of the nonpeptide compounds would likely provide additional information about their molecular mechanism of action. In the present study we have focused on the structural basis for the significantly higher affinity of three chemically distinct, nonpeptide, tachykinin antagonists, CP 96,345, FK 888, and SR 48,968, for the human receptor, compared with the corresponding rat receptor. Surprisingly, we found by gradual humanization of the rat NK-1 receptor that the species selectivities of FK 888 and SR 48,968, independently of their different chemical structures and means of discovery, have the same structural basis as that described previously for CP 96,345 and RP 67,580 (16, 17), being primarily dependent on the same divergent residues located in TM III and VII.

Materials and Methods

Peptide and nonpeptide ligands. Substance P was purchased from Peninsula (St. Helens, Merseyside, UK). CP 96,345, (2*S*,3*S*)-(*cis*)-(2-diphenylmethyl)-*N*-[(2-methoxyphenyl)methyl]-1-azabicyclo[2.2.2]-octan-3-amine, was kindly provided by Drs. R. M. Snider and J. A. Lowe III, Pfizer Inc. (Groton, CT) (5). FK 888, N^2-[(4*R*)-4-hydroxy-1-[(1-methyl-1*H*-indole-3-yl)carbonyl]-L-propyl]-*N*-methyl-*N*-(phenylmethyl)-3-(2-naphtyl)-L-alaninamide, was kindly provided by Dr. T. Fujii, Fujisawa Pharmaceuticals Co. Ltd. (Osaka, Japan) (18–20). SR 48,968, (*S*)-*N*-methyl-*N*-[(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)-butyl]benzamide, was kindly provided by X. Emonds-Alt and J.-C. Bréliere, Sanofi Recherche (Montpellier, France) (21).

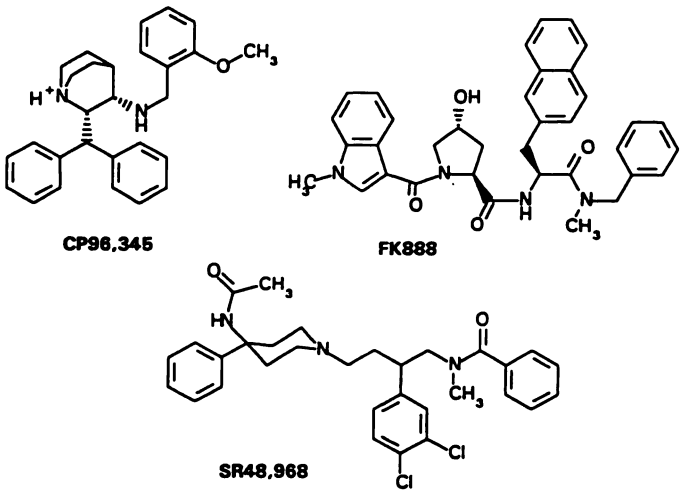

Fig. 1. Structures of tachykinin nonpeptide antagonists. Lead compounds for the development of the NK-1-selective compound CP 96,345 and the NK-2-selective compound SR 48,968 were identified by screening of chemical files (5, 7). The lead compound for the development of FK 888 was a tripeptide identified as the minimally active element in the structure of an octapeptide substance P antagonist substituted with three D-tryptophan residues (9).

Construction of mutated and chimeric NK-1 receptors. The cDNA encoding the rat NK-1 receptor was generously provided by Dr. S. Nakanishi, Institute for Immunology, Kyoto University (Kyoto, Japan) (12). Mutations in the rat NK-1 receptor, yielding r(h190–195), r(h266), r(h116), and r(h290), were introduced by use of site-directed mutagenesis in the M13 system, according to previously described methods (22). The mutations were verified by DNA sequencing analysis. The cDNAs encoding the wild-type rat NK-1 receptor and mutated receptors were cloned into the pTEJ-8 expression vector as 2049-bp *Hind*III-*Eco*RI fragments (23, 24). The cDNA encoding the wild-type human NK-1 receptor (13) was cloned into the pTEJ-8 expression vector as a 1273-bp *Hind*III-*Bam*HI fragment. Chimeric receptors were constructed using unique equivalent restriction sites in the cDNAs. Chimeric receptors r(h190–266) and r(h116,290) were constructed by joining the 1158-bp *Hind*III-*Mlu*I fragment of mutant r(h190–195) or r(h116), respectively, with the 891-bp *Mlu*I-*Eco*RI fragment of mutant r(h266) or r(h290), respectively. Chimeric receptor r(h1–54,190–266) was constructed by joining the 277-bp *Hind*III-*Stu*I fragment of the human NK-1 receptor with the 1297-bp *Stu*I-*Eco*RI fragment of r(h190–266). Chimeric receptors r(h190–407) and r(h290–407) were constructed by joining the 1380-bp *Hind*III-*Bgl*II fragment of r(h190–266) or the rat NK-1 receptor, respectively, with the 406-bp *Bgl*II-*Bam*HI fragment of the human NK-1 receptor. The appropriate restriction endonuclease fragments encoding the chimeric constructs were cloned into the pTEJ-8 expression vector (23).

Transfections and cell culture. The receptor constructs were transiently transfected into COS-7 cells by the calcium phosphate precipitation method, acccording to previously reported methods (22–24). COS-7 cells were grown as described (22).

Binding assay. Monoiodinated ^{125}I-BH-substance P were prepared and purified by reverse phase high performance liquid chromatography, according to methods described earlier (24, 25). The binding assay was performed as described (24). Briefly, the transfected COS-7 cells were transferred to 12-well culture plates (0.3–1.0 × 10^5 cells/well) 1 day after transfection and 24 hr before the binding experiments were performed. The number of cells was determined by the expression efficiency of the individual plasmid, aiming at 5–10% binding of added radioligand in competition binding experiments. Binding experiments were performed for 3 hr at 4° with 50 pM ^{125}I-BH-substance P and the indicated concentrations 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 (Sigma), and 10 μg/ml chymostatin (Sigma). All determinations were performed in triplicate. The nonspecific binding was determined as the binding of radioligand in the presence of 1 μM substance P. The specific binding constituted >80% of total bound radioligand. The binding data were analyzed and IC_{50} values were determined by computerized nonlinear regression analysis using InPlot (GraphPad Software, San Diego, CA).

Results

CP 96,345 showed, as expected from previous studies (10, 11), a marked preference for the human receptor, compared with the rat variant, having 22-fold higher affinity for the human than for the rat NK-1 receptor, as determined by transient expression of the cloned receptors in COS-7 cells (Table 1). FK 888 possessed an even more remarkable species selectivity than CP 96,345, with 76-fold higher affinity for the human than for the rat receptor, whereas the newly developed compound SR 140,333 (8) showed no species selectivity (Table 1). However, the NK-2-selective compound SR 48,968, which is chemically related to SR 140,333 and binds to the NK-2 receptor with nanomolar affinity (15), showed selectivity be-

TABLE 1

Comparison of substance P and nonpeptide tachykinin antagonist affinities between the rat and human NK-1 receptors

Data are IC_{50} values (mean ± standard error, three to five experiments) for inhibition of ^{125}I-BH-labeled substance P binding. The binding experiments were performed on transfected intact COS-7 cells and IC_{50} values were determined as described in Materials and Methods. The relative affinity for the rat receptor, compared with the human receptor, is indicated [r/h = IC_{50} (rat)/IC_{50} (human)].

	IC_{50} values		
	Human NK-1	Rat NK-1	r/h
	nM	nM	
Substance P	0.19 ± 0.07	0.27 ± 0.04	1.4
CP 96,345	0.38 ± 0.03	8.1 ± 1.2	21
FK 888	1.08 ± 0.18	82 ± 15	76
SR 140,333	0.51 ± 0.07	1.0 ± 0.2	2.0
SR 48,968	320 ± 80	4900 ± 700	15

tween the human and rat NK-1 receptors. Thus, SR 48,968 inhibited binding of radiolabeled substance P to the human NK-1 receptor with 15-fold higher potency, compared with the rat receptor (Table 1). To identify residues that determine this selectivity of CP 96,345, FK 888, and SR 48,968 for the human NK-1 receptor, we gradually mutated the divergent residues of the rat receptor to the corresponding residues of the human receptor. Importantly, using this strategy in our mutational analysis binding energy was gained instead of lost. Because mutational studies have been performed to investigate the selectivity of CP 96,345 (16, 17), we focused on the newly developed compounds FK 888 and SR 48,968, using CP 96,345 as our control ligand.

FK 888 and CP 96,345. Recently, by construction of chimeric receptors between NK-1 and NK-3 receptors we have localized the apparent binding site for both FK 888 and CP 96,345 to a region around the top of TM V and VI of the NK-1 receptor (14).[2] In this part of the receptor, in the extracellular loop just adjacent to TM V, three residues (residues 190, 191, and 195) differ between the rat and human receptors, and in the outer part of TM VI one residue is divergent (residue 266) (Fig. 2). Surprisingly, neither the exchange of the residues at the top of TM V [r(h190–195)], the exchange of the residue in TM VI [r(h266)], nor the combined exchange of all four divergent residues at the top of TM V and VI [r(h190–266)] increased the ability of CP 96,345 or FK 888 to inhibit binding of radiolabeled substance P (Table 2).

Additional humanization of r(h190–266) by incorporation of the extracellular amino terminus and TM I from the human receptor [r(h1–54,190–266)] was likewise without significant effect (Table 2). However, incorporation of TM VII and the intracellular carboxyl-terminal part from the human receptor [r(h190–407)] markedly increased the affinities of both CP 96,345 and FK 888, i.e., to affinities that were only approximately 4-fold less than the affinities for the human receptor (Table 2). This was solely due to the exchange of TM VII and the intracellular carboxyl terminus of the rat receptor with the corresponding part of the human receptor [r(h290–407)], because this exchange resulted in the same increase in affinity (Table 2). Several amino acid residues differ between the rat and human NK-1 receptors in the intracellular carboxyl terminus, whereas only one residue (residue 290) differs in TM VII (Fig. 2). Mutating this residue from serine to isoleucine caused an increase in affinity of CP 96,345 and FK 888 similar to that found for r(h290–407) (Fig. 3; Table 2), indicating that residue 290 is a major determinant of both CP 96,345 and FK 888 species selectivity.

Only four divergent residues (residues 80, 95, 116, and 152) were remaining candidates for the selectivity not determined by residue 290 (Fig. 2). Mutation of residue 116, which recently was found to partially contribute to CP 96,345 selectivity (16), from leucine to valine resulted, as expected, in a modest 2-fold increase in CP 96,345 affinity, whereas a 5-fold increase in affinity was observed for FK 888 (Fig. 3; Table 2). Importantly, the combined conversion of both residues 116 and 290 [r(h116,290)] conveyed full affinity for FK 888 and essentially full human-like affinity for CP 96,345 (Fig. 3; Table 2). Thus, the selectivity of both CP 96,345 and FK 888 between the rat and human receptors can apparently be determined by residue 290, with some additional contribution from residue 116.

SR 48,968. Through systematic generation of chimeric constructs between the NK-1 receptor and the homologous NK-2 receptor, we have recently localized the apparent binding site for SR 48,968 to a region around TM VI and VII (15). Two residues differ between the rat and human NK-1 receptors in this region (residues 266 and 290) (Fig. 2). The mutation of Val^{266} to isoleucine [r(h266)] did not change the affinity of SR 48,968 (data not shown). However, conversion of Ser^{290} to isoleucine [r(h290)] conveyed full human-like affinity for SR 48,968, i.e., the IC_{50} value decreased to 220 ± 60 nM (mean ± standard error, three experiments), similar to the affinity for the wild-type human receptor (IC_{50} = 320 ± 80 nM) (Fig. 3; Table 1). Thus, residue 290 alone can apparently account for the species selectivity of SR 48,968. It should be noted that mutating Leu^{116} to valine did not increase SR 48,968 affinity (IC_{50} = 9100 ± 1300 nM, mean ± standard error, three experiments) and the combined exchange of 116 and 290 [r(h116,290)] resulted in SR 48,968 affinity (IC_{50} = 370 ± 40 nM, mean ± standard error, three experiments) similar to that observed for r(h290) (data not shown).

Discussion

In the present study we have investigated the molecular mechanism underlying the species selectivity of a series of tachykinin nonpeptide antagonists that are very different both in their chemical structure and in their means of discovery (5, 7, 9). We found that, despite these overt chemical differences, the selectivity between the rat and human NK-1 receptors for two nonpeptide NK-1 receptor antagonists, CP 96,345 and FK 888, and one NK-2 receptor antagonist, SR 48,968, was dependent on the same amino acid residue in TM VII (residue 290), with additional contributions from residue 116 in TM III for CP 96,345 and FK 888. Thus, the recently demonstrated importance of residue 290 for CP 96,345 and RP 67,580 selectivity (16, 17) can be extended to nonpeptide compounds without any similarity to CP 96,345 and RP 67,580. In other words, full human NK-1 receptor pharmacology with respect to nonpeptide antagonist binding could be conveyed to the rat receptor by the combined mutation of Ser^{290} to isoleucine and Leu^{116} to valine. In parallel with these data, it has also been found in systems other than the tachykinin system that the species selectivity of small antagonist ligands is dependent on residues localized in the carboxyl-terminal part of the receptor. For example, in the cholecystokinin system a conservative substi-

[2] U. Gether, X. Emonds-Alt, J.-C. Brelière, T. Fuji, D. Hagiwara, L. Pradier, G. Garret, T. E. Johansen, and T. W. Schwartz, unpublished observations.

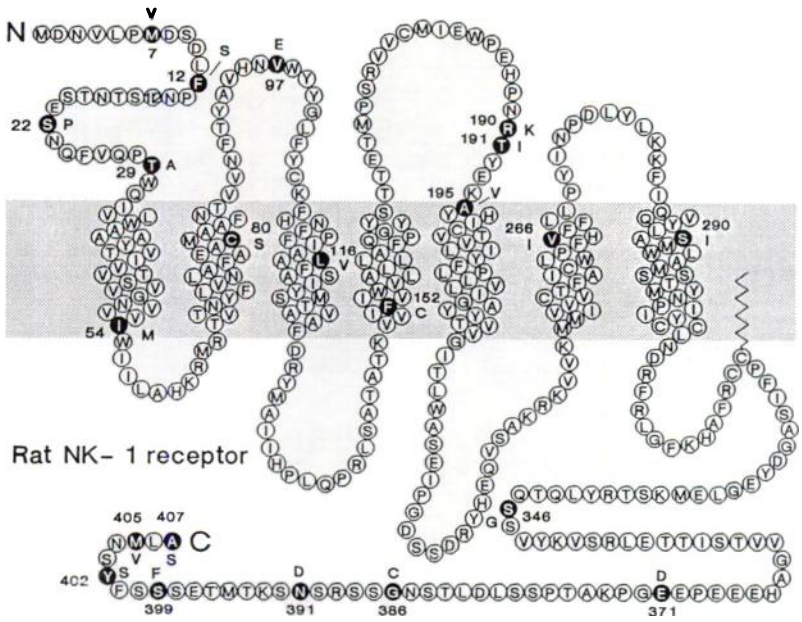

Fig. 2. Proposed two-dimensional structure and amino acid sequence of the rat NK-1 receptor embedded in the cell membrane (12). ○, Amino acids conserved between the rat and human NK-1 receptors; ●, divergent residues. The corresponding residues in the human receptor are indicated.

TABLE 2

Binding affinities of substance P, FK 888, and CP 96,345 for the wild-type rat and human NK-1 receptors and mutated NK-1 receptors

Data are IC_{50} values (mean ± standard error, three to five experiments). The binding experiments were performed on transfected intact COS-7 cells and IC_{50} values were determined as described in Materials and Methods. The relative affinities, compared with the human receptor, are indicated in parentheses [IC_{50} (mutant or rat receptor)/IC_{50} (human receptor)]. Nomenclature of mutant receptors is as follows: mutant r(h190-195), for example, indicates a rat NK-1 receptor in which the divergent residues between residues 190 and 195 (both included) have been substituted with the corresponding residues of the human NK-1 receptor.

	IC_{50} values		
	Substance P	FK 888	CP 96,345
		nM	
Rat NK-1	0.27 ± 0.04	82 ± 15 (76)	8.1 ± 1.2 (21)
r(h190-195)	0.18 ± 0.05	100 ± 27 (93)	11 ± 3 (29)
r(h266)	0.33 ± 0.17	120 ± 20 (111)	14 ± 4 (37)
r(h190-266)	0.21 ± 0.07	60 ± 10 (56)	10 ± 2 (26)
r(h1-54,190-266)	0.10 ± 0.03	50 ± 6 (46)	13 ± 4 (34)
r(h190-407)	0.33 ± 0.23	3.4 ± 0.6 (3.1)	1.26 ± 0.19 (3.3)
r(h290-407)	0.16 ± 0.09	5.6 ± 1.0 (5.2)	1.50 ± 0.14 (3.9)
r(h290)	0.31 ± 0.13	5.0 ± 1.0 (4.6)	1.6 ± 0.2 (4.2)
r(h116)	0.42 ± 0.13	16 ± 3 (15)	4.3 ± 0.7 (11)
r(h116,290)	0.47 ± 0.12	1.18 ± 0.07 (1.1)	0.67 ± 0.06 (1.8)
Human NK-1	0.19 ± 0.07	1.08 ± 1.18 (1.0)	0.38 ± 0.03 (1.0)

tution (leucine to valine) in TM VI can fully account for the species selectivity of two nonpeptide cholecystokinin antagonists between the human and canine cholecystokinin type B/gastrin receptors (26). In addition, a single amino acid residue in TM VII confers species selectivity to the 5-hydroxytryptamine type 1B receptor (27). The same residue has previously been found to be important for the differentiation of a class of β-adrenergic receptor antagonists between the α_2- and β_2-adrenergic receptors and for binding of the same antagonists to the 5-hydroxytryptamine type 1A receptor (28, 29).

In our previous studies we have localized the apparent binding site for CP 96,345 and FK 888 to epitopes around the outer part of TM V and VI (14).[3] The amino acid residues that differ between the rat and human receptors in this region surprisingly did not seem to be involved in determining the species selectivity of CP 96,345 and FK 888 (Table 2). Thus, we could not reproduce the weak effect on CP 96,345 affinity that previously has been found by substitution of residues 190, 191, 195, and 266 (17). In contrast, the single amino acid residue that differs in TM VII (residue 290) was identified as the most important for the observed species selectivities of CP 96,345 and FK 888. The same residue in TM VII was also responsible for the NK-1 receptor selectivity of SR 48,968 (Fig. 3). However, this was not surprising, because we earlier localized important points of interaction of SR 48,968 around TM VI and VII of the NK-2 receptor (15).

The change in binding affinity of the nonpeptide compounds produced by mutating residue 290 could be due to either a direct interaction of the nonpeptide compounds with this residue or an indirect structural effect on the nonpeptide antagonist binding site. We tend to believe that the effect is indirect. First, it is less likely that compounds with no similarity in their chemical structure all have specific interactions with the same single amino acid residue. From our chimeric studies we also know that, although the compounds may bind within the same general domain, they interact with different subepitopes (14, 15).[4] The gain of binding energy obtained by mutating Ser^{290} to isoleucine corresponds well to the energy of a hydrogen bond (approximately 1–1.5 kcal/mol). This binding energy is gained by removing a putative hydrogen bond donor, serine, and inserting the hydrophobic isoleucine, with which only van der Waals interactions are likely to occur. It is more likely that this mutation could induce a small but not overall change in the packing of the transmembrane helices and thereby indirectly disrupt the geometry of a hydrogen bond between the ligands and an evolutionarily conserved residue located in the same general area of the receptor. Alternatively, it could be speculated that the substitution of serine with isoleucine disrupts a hydrogen bond within the receptor. This could lead to

[3] U. Gether, X. Emonds-Alt, J.-C. Brelière, T. Fuji, D. Hagiwara, L. Pradier, C. Garret, T. E. Johansen, and T. W. Schwartz, unpublished obeservations.

[4] U. Gether, X. Emonds-Alt, J.-C. Brelière, T. Fuji, D. Hagiwara, L. Pradier, C. Garret, T. E. Johansen, and T. W. Schwartz, unpublished obeservations.

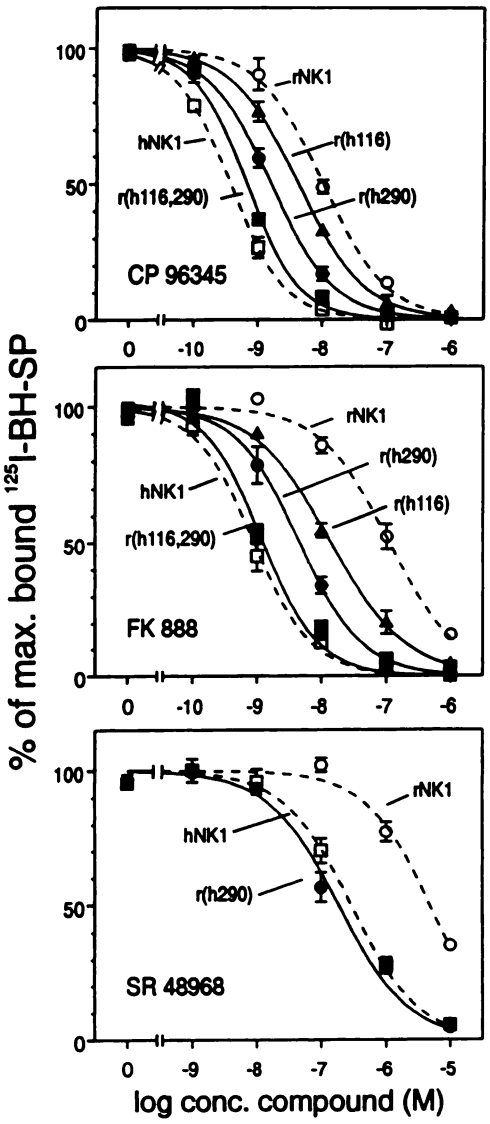

Fig. 3. Identification of residues 290 and 116 as critical for nonpeptide antagonist selectivity. *Upper* and *middle*, competition binding of ^{125}I-BH-substance P with CP 96,345 (*upper*) and FK 888 (*middle*) to the human (□) and rat (○) NK-1 receptors and the mutated receptors r(h116) (▲), r(h290) (●), and r(h116,290) (■). *Lower*, competition binding of ^{125}I-BH-substance P with SR 48,968 to the human (□) and rat (○) NK-1 receptors and the mutant receptor r(h290) (●). The binding experiments were performed on transfected intact COS-7 cells, as described in Materials and Methods. Data are percentage of maximum bound ^{125}I-BH-substance P (mean ± standard error, three to five experiments).

release of a conformational constraint, which may result in a more favorable conformation of a common putative nonpeptide antagonist binding pocket. It should be noted that point mutations in TM VII of, for example, adrenergic receptors are known to profoundly affect the helical packing and eventually the proper folding of the receptor (30). The highly conservative substitution of Leu^{116} in TM III with valine could also be due to both a direct effect and an indirect effect. The involvement of residue 116 in both CP 96,345 and FK 888 species selectivity suggests that the effect is indirect. However, both a direct or an indirect effect are likely to occur, according to the crystallographic projection map of rhodopsin (31) that positions TM III centrally in the molecule in close proximity to TM V and VI, which previously have been found to comprise an important part of the nonpeptide antagonist binding domain (14, 15, 32).

Regardless of whether the effect of substituting residues 116 and 290 is direct or indirect, it is interesting to find that the species selectivity of several chemically unrelated nonpeptide antagonists has the same structural basis, being critically dependent on primarily the same amino acid residues, which are not involved in peptide agonist binding. In this way, the present results support our previous data on chimeric NK-1/NK-3 and NK-1/NK-2 receptors (14, 15), suggesting that chemically distinct nonpeptide antagonists act through different subepitopes within a common general binding domain, which may be largely different from the peptide agonist binding site.

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