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Both Extracellular and Transmembrane Residues Contribute to the Species Selectivity of the Neurokinin-1 Receptor Antagonist WIN 51708

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SUMMARY

WIN 51708 is a nonpeptide antagonist of the neurokinin (NK)-1 (substance P) receptor that possesses a dramatically higher affinity for the rat NK-1 receptor, compared with the human NK-1 receptor. This selectivity is the opposite of the selectivity displayed by CP-96,345 and is much greater in magnitude than the selectivity of RP 67580. The naturally occurring peptide agonist substance P shows no such species selectivity. To determine the molecular basis for the species selectivity of WIN 51708, a series of chimeric and point-mutated NK-1 receptors were created and functionally expressed in Chinese hamster ovary cells. Residue 97 in the first extracellular loop and residue 290 in the seventh putative transmembrane domain are critical determinants for the selectivity of WIN 51708 for the rat over the human NK-1 receptor. Although mutation of either residue 97 or residue 290 in the rat NK-1 receptor is sufficient for a low, human-like affinity for WIN 51708, both of these residues must be simultaneously mutated in the human NK-1 receptor to allow nearly rat wild-type affinity for this antagonist. This suggests that the binding environment for WIN 51708 in the rat NK-1 receptor differs, at least in part, from the binding environment in the human NK-1 receptor. In addition, although residue 290 is critical for the species selectivity of WIN 51708 and CP-96,345, residue 97 does not play a role in the species selectivity of CP-96,345. These data support a model in which the binding environments for WIN 51708 and CP-96,345 in part differ.

The NK-1 receptor, also known as the SP receptor, is a member of the G protein-coupled superfamily of receptors. It is distinguished from other NK receptors (NK-2 and NK-3) by its rank order of potency of SP > NKA > NKB for naturally occurring tachykinin peptide agonists. The NK-1 receptor has been cloned from several species (1–6) and has been found to contain seven putative membrane-spanning regions, consistent with its pharmacological identification as a G protein-coupled receptor (7, 8). The tachykinin peptide/receptor system has been shown to be involved in a variety of physiological functions, including pain transmission, salivary secretion, and smooth muscle contraction (9–11). Furthermore, these receptors have also been implicated in several disease states, including asthma, arthritis, and inflammatory bowel syndrome (12–14). Consequently, the desire for nonpeptide antagonists for tachykinin receptors is strong, and several classes have recently been developed (see Refs. 15 and 16 for reviews).

Several high affinity nonpeptide antagonists of the NK-1 receptor that display dramatic species selectivity have been identified. These include CP-96,345 (17, 18), RP 67580 (19), and WIN 51708 (20). Although these antagonists show a marked selectivity for NK-1 receptors from some species, compared with others (17, 19, 21, 22), SP shows no such species selectivity. The molecular basis for the species selectivity of CP-96,345 (23, 24) and RP 67580 (24) has recently been elucidated. Additionally, several residues of the NK-1 receptor have recently been implicated in direct interactions with CP-96,345 (25, 26). The information from such studies not only will aid in the design of new and improved antagonists but also should contribute to the understanding of the structural basis of receptor function.

WIN 51708, a steroid-based molecule with a nitrogen-containing heterocycle attached to the steroid A-ring (Fig. 1), is a competitive NK-1 receptor antagonist (20) and belongs to a different chemical class than CP-96,345 or RP 67580. WIN 51708 has been shown to be a functional antagonist *in vivo* for SP-induced hindpaw plasma extravasation as well as SP-induced salivation in rats (20). This antagonist also displays a dramatic preference for the NK-1 receptor from rat tissue, compared with human tissue (22), which is the converse of CP-96,345, which interacts with higher affinity at the human NK-1 receptor than at the rat NK-1 receptor. Because the cloned NK-1 receptors from these two species differ in only 22 of 407

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ABBREVIATIONS: NK, neurokinin; SP, substance P; CHO, Chinese hamster ovary.

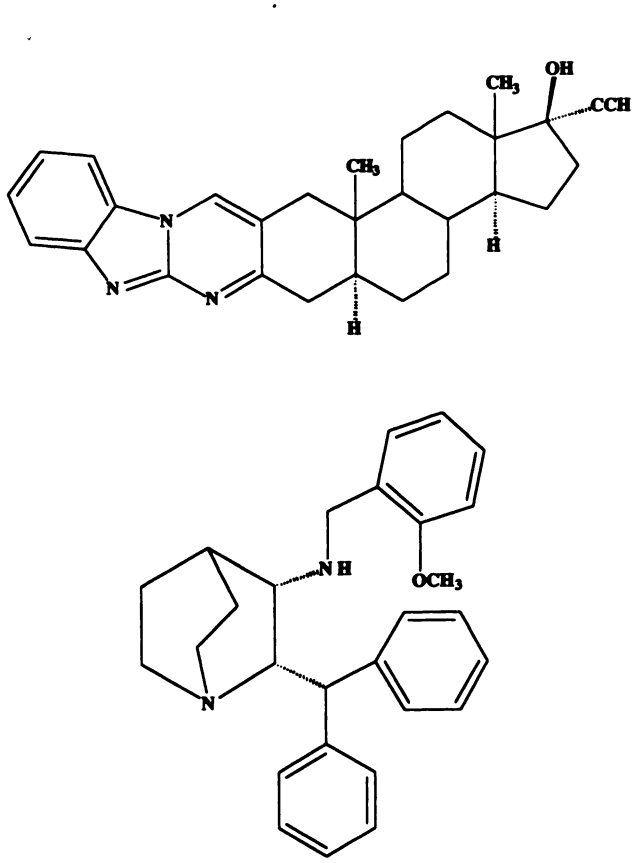

Fig. 1. Structural formulae of NK-1 receptor antagonists. A, WIN 51708; B, CP-96,345.

residues, it is likely that one or more of these divergent residues are responsible for the species selectivity of WIN 51708. In the present studies, we have determined that several residues, located both extracellularly and within putative transmembrane domains, contribute to the species selectivity of WIN 51708 at the rat and human NK-1 receptors. These results are discussed with regard to molecular determinants for the species selectivity for CP-96,345.

Experimental Procedures

Materials. PRISM T7 terminator single-stranded DNA sequencing kit was obtained from Applied Biosystems (Foster City, CA). Geneticin (G418) was obtained from GIBCO-BRL. Other materials and reagents have been described previously (23, 27).

Construction of chimeric and point-mutated NK-1 receptors. Construction of the chimeric NK-1 receptors has been described previously (23). Site-directed mutagenesis was performed as described previously (23), using the M13-based oligonucleotide-directed *in vitro* mutagenesis system from Amersham. For mutation of the human NK-1 receptor the following oligonucleotides were used: 5′-AGCCATGCAGGCCTC-3′ for S80→C, 5′-GTACCACACGTTGTG-3′ for E97→V, 5′-GGCGAAGAGAGCGGC-3′ for V116→L, 5′-GATGACAAAGATGAC-3′ for C152→F, and 5′-CAACATGCTGGCCAG-3′ for I290→S. For mutation of the rat NK-1 receptor the following oligonucleotides were used: 5′-AGCCATGGAGGCCTC-3′ for C80→S, 5′-GTACCATTCATTGTG-3′ for V97→E, 5′-GGCGAAGACAGCAGC-3′ for L116→V, 5′-GATGACACAGATGACCA-3′ for F152→C, and 5′-CAACATGATGGCCAG-3′ for S290→I. Wild-type human NK-1 receptor was used as the template for mutagenesis in the construction of H-S80C, H-E97V, H-V116L, H-C152F, and H-I290S. Wild-type rat NK-1 receptor was used as the template for mutagenesis in the construction of R-C80S, R-V97E, R-L116V, R-F152C, and R-S290I. H-I290S was used as the template in the construction of H-S80C,I290S, H-E97V,I290S, H-V116L,I290S, and H-C152F,I290S. H-S80C,I290S was used as the template in the construction of H-S80C,V116L,I290S and H-S80C,C152F,I290S. H-V116L,I290S was used as the template for construction of H-E97V,V116L,I290S, H-V116L,C152F,I290S, and H-S80C,V116L,C152F,I290S. H-S80C,V116L,C152F,I290S was used as the template for construction of H-S80C,E97V,V116L,C152F,I290S. The nucleotide sequences of all mutated cDNAs used were verified by the chain termination method of Sanger (28), using the PRISM T7 terminator single-stranded DNA sequencing kit (Applied Biosystems), and sequences were analyzed on an automated 373A DNA sequencer (Applied Biosystems). Mutant NK-1 receptor cDNAs were isolated from M13 mp19 replicative form using *Hind*III and *Bam*HI and were subcloned into pBS. The cDNAs were then subcloned into pM^2-AH (23) using *Xho*I and *Bam*HI. Plasmid DNA was purified using an anion exchange procedure (QIAGEN Inc.)

Stable transfection of CHO cells. One day before transfection, CHO cells were plated at a density of 3×10^5 cells/10-cm diameter tissue culture dish. The cells were transfected with 10 μg of pM^2-AH plasmid containing the appropriate NK-1 receptor cDNA insert by using a calcium phosphate transfection procedure, as described previously (27). Cells were grown in minimal essential medium-α containing 10% fetal bovine serum, with 0.8 mg/ml G418, for 7–10 days. For each plasmid, all colonies were collected in a single pool and propagated. We refer to this pool for a single DNA construct as a population stable cell line. Population stable cell lines provide the convenience of stable cell lines while avoiding the labor-intensive characterization of individual clonal cell lines and the potential problems associated with the site of integration.

Radioligand binding and data analysis. Ligand binding was performed as described previously (23, 27). Briefly, incubation of transfected CHO cells with 0.07 nM ^{125}I-Tyr^{-1}-SP was performed at 4° for 2 hr (to equilibrium) in the presence of increasing amounts of unlabeled competitor. Due to the limited solubility of WIN 51708, dimethylsulfoxide was present at a final concentration of 1% (v/v). Under these conditions the maximal final concentration of WIN 51708 that could be obtained was 5 μM. Total binding was determined by rapid filtration over no. 32 glass filters (Schleicher and Schuell). Nonspecific binding was defined as the cpm bound in the presence of 1 μM SP and was <10% of total binding. IC_{50} values were determined by the equation log $[B_I/(B_o - B_I)] = (n_H \cdot \log [I]) + (n_H \cdot \log IC_{50})$, where B_I is the cpm of ^{125}I-Tyr^{-1}-SP specifically bound in the presence of unlabeled ligand, B_o is the cpm specifically bound in the absence of unlabeled ligand, [I] is the concentration of unlabeled ligand, and n_H is the indirect Hill coefficient (29).

Results

The selectivity of WIN 51708 for the cloned rat and human NK-1 receptors was evaluated using stably transfected CHO cell lines that had been established previously (27).[1] These cell lines express either the rat NK-1 or human NK-1 receptor at levels of 2×10^5 to 5×10^5 high affinity sites/cell. The structural formula of WIN 51708 is shown in Fig. 1. WIN 51708 displaced ^{125}I-Tyr^{-1}-SP from the rat NK-1 receptor with an IC_{50} value of 25 nM, whereas under the same conditions this antagonist had an IC_{50} value of ≥10,000 nM for the human NK-1 receptor (Fig. 2; Table 1). These and subsequent displacement studies were performed with a 2-hr incubation period, because time course studies demonstrated that binding was at steady state at this time (data not shown). These data show that WIN 51708 displays at least a 400-fold higher affinity for the rat, compared with the human, NK-1 receptor. Because the primary sequences

[1] Y. Takeda and J. E. Krause, manuscript in preparation.

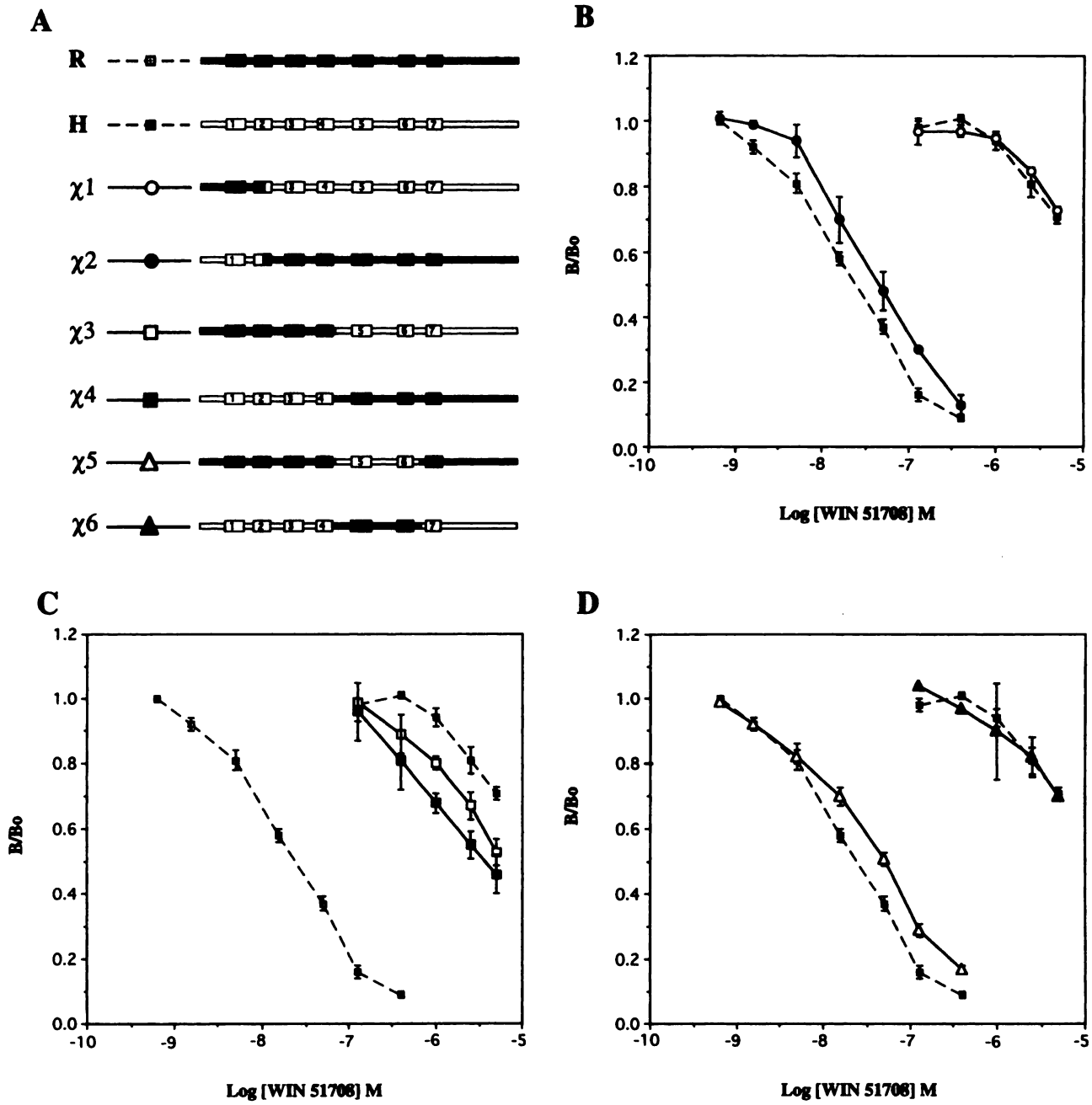

Fig. 2. Analysis of WIN 51708 binding to wild-type and chimeric NK-1 receptors. A, Schematic illustration of wild-type and chimeric rat/human NK-1 receptors. *Filled areas*, sequences from the rat receptor (*R*); *open areas*, sequences from the human receptor (*H*). *Boxes 1–7*, putative transmembrane domains. Abbreviated names and symbols are shown at the *left*. Full names are as follows: χ1, R(1–79)χH(80–407); χ2, H(1–79)χR(80–407); χ3, R(1–185)χH(186–407); χ4, H(1–155)χR(156–407); χ5, R(1–185)χH(186–275)χR(276–407); χ6, H(1–155)χR(156–275)χH(276–407). B-D, Displacement of 0.07 nM ^{125}I-Tyr^{-1}-SP by WIN 517087 at wild-type and chimeric NK-1 receptors. Each *curve* represents three independent experiments performed in duplicate, and each *data point* is the mean ± standard error.

of these two cloned receptors differ in only 22 of 407 residues (3), we hypothesized that one, or some combination, of these divergent residues must be responsible for this dramatic affinity difference.

As a first step in the identification of the residue(s) responsible for the difference in the affinity of WIN 51708 for the rat and human NK-1 receptors, three sets of complementary rat/human chimeric receptors were constructed (23) and stably expressed in CHO cells. These chimeric receptors are represented schematically in Fig. 2A. Results for χ1 and χ2 show that residues amino-terminal to Ala^{79} (located in the second putative transmembrane domain) do not alter WIN 51708 affinity, because this antagonist displayed a similar affinity for these chimeric receptors, compared with the human and rat wild-type receptors, respectively (Fig. 2B; Table 1). The same can be said for residues located between Trp^{155} (in the fourth transmembrane domain) and Pro^{275} (in the third extracellular loop), based on the displacement data for chimeric receptors χ5 and χ6 (Fig. 2D; Table 1). WIN 51708 displayed IC_{50} values of 5500 nM and 4500 nM for χ3 and χ4, respectively (Fig. 2C; Table 1). The affinity of WIN 51708 for both of these chimeric receptors is similar to that for the human NK-1 receptor. The

TABLE 1

WIN 51708, SP, and CP-96,345 displacement of ^{125}I-Tyr^{-1}-SP from wild-type, chimeric, and mutated NK-1 receptors

IC_{50} values are expressed as the mean ± standard error; when only two independent experiments were performed, the error value shown is the standard deviation. The numbers in parentheses are the numbers of independent experiments.

Receptor	WIN 51708		SP		CP-96,345	
	IC_{50}	n_H	IC_{50}	n_H	IC_{50}	n_H
	nM		nM		nM	
Rat	25 ± 3 (3)	0.87	1.5 ± 0.2 (4)[a]	1.00	40 ± 5 (8)[a]	0.90
Human	≥10,000 (3)	0.95	0.67 ± 0.09 (3)[a]	0.96	0.44 ± 0.04 (8)[a]	1.00
χ^1	≥10,000 (3)	1.10	0.81 ± 0.27 (2)[a]	0.96	0.54 ± 0.04 (3)[a]	1.00
χ^2	45 ± 11 (3)	0.93	1.0 ± 0.5 (2)[a]	0.74	53 ± 8 (3)[a]	1.00
χ^3	5,500 ± 900 (3)	0.96	0.73 ± 0.26 (2)[a]	1.20	1.2 ± 0.1 (3)[a]	0.80
χ^4	4,500 ± 1,400 (3)	0.68	1.5 ± 0.2 (2)[a]	0.91	38 ± 9 (3)[a]	0.96
χ^5	46 ± 8 (3)	0.77	0.82 ± 0.30 (2)[a]	1.00	12 ± 2 (4)[a]	0.85
χ^6	≥10,000 (3)	1.00	0.71 ± 0.40 (2)[a]	0.89	1.2 ± 0.3 (4)[a]	1.10
H-S80C	≥10,000 (2)	0.98	0.50 ± 0.04 (2)	0.93	1.2 ± 0.1 (2)	0.96
H-E97V	≥10,000 (3)	1.30	0.60 ± 0.21 (2)	0.87	0.52 ± 0.08 (3)	0.98
H-V116L	2,400 ± 100 (2)	1.20	0.79 ± 0.12 (2)	0.79	1.4 ± 0.4 (2)	1.10
H-C152F	≥10,000 (2)	0.79	0.70 ± 0.12 (2)	0.79	1.4 ± 0.4 (2)	1.10
H-I290S	5,600 ± 800 (2)	0.97	1.1 ± 0.1 (2)[a]	0.96	8.4 ± 0.5 (3)[a]	0.94
R-C80S	93 ± 0 (2)	1.00	1.5 ± 0.2 (2)	0.93	22 ± 4 (3)	0.87
R-V97E	1,500 ± 170 (3)	0.82	1.8 ± 0.6 (2)	0.90	74 ± 11 (3)	0.82
R-L116V	340 ± 50 (2)	0.86	1.8 ± 1.0 (2)	1.00	17 ± 2 (3)	0.77
R-F152C	43 ± 12 (2)	0.79	1.1 ± 0.4 (2)	0.96	32 ± 3 (3)	0.89
R-S290I	5,600 ± 600 (2)	1.10	0.90 ± 0.49 (2)[a]	0.90	2.2 ± 0.2 (3)[a]	0.98
H-S80C,I290S	4,600 ± 1,200 (2)	0.73	1.4 ± 0.1 (2)	1.10	13 ± 1	0.95
H-E97V,I290S	220 ± 10 (3)	0.70	1.1 ± 0.15 (2)	1.00	4.1 ± 0.09 (3)	0.80
H-V116L,I290S	2,100 ± 600 (2)	0.80	1.3 ± 0.4 (2)	1.00	22 ± 1 (4)	0.97
H-C152F,I290S	9,000 ± 200 (2)	0.82	2.1 ± 0.8 (2)	1.10	14 ± 2 (2)	1.10
H-S80C,V116L,I290S	1,600 ± 200 (3)	0.88	0.88 ± 0.07 (2)	1.00	33 ± 11 (2)	0.77
H-E97V,V116L,I290S	1,200 ± 100 (3)	0.94	1.0 ± 0.1 (2)	0.79	21 ± 3 (3)	0.85
H-S80C,C152F,I290S	3,400 ± 300 (3)	0.78	1.1 ± 0.1 (2)	1.10	15 ± 3 (2)	0.84
H-V116L,C152F,I290S	1,600 ± 200 (3)	0.78	1.0 ± 0.1 (2)	0.98	26 ± 10 (2)	0.87
H-S80C,V116L,C152F,I290S	730 ± 70 (3)	0.89	0.61 ± 0.13	1.10	31 ± 8 (2)	0.85
H-S80C,E97V,V116L,C152F,I290S	40 ± 9 (3)	0.74	0.67 ± 0.09 (3)	1.1	18 ± 1 (3)	0.85

[a] From Ref. 23.

results from the chimeric receptors suggest that only one of several residues from the human NK-1 receptor sequence is required to diminish the affinity of WIN 51708 to micromolar levels, whereas at least two residues from the rat NK-1 receptor sequence may be required to allow nanomolar interaction of this antagonist with the NK-1 receptor. Furthermore, at least one of these residues must come from sequences amino-terminal to Trp^{155}, and at least one must come from sequences carboxyl-terminal to Trp^{155}. The affinity of WIN 51708 observed using a deletion mutant of the rat NK-1 receptor [NK-1(1–325)] (30) was identical to that for the rat NK-1 receptor (data not shown). Taken together, these results leave one candidate residue, residue 290, carboxyl-terminal to Trp^{155} and four candidate residues, residues 80, 97, 116, and 152, amino-terminal to Trp^{155} that could be responsible for the difference in the affinity of WIN 51708 for the rat and human NK-1 receptors.

To assess the contribution of each of these five residues to the species selectivity of WIN 51708, each was mutated individually in the rat NK-1 receptor to its human equivalent and conversely in the human NK-1 receptor to its rat equivalent. WIN 51708 displayed micromolar affinity for all of the single mutants of the human NK-1 receptor (Table 1). This is consistent with the hypothesis generated from the chimeric receptors, that at least two residues from the rat NK-1 receptor are required to impart higher affinity binding of WIN 51708 at the human NK-1 receptor. Beginning with the rat NK-1 receptor, mutation of either Val^{97} to glutamate or Ser^{290} to isoleucine was sufficient to lower the affinity of WIN 51708 to micromolar levels (Fig. 3; Table 1). On the other hand, mutation of the other three candidate residues (Cys^{80}, Leu^{116}, and Phe^{152}) in the rat NK-1 receptor to their human equivalents resulted in little or no decrease in the affinity of WIN 51708 (Table 1).

A mutant of the human NK-1 receptor in which residues 97 and 290 were simultaneously mutated (H-E97V,I290S) displayed close to rat wild-type affinity for WIN 51708 (Fig. 3B; Table 1). The IC_{50} of WIN 51708 for this mutant receptor was 220 nM, only ~9-fold higher than that for the wild-type rat NK-1 receptor and ≥45-fold lower than that for the wild-type human NK-1 receptor. Other double mutants of the human NK-1 receptor (H-S80C,I290S, H-V116L,I290S, and H-C152F,I290S) displayed IC_{50} values for WIN 51708 similar to that of the wild-type human NK-1 receptor (Table 1). These data are consistent with the data from chimeric receptors and show that, whereas mutation of either Val^{97} to glutamate or Ser^{290} to isoleucine in the rat receptor is sufficient to lower the affinity of WIN 51708 to levels similar to that seen with the wild-type human NK-1 receptor, simultaneous mutation of Glu^{97} to valine and Ile^{290} to serine in the human NK-1 receptor is required to create an environment in which WIN 51708 can interact with an affinity close to that for the wild-type rat NK-1 receptor.

In an attempt to impart wild-type rat affinity for WIN 51708 to the human NK-1 receptor, Val^{116} was mutated to leucine in

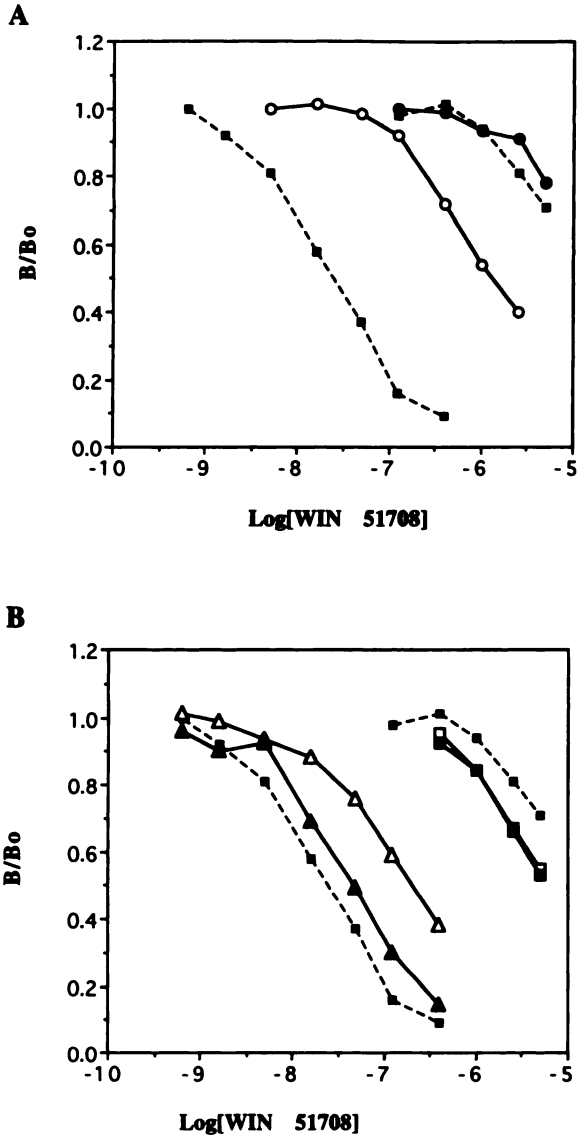

Fig. 3. Displacement of 0.07 nM ^{125}I-Tyr^{-1}-SP by WIN 51708 at wild-type and point-mutated NK-1 receptors. Each *curve* represents two or three independent experiments performed in duplicate, and each *data point* represents the mean ± standard deviation (for two independent experiments) or the mean ± standard error (for three independent experiments). The receptors shown are rat NK-1 (⊡) and human NK-1 (■) (A and B), R-V97E (○) and H-E97V (●) (A), and R-S290I (□), H-I290S (■), H-E97V,I290S (△), and H-S80C,E97V,V116L,C152F,I290S (▲) (B).

the residue 97/residue 290 double-mutant, giving H-E97V,V116L,I290S. To our surprise, the affinity of WIN 51708 was 5-fold lower for this receptor than for H-E97V,I290S (Table 1). Further addition of the mutations Ser^{80} to cysteine and Cys^{152} to phenylalanine resulted in a receptor for which WIN 51708 displayed an IC_{50} value equivalent to that for the rat wild-type NK-1 receptor (H-S80C,E97V,V116L,C152F,I290S; see Fig. 3B and Table 1). These data show that, together, these five residues are sufficient to impart an affinity of WIN 51708 for the human receptor that is identical to that for the wild-type rat NK-1 receptor. Furthermore, the data for H-E97V,V116L,I290S, compared with H-E97V,I290S and H-S80C,E97V,V116L,C152F,I290S, suggest that a molecular incompatibility is present in H-E97V,V116L,I290S that results in the lower affinity of WIN 51708 for this receptor. Whereas the mutant NK-1 receptors described above displayed differences in WIN 51708 binding, SP binding to all receptor mutants was similar to that to the wild-type rat and human NK-1 receptors.

The antagonist CP-96,345 is known to exhibit an affinity for the human NK-1 receptor that is approximately 90-fold greater than that for the rat NK-1 receptor (17). The molecular basis for this difference has been elucidated (23, 24). To examine the role of residues identified in this study in the binding of CP-96,345, CP-96,345 displacement of ^{125}I-Tyr^{-1}-SP was performed (Table 1). Residue 290 is largely responsible for the species difference in the affinity of CP-96,345, as has been previously reported and is seen with R-S290I and H-I290S (Table 1) (23). Residue 290 is therefore responsible in part for the species differences in the affinity of both CP-96,345 and WIN 51708.

The affinity of CP-96,345 for R-V97E and H-E97V was similar to that for rat and human wild-type NK-1 receptors, respectively (Table 1), failing to support a role for residue 97 in the species difference exhibited by CP-96,345. In addition, the affinity of CP-96,345 for H-E97V,I290S was similar to that for H-I290S and the affinity for H-E97V,V116L,I290S was similar to that for H-V116L,I290S. These data support the notion that residue 97 does not contribute to the species difference of CP-96,345, whereas it is critical for the species difference of WIN 51708. The binding environments of these two antagonists must therefore differ, at least in part.

Discussion

We have shown that the affinity of WIN 51708 for the cloned rat NK-1 receptor is ≥400-fold higher than that for the cloned human NK-1 receptor. Although this affinity difference has been observed previously using receptors from tissues derived from these species (22), our data demonstrate this effect on cloned receptors expressed recombinantly in CHO cells. These data support the hypothesis that the difference in affinity of these receptors for WIN 51708 is the result of specific receptor amino acid residue differences and is not due to other physicochemical properties of the receptor environment.

It is residue 97 in the first extracellular loop and residue 290 in the seventh putative transmembrane domain that are primarily responsible for the dramatic species selectivity seen with WIN 51708. Residues 80, 116, and 152 also contribute to this phenomenon. Although mutation of either Val^{97} or Ser^{290} in the rat NK-1 receptor to Glu^{97} or Ile^{290}, respectively, is sufficient to lower the affinity of WIN 51708 to a level similar to that seen with the human NK-1 receptor, both of these residues must be simultaneously mutated in the human NK-1 receptor for the affinity of WIN 51708 to increase to nearly rat wild-type affinity. This suggests that the binding environments for WIN 51708 at the rat and human NK-1 receptors differ. If the binding environments were identical at the two wild-type receptors, then mutation of either Glu^{97} or Ile^{290} in the human NK-1 receptor would only change interactions between WIN 51708 and the mutated amino acid side chain. Because the change in binding energy would be equal to the change in enthalpy from the residue 97 (or 290) side chain/WIN 51708 interaction, this should lead to an increase in affinity for WIN 51708 comparable to the loss seen with the converse mutant receptor. Because this is clearly not the case, other molecular contacts at the rat and human receptors must differ.

One possible model to explain these data is that mutation of

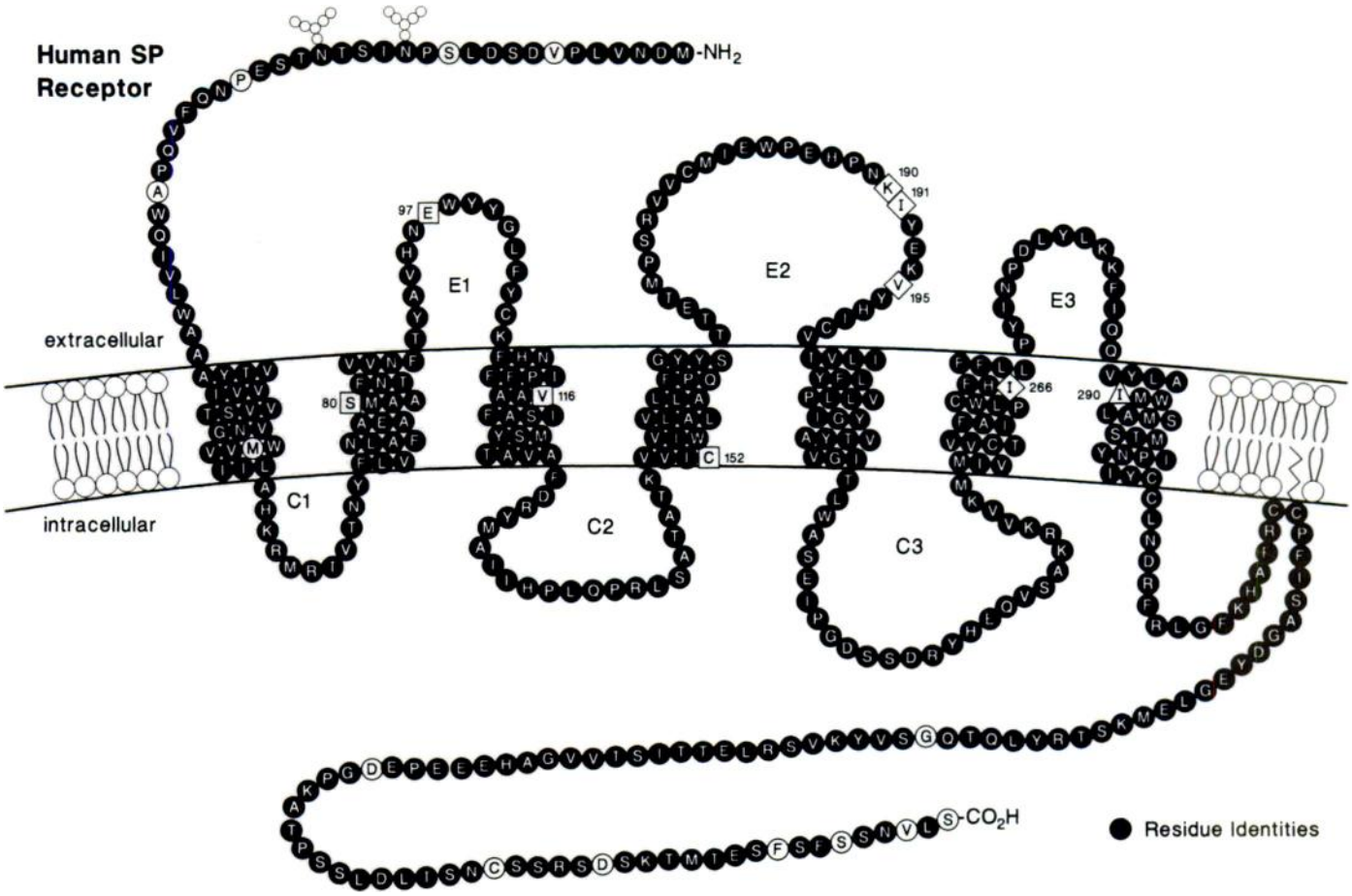

Fig. 4. Summary of residues involved in species selectivity of WIN 51708 and CP-96,345. Pictured is a two-dimentional model of the human NK-1 receptor. *Black circles*, residue identities with the rat NK-1 receptor. *White symbols*, residue differences; *squares*, residues identified in this study as being important for the species selectivity of WIN 51708; *diamonds*, residues identified in Ref. 23 as being important for the species selectivity of CP-96,345. Residue 290 (*triangle*) is important for the species selectivity of both of these antagonists.

residues 97 and/or 290 changes the shape of the WIN 51708 binding site. Starting with the rat NK-1 receptor, mutation of Val^{97} (or Ser^{290}) introduces one or more negative interactions (or removes positive interactions) between WIN 51708 and the receptor due to local conformational changes in the WIN 51708 binding pocket. The orientation of WIN 51708 in this scenario stays the same with respect to the receptor protein as a whole. To recreate (more or less) the rat binding environment in the human NK-1 receptor and therefore restore the lost positive (or lose the gained negative) interactions at the human receptor, both residues 97 and 290 must be those present in the rat receptor. In this model, residues 97 and 290 may or may not interact directly with WIN 51708. In an alternative model, if residues 97 and 290 do interact directly with WIN 51708, then it is possible that mutation of these residues alters the orientation of WIN 51708 within its binding pocket while leaving the shape of the binding pocket intact. In this second model, aside from the side chains of residues 97 and 290, the receptor protein retains its local conformation at the binding site for WIN 51708. This situation also creates a different binding environment for WIN 51708 at the rat, compared with the human, NK-1 receptor, because different points of contact with the receptor result from the different orientations of the antagonist in the binding pocket. It is not possible to distinguish between these two models from the data presented. It also is likely that a combination of these models may best describe the mechanism of the species selectivity of WIN 51708 at the rat and human NK-1 receptors.

Although mutation of residues 97 and 290 dramatically increases the affinity of WIN 51708 for the human NK-1 receptor (H-E97V,I290S), the affinity is still ~9-fold lower than that for the rat NK-1 receptor. In examining the contributions of the other candidate residues, we discovered that additionally mutating Val^{116} to leucine resulted in a receptor (H-E97V,V116L,I290S) with a 5-fold lower affinity for WIN 51708, compared with H-E97V,I290S, whereas a receptor with mutations at all five of the candidate residues (H-S80C,E97V,V116L,C152F,I290S) displayed wild-type rat receptor affinity for WIN 51708. These results show that residue 116 can affect WIN 51708 affinity, and they suggest that a molecular incompatibility exists in the H-E97V,V116L,I290S receptor that disrupts the WIN 51708 binding site. This incompatibility is corrected in the H-S80C,E97V,V116L,C152F,I290S receptor, suggesting that Leu^{116} interacts unfavorably (either directly or indirectly) with Ser^{80} and/or Cys^{152}.

Comparing the effects of mutations on the binding of WIN 51708 with those on the binding of CP-96,345, several observations can be made based on the fact that various residues differentially contribute to the observed species selectivity (Fig. 4). First, although the species preference for these antagonists

is reversed, residue 290 plays a critical role in defining the species selectivity of both WIN 51708 and CP-96,345. Although mutation of residue 290 alone in the lower affinity interaction with the rat NK-1 receptor is sufficient to increase the affinity of CP-96,345, simultaneous mutation of residue 97 in the human NK-1 receptor is required to increase the affinity of WIN 51708. Residue 97, on the other hand, is critically involved in the species selectivity of WIN 51708, whereas it does not appear to play a role in the selectivity of CP-96,345. In addition, it is of note that, although the affinity of WIN 51708 is lower for the H-E97V,V116L,I290S mutant than for the H-E97V,I290S mutant, the affinity of CP-96,345 is identical for these two mutant receptors. These data support the notion that WIN 51708 and CP,96,345 bind at sites that are at least in part different. These sites may or may not overlap in physical space. Although both of these antagonists behave competitively with respect to SP, it is not known whether they bind to the receptor in a competitive manner with respect to each other. Due to the chemical structure of WIN 51708, it is possible that this antagonist makes significant contact with the lipid bilayer. Understanding whether the binding of each of these antagonists affects the binding of the other may provide important insights into their mechanism of antagonism at the NK-1 receptor.

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