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Relative Contribution of Polar Interactions and Conformational Compatibility to the Binding of Neurokinin-1 Receptor Antagonists

TUNG MING FONG, HONG YU, RUEY-RUEY C. HUANG, MARGARET A. CASCIERI, and CHRISTOPHER J. SWAIN

Merck Research Laboratories, Rahway, New Jersey 07065 (T.M.F., H.Y., R.C.H, M.A.C.), and Merck Sharp & Dohme Research Laboratories, Harlow, Essex CM20 2PT, UK (C.J.S.)

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SUMMARY

Based on single residue substitutions, previous studies suggested that Gln165, His197, and His265 of the neurokinin-1 receptor interact directly with many nonpeptide antagonists, including CP-96,345. To further test this model, all three residues have been substituted simultaneously with alanine. The Q165A-H197A-H265A triple mutant bound CP-96,345 and eight analogs with similar affinity (2–20 μM), even though the same series of compounds bound to the wild-type receptor with affinities over a range of 1000-fold. These observations correspond exactly to the prediction of the binding site model. The micromolar binding affinity of all tested CP-96,345 analogs for the triple mutant seems to reflect solely van der Waals interactions, which suggests a significant contribution of conformational compatibility (or shape complementarity) to binding affinity. The primary role of conformational compatibility in ligand binding was consistent with the observation that simply transferring the residues involved in polar interactions with β_2-agonists into the neurokinin-1 receptor did not lead to increased binding affinity for the β_2-agonists. Taken together, these results support a general principle of ligand-receptor binding in which specific polar interactions can take place only if the overall ligand conformation is compatible with the stereochemistry of the binding pocket. In addition, double-residue and triple-residue substitutions, in combination with single-residue substitutions, can provide an alternative route to reveal multiple interactions that may not be detectable by single-residue substitutions and represent a novel approach to examine ligand-receptor interactions in the absence of high-resolution structural data.

Elucidating the molecular basis of ligand-receptor binding and recognition remains a major challenge in membrane receptor research. The difficulty of obtaining high-resolution structural data for membrane proteins has led to the popular use of site-directed mutagenesis to map the ligand binding site on membrane receptors (1–3). Because analyzing receptor mutants alone is not sufficient to map the ligand binding site, a complementary modification approach has been used frequently to support direct interactions between a ligand and a receptor. For instance, if a direct interaction between a specific residue and a specific group on the ligand is proposed, the residue can be substituted with alanine and the specific group of the ligand can be substituted with nonpolar atoms. Four affinity constants are then determined for the parent compound and a modified compound, each binding to the WT receptor and a mutant receptor. In an ideal case of one-to-one interaction, the mutual dependence of donor-acceptor would predict that the parent compound will bind to the WT receptor with high affinity, whereas the other three affinity constants will be equal and of lower affinity than the constant of the parent compound for the WT receptor. However, such an ideal situation is rarely observed. It is more common to observe that only two of the constants are equal, as exemplified by affinities of L-703,766, which contains a benzyl in place of the benzhydryl in CP-96,345, for the neurokinin-1 receptor and the H197A mutant (4).

The NK1R is a G protein-coupled receptor, the natural peptide agonists of which include SP, neurokinin A, and neurokinin B. The NK1R activates the phosphatidyl inositol second-messenger pathway, and it plays an important role in neurogenic inflammation (5–9). Extensive studies have been carried out to map the antagonist binding site of the NK1R, suggesting that Gln165, His197, and His265 in helices 4, 5, and 6 are the most critical residues in forming polar interactions with many nonpeptide antagonists. Specifically for quinuclidine antagonists (such as CP-96,345), Gln165 interacts with the C3 heteroatom, His197 interacts with the benzhydryl, and His265 interacts with certain substituted benzyl moieties. The current model of nonpeptide antagonist binding site is based on several single residue substitutions and the analysis of a series of antagonist analogs (10). For example, L-703,766 has the same binding affinity for the WT

ABBREVIATIONS: ^{125}I-SP, [^{125}I-Tyr^8]-substance P; NK1R, neurokinin-1 receptor; SP, substance P; WT, wild type.

and the H197A mutant receptor, whereas the affinity of CP-96,345 is very sensitive to the chemical nature of side chains at position 197. These data suggest that His197 in the fifth transmembrane segment of the NK1R interacts directly with the benzhydryl group of CP-96,345. However, our previous data showed that the affinity of L-703,766 for the H197A mutant is still lower than the affinity of CP-96,345 for the H197A mutant (4). One interpretation is that additional receptor residues also interact with the benzhydryl group (4, 11). In other examples of soluble proteins, it is also known that one receptor residue can interact with more than one group in the ligand (12, 13). These multiple interactions mediated by one receptor residue or one group in the ligand can certainly complicate data interpretation of single residue substitutions. Under these nonideal conditions, the possibility that His197 may interact indirectly with CP-96,345 through some complicated schemes cannot be ruled out with absolute certainty in the absence of structural data.

To support the proposed antagonist binding site in the NK1R (10), we have formulated another approach to test the hypothesis that Gln165, His197, and His265 interact directly with quinuclidine antagonists. In the present study, we have substituted all three side chains simultaneously with alanine, which should lead to the disruption of all known polar interactions. This mutant receptor was found to bind CP-96,345 and eight other substituted analogs with similar affinity. These results also provide an estimate for the relative contributions of polar interactions and conformational fit to small molecule binding. The energetic contribution of collective van der Waals interactions is as large as that of polar interactions, which indicates the importance of conformational compatibility between the ligand and the binding pocket. These results are discussed within the framework that selective ligands need to have the appropriate conformation to fit into the binding site first, and optimal binding is achieved by maximizing polar interactions with the closest receptor residues.

Materials and Methods

All mutations were constructed from the human NK1R by the uracil selection method of site-directed mutagenesis (BioRad, Richmond, CA). All mutated sequences were confirmed by DNA sequencing. All receptors were expressed in COS-7 cells for determination of ligand binding affinity and receptor activation, using 10 μg of plasmid DNA to transfect 10 million cells by electroporation (4). The nonpeptide antagonists SR140333 and RP67580 and analogs of CP-96,345 were synthesized as described previously (14–17). All peptide agonists were purchased from Peninsula Laboratories (Belmont, CA). Radiolabeled peptides were obtained from DuPont-New England Nuclear (Boston, MA).

The binding affinities of various ligands for the WT and mutant receptors were determined from the inhibition of 0.2 nM ^{125}I-SP binding to intact transfected COS cells at 4° in the presence of varying concentrations of unlabeled ligands (10). IC_{50} values were calculated from these experiments, which represent the ligand concentrations at which 50% inhibition of the specific binding of ^{125}I-SP was observed. The formation of total inositol phosphates was measured as described previously (18).

Results

Based on three different single-residue substitution mutants of the NK1R and complementary modification of quinuclidine antagonists, we have proposed previously that Gln165, His197, and His265 interact directly with quinuclidine NK1 antagonists (4, 10, 19). To further test this model, a triple mutant of the NK1R was constructed in which Gln165, His197, and His265 were substituted simultaneously with alanine. The triple-mutant Q165A-H197A-H265A bound ^{125}I-SP in a manner similar to the Q165A mutant, with an IC_{50} value of 4 nM for SP (Fig. 1A). The lower affinity of the triple mutant for SP compared with the WT is due to the lower affinity of the Q165A mutant for SP (10). The triple mutant was functionally active (Fig. 1B), which suggests that the simultaneous substitution of three residues does not impair the overall receptor conformation. A series of analogs of CP-96,345 with modification at the bridgehead nitrogen (L-706,164), C3 linking amine (L-705,084), benzhydryl (L-703,766) and/or substituted benzyl moiety (L-703,605, L-709,232, L-703,776, L-703,625, and L-706,125) were tested at both the WT receptor and the triple mutant (Fig. 2A). The triple mutant bound these CP-96,345 analogs with similarly low affinity (2–20 μM). In contrast to the Q165A-H197A-H265A triple mutant, the WT receptor exhibited varying binding affinities for different antagonist molecules, ranging from 0.5 nM to 200 nM (Fig. 2B). The similar affinities of various quinuclidine compounds for the triple mutant strongly support the model in which Gln165, His197, and His265 directly interact with the polar groups and aromatic groups in these quinuclidine antagonists.

On the other hand, if some residues are not involved in direct ligand interaction, simultaneously removing those side chains is not expected to produce a mutant receptor that binds different CP-96,345 analogs with similar affinity. Pre-

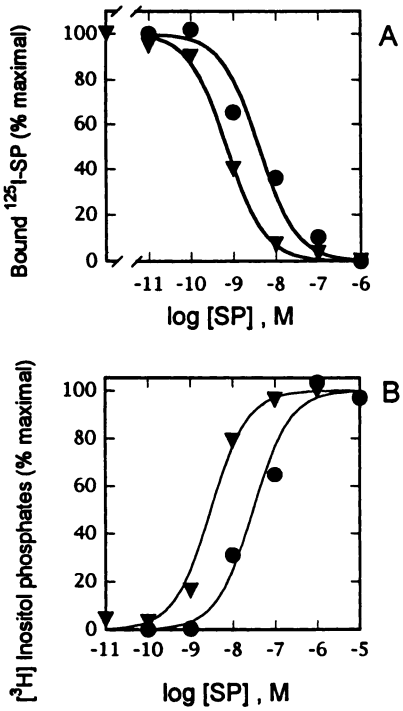

Fig. 1. A, Inhibition of ^{125}I-SP binding to the WT human NK1R (▲) or the Q165A-H197A-H265A mutant (●) by SP. In all cases, the amount of cells used in the binding was adjusted such that < 10% of the added radioligand was bound. B, Stimulation of the synthesis of inositol phosphates. Symbols are same as in A. The data represent the average of two independent experiments.

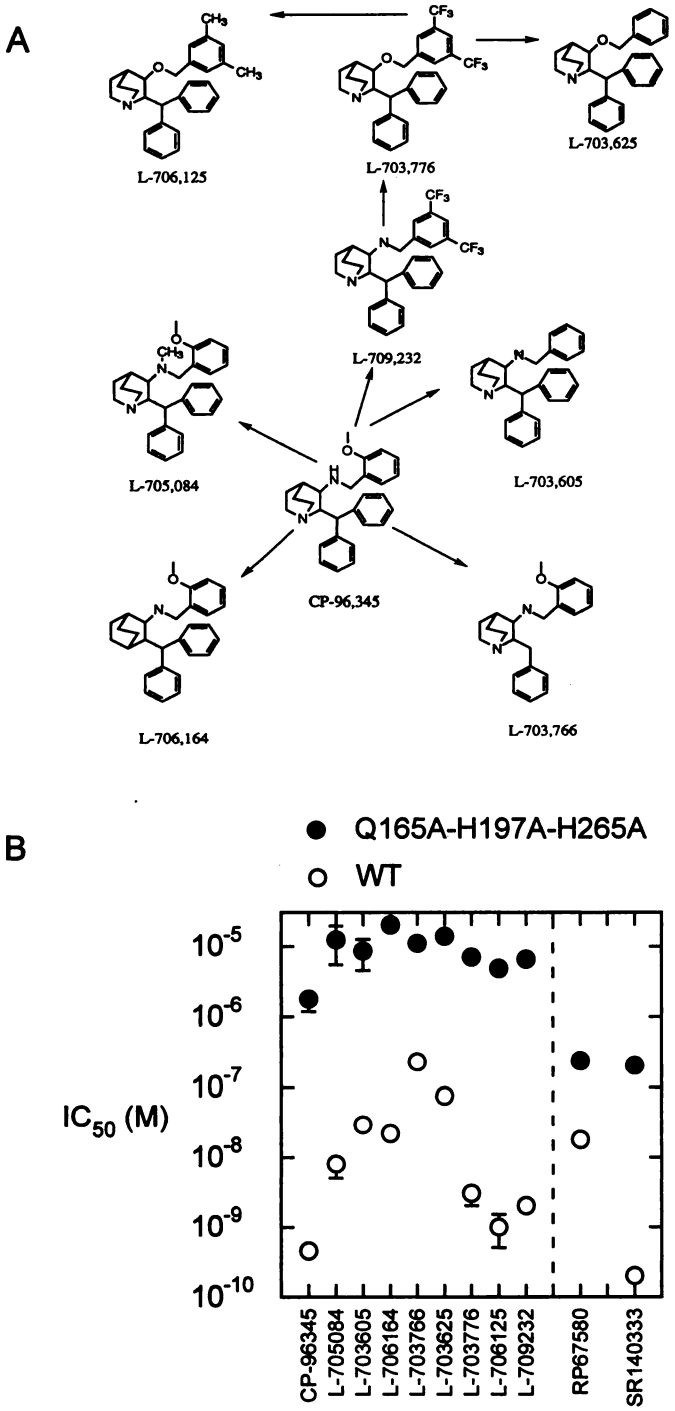

Fig. 2. A, Structure of antagonists. *Arrows* start from the portion of the parent compound that is modified to generate the new compound and do not represent synthetic route. B, Apparent binding affinity of a series of quinuclidine antagonists, RP67580 and SR140333, for the WT and the Q165A-H197A-H265A mutant of human NK1R. The data represent the average of at least two independent experiments. *Error bar*, mean ± standard error.

vious studies proposed that residues 116 and 290 of the NK1R are indirectly involved in determining the conformation of the antagonist binding pocket (20). When the V116L-I290S double mutant was tested on two analogs, their binding affinities for the double mutant differed by about 100-fold [CP-96,345, IC_{50} = 12 ± 0.3 nM (three experiments); L-703,776, IC_{50} = 1500 ± 230 nM (two experiments)]. This is in contrast to the effect of the Q165A-H197A-H265A triple mutation on the binding affinities of these two compounds (Fig. 2B).

The data in Fig. 2B also provide an estimate of the relative contribution of polar interactions and van der Waals interactions to the high affinity binding of CP-96,345. With a binding affinity of 0.5 nM, the binding energy of CP-96,345 to the human NK1R was calculated to be −12.1 kcal/mol [$\Delta G = 2.303 \times RT \times \log(K_d)$, where R is molar gas constant and T is temperature]. Because all CP-96,345 analogs tested here bound to the Q165A-H197A-H265A triple mutant with an affinity in the range of 2–20 μM, the micromolar binding affinity for the triple mutant apparently reflects the contribution of van der Waals interactions and corresponds to an average binding energy of −6.6 kcal/mol. Thus, polar interactions mediated by Gln165, His197, and His265 contribute only −5.5 kcal/mol of binding energy [i.e., −12.1 − (−6.6)] for CP-96,345.

Although single-residue substitutions are valuable in mapping the ligand binding site, they may not detect multiple interactions mediated by one particular group. For example, the Q165A, H197A, or H265A mutation in the human NK1R led to reduced binding affinity for several nonpeptide antagonists (4, 10, 19). The reduction in affinity for each point mutant can be used to predict the binding affinity for the Q165A-H197A-H265A triple mutant. As shown in Table 1, for three of the compounds we tested, the predicted affinity deviated from the measured value by more than 10-fold. L-705,084 bound to the triple mutant with a lower binding affinity than was predicted from the three single-residue mutations, whereas L-703,605 and L-703,776 bound to the triple mutant with higher binding affinities than were predicted from the three single-residue mutations. Similarly, it has been demonstrated that His265 is very critical for the binding of certain quinuclidine antagonists, yet the H265A mutation does not affect the binding affinity of CP-96,345 (Table 2) (19). When the H197A-H265A double mutant was tested, it was found that the binding affinity of CP-96,345 (233 nM) was lower than was predicted from the two point mutants (17 nM for H197A, 0.7 nM for H265A, and 0.5 nM for WT). In addition, SR140333 bound to the H197A-H265A double mutant with an lower affinity than was predicted from the two point mutants. On the other hand, such a discrepancy was not observed for RP67580 (Table 2).

In addition to quinuclidine NK1 antagonists, other struc-

TABLE 1

Predicted and measured binding affinities for the Q165A-H197A-H265A triple mutant

The measured binding affinity is calculated as $K_i = IC_{50}/(1 + [^{125}I\text{-SP}]/K_{d(SP)})$. The predicted binding affinity is calculated based on the three single-residue mutants: $K_{i(\text{triple mutant})}/K_{i(WT)} = [K_{i(Q165A)}/K_{i(WT)}] \times [K_{i(H197A)}/K_{i(WT)}] \times [K_{i(H265A)}/K_{i(WT)}]$.

	K_i for the triple mutant	
	Predicted	Measured
	μM	
CP-96,345	0.598	2
L-705,084	0.81	12
L-703,605	140	8
L-706,164	9	20
L-703,766	2	11
L-703,776	364	7

TABLE 2
Apparent binding affinities of NK1R ligands for the WT human NK1R or mutants

Receptor	IC_{50}			
	CP-96,345	SR140333	RP67580	SP
		nM		
hNK1R	0.5 ± 0.1 (4)	0.2 ± 0.02 (8)	18 ± 3 (4)	0.6 ± 0.2 (7)
H197A	17 ± 2 (2)	3.7 ± 0.7 (4)	22 ± 3 (2)	0.7 ± 0.1 (3)
H265A	0.7 ± 0.1 (3)	0.3 ± 0.03 (4)	272 ± 22 (3)	0.6 ± 0.2 (3)
H197A-H265A	233 ± 38 (3)	99 ± 30 (3)	257 ± 42 (3)	0.5 ± 0.1 (2)
Q284A	0.3 ± 0.1 (2)	3.6 ± 1.0 (2)	22 ± 9 (2)	0.7 ± 0.1 (2)

tural classes of NK1 antagonists have also been discovered. To test whether different types of antagonists interact with different sets of receptor residues within a common binding site, the binding affinities of the triple mutant for various antagonists were measured. As shown in Fig. 2B, whereas RP67580 and SR140333 did exhibit reduced affinity for the Q165A-H197A-H265A triple mutant compared with the WT receptor, their binding affinities for the triple mutant were at least 10-fold higher than all tested CP-96,345 analogs for the triple mutant. This is consistent with previous studies indicating that Ser169 and Tyr287 of the NK1R are required for the high-affinity binding of RP67580 but not quinuclidine antagonists (10, 18). In addition, we have found that Gln284 is required specifically for the high-affinity binding of SR140333 but not CP-96,345, RP67580, or SP (Table 2).

The relative contribution of polar interactions and compatible stereochemistry to ligand binding was further investigated by comparing the ligand binding sites of the β_2-adrenergic receptor and the NK1R. The agonist binding site of the β_2-adrenergic receptor contains at least three residues (Asp113, Ser204, and Ser207) that are known to be critical for the direct interaction of β_2-agonists (21). When these three residues were introduced into the NK1R at the equivalent positions (see Fig. 3 for alignment), a triple mutant P112D-T201S-I204S of the human NK1R was obtained. The P112D-T201S-I204S mutant bound SP with high affinity, which suggests that the substitution of these three residues

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Helix  3
          113
     .     *
β2R    FwtsidvlcVtASIetlcviAvDRY
       :         : :|:       : |||
NK1R   FhnffpiaaVFASIysmtavAfDRY
            *
          112


Helix  5
          204 207
            *   *
β2R    aIassivsfyvPLvVmvfvY
        :           |: :     |
NK1R   hIcvtvliyflPLlVigyaY
            *   *
          201 204
```

Fig. 3. Sequence alignment of the hamster β_2-adrenergic receptor and the human NK1R in the helix 3 and helix 5 regions. The alignment is based on the highly conserved residues in the rhodopsin subfamily of G protein-coupled receptors (SxxxLxxxxxDRY in helix 3 and PxxxxxxxY in helix 5) (9).

does not impair the overall receptor conformation. Despite the presence of these three crucial residues in the triple mutant, the β_2-agonist isoproterenol did not inhibit the binding of ^{125}I-SP (Fig. 4A). This triple mutant did not exhibit any detectable level of binding for the β_2 antagonist ^{125}I-cyanopindolol. Furthermore, isoproterenol did not elicit any functional response as measured by the level of inositol phosphates (Fig. 4B). These data suggested that the stereochemistry in the central cavity of the NK1R is not compatible with β_2 specific ligands, and therefore, introducing the Asp and Ser residues is not sufficient for increased binding affinity of β_2 ligands for the NK1R mutant.

Discussion

Based on single-residue substitutions and complementary modification of ligands, we have proposed that Gln165,

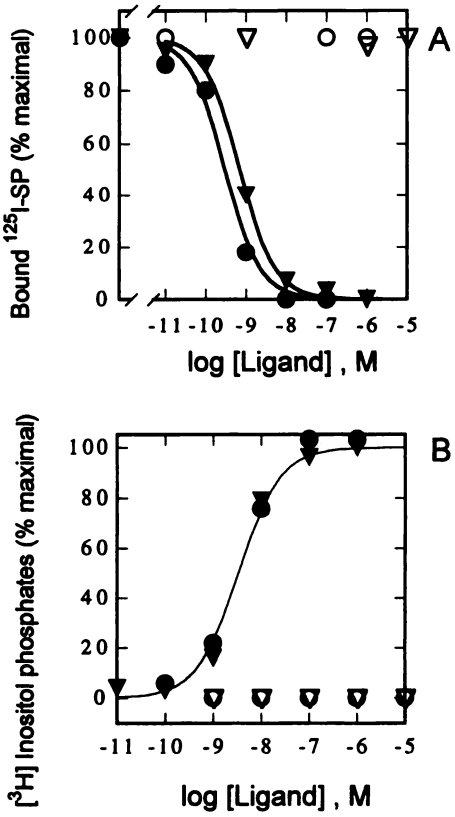

Fig. 4. A, Inhibition of ^{125}I-SP binding to the WT NK1R (▲, △) or the P112D-T201S-I204S mutant (●, ○). ▲ and ●, SP; △ and ○, isoproterenol. In all cases, the amount of cells used in the binding was adjusted such that < 10% of the added radioligand was bound. B, Stimulation of the synthesis of inositol phosphates. Symbols are same as in A. The data represent the average of two independent experiments.

His197, and His265 define a minimal part of the antagonist binding site in the NK1R (10). The proposed amino-aromatic interaction between the benzhydryl of CP-96,345 and His197 of the NK1R has been further supported using conformationally constrained analogs (4, 22). Nonetheless, mutational analysis cannot be equated with structural analysis, and a model proposing direct interactions between specific receptor residues and a ligand requires support from multiple experimental approaches. To further test the direct interactions mediated by the three residues, a triple mutant was constructed in which Gln165, His197, and His265 were substituted simultaneously with alanine. As in most mutational analyses of proteins, it is assumed that the amino acid substitutions do not lead to a change of receptor conformation. In the case of point mutations, this assumption is supported by the observation that at least one other ligand binds to both the mutant and WT receptors with the same affinity (4, 10, 19). For the current triple mutant, the lack of gross conformational change as a result of the mutations is supported by the data in Fig. 1. When nine quinuclidine analogs with various functional group modifications were tested on the triple mutant, all had similar binding affinity (Fig. 2B). These data suggest that without the Gln165, His197, and His265 side chains, all tested quinuclidine analogs will have essentially the same binding affinity for the NK1R mutant. In the WT NK1R, however, some quinuclidine antagonists will have high affinity, presumably because they can maximize polar interactions with Gln165, His197, and His265. These results confirm the model that Gln165, His197, and His265 are the most critical residues in forming direct specific polar interactions with CP-96,345.

Photoaffinity labeling experiments using a CP-96,345 analog have indicated that an antibody directed against the third extracellular loop (residues 273–287) of the NK1R no longer immunoprecipitates the receptor when covalently labeled (23). It is possible that the photoreactive analog may attach to one of the residues in the top of the seventh transmembrane segment (i.e., residues 281–287). This interpretation would be consistent with the current model of CP-96,345 binding in which the His265 residue is in the vicinity of the substituted benzyl moiety and the His265 residue is at the same horizontal level as residues 283–287 (10, 19).

The strength of polar interactions depends on interatomic distance, r, ranging from $1/r$ to $1/r^5$ (24). These interactions are relatively strong in ligand-protein interactions, making them amenable to mutational analysis. On the other hand, other factors such as van der Waals interactions and conformational fit are difficult to quantitate by single-residue substitutions. This is because the energetic contribution of van der Waals interactions (with their $1/r^6$ to $1/r^{12}$ dependence) from one group is so small that substituting one residue will not change the binding affinity significantly if that residue is involved only in van der Waals interaction and if there is no conformational change. However, the sum of van der Waals interactions from all relevant groups can be quite large. This is evident in the Q165A-H197A-H265A triple mutant, which binds many CP-96,345 analogs with an affinity of 2–20 μM. The micromolar binding affinity is apparently attributable to van der Waals interactions between a minimal ligand scaffold and the receptor binding pocket because various functional groups in the tested compounds have minimal effect on binding affinity.

When using site-directed mutagenesis to detect ligand-receptor interactions (4, 25–28), single-residue substitutions may underestimate the contribution of a particular residue due to multiple interactions, coupled interactions, or more complicated modes of interactions. For example, when one residue is substituted, the ligand binding affinity may not change, possibly due to a new interaction with another residue or strengthening another interaction. This explanation is consistent with the observation that the H265A substitution in the human NK1R does not change the binding affinity of CP-96,345, and yet, other analogs of CP-96,345 are very sensitive to the H265A substitution (19). Further comparison of the H197A-H265A double mutation with the respective single-residue mutations suggests that His265 may indeed interact with CP-96,345 (Table 2), and the effect of H265A substitution may be masked by a stronger interaction between His197 in the H265A mutant and the benzhydryl group of CP-96,345. Such an explanation may also be applicable to the lower affinity of L-705,084 for the Q165A-H197A-H265A triple mutant than what is predicted based on the three point mutants. Irrespective of how His265 interacts with CP-96,345, the similar affinities of many CP-96,345 analogs for the Q165A-H197A-H265A triple mutant provided further confirmation that these three residues are the most critical residues in forming polar interactions with CP-96,345.

On the other hand, point mutation may also overestimate the direct energetic contribution for a particular residue (29). As shown in Table 1, L-703,605 and L-703,776 bind to the Q165A-H197A-H265A triple mutant with a higher affinity than what is predicted based on the three-point mutants. One possibility would be that one group of the ligand interacts with two-receptor residues. When one residue is mutated, the second interaction may also be weakened due to reduced constraint, thereby overestimating the energetic contribution of the first residue. Therefore, analyzing the triple mutant along with the single-residue substitutions can guard against attributing an unrealistically large, direct energetic contribution to a single residue.

The present study provides further evidence that although antagonists in different structural classes can bind to the same central cavity of the transmembrane region, the basic scaffold of each structural class provides a means to interact with different transmembrane residues within the same binding region (4, 10, 30, 31). For RP67580, the most important residues seem to be Ser169, His265, and Tyr287 (10, 18), with Gln165 and His197 contributing moderately in the human NK1R and rat NK1R, respectively (10, 19). In contrast, His197, His265, and Gln284 are most critical for the binding of SR140333. These results demonstrate that different nonpeptide antagonists can bind to the same central cavity, with each individual ligand interacting with a specific set of residues from helices 4–7 (Fig. 5). The consequences of such a conclusion are analogous to drug-hemoglobin interactions in which compound binding is determined by the available van der Waals space and, within this space, by a tendency to maximize polar interactions (13).

The importance of available van der Waals space as a first step in ligand binding can be further illustrated by the difficulty of transplanting ligand binding properties between two distantly related receptors and the ease of transplantation between two closely related receptors. The β_2-adrenergic re-

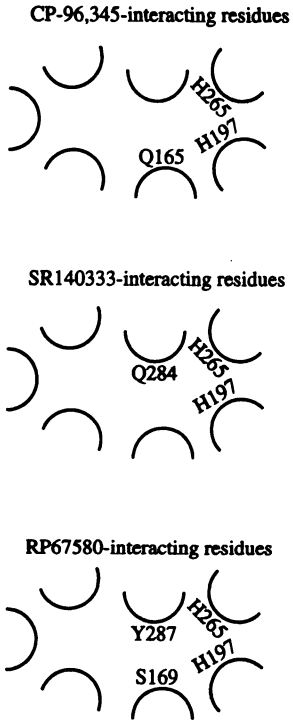

Fig. 5. Schematic drawings depicting the central cavity of the NK1R and different sets of side chains interacting with different antagonists. The highlighted residues represent those that have been identified by mutational analysis.

ceptor is another well characterized example in which three residues are known to be critical for direct agonist interaction (21). When one aspartate and two serine residues are introduced into the NK1R at positions equivalent to those in the β_2-adrenergic receptor, the mutant NK1R did not exhibit increased binding affinity for any β_2-agonist. These data suggest that the stereochemistry of the tested β_2 ligands, even within its flexibility limit, is not compatible with the stereochemistry of the central cavity of the NK1R. Therefore, the aspartate and serine residues are not within the effective range to interact strongly with the β_2 ligands. The difficulty of transplanting β_2 binding properties into the NK1R is apparently caused by the the relatively distant relationship of the two receptors. On the other hand, closely related receptors are more likely to exhibit similar stereochemistry of van der Waals space in the central cavity. For example, reversing the species selectivity for nonpeptide ligands has been demonstrated with the substitution of two residues between the human and rat NK1R (20, 32–34), and transplanting the CP-96,345 binding property into the NK3R has been demonstrated with the substitution of helices 5 and 6 (35). Because Gln165, His197, and His265 are conserved in all neurokinin receptors cloned to date, these data suggest that the pharmacologically observed conversions are actually due to changes in the shape of the binding pocket, which in turn determines the strength of interactions. Taken together, the present study supports a model of ligand-receptor interaction in which conformational compatibility plays a primary role (20, 36).

The current binding site model of CP-96,345 (encompassing Gln165, His197, and His265) is consistent with a steric hindrance, competitive mechanism for the antagonistic activity of CP-96,345. First, Gln165 is required for the high-affinity binding of both SP and CP-96,345 (10). Second, His197 and His265 seem to be in the vicinity of the bound SP molecule (18), such that a bound CP-96,345 molecule is expected to block the binding of SP. Third, the dissociation rate constant of SP is not affected by L-703,606 (a close analog of CP-96, 345), and the dissociation rate constant of L-703,606 is not affected by SP (37). Thus, the SP binding site and CP-96,345 binding site overlap spatially, and each ligand uses a different set of side chains for polar interactions. It is well known that structurally different and competitive ligands can bind to the same site on an antibody, but only one residue is involved in interacting with all ligands whereas each individual ligand interacts with a second uncommon residue (38). Alternatively, an allosteric model has been proposed to explain the antagonistic activity of CP-96,345 (39).

In summary, the present study demonstrates that multiple-residue substitution provides another route to test a proposed model of ligand binding site. The energetic contribution of collective van der Waals interactions (or conformational compatibility) is quite large, and the conformational compatibility should determine whether specific polar interactions can take place. These results stress the need for accurately predicting the binding site conformation in the receptor and the bound conformation of a ligand in the process of developing novel receptor ligands when structural data are not available.

Acknowledgments

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Send reprint requests to: Dr. T.M. Fong, Merck Research Labs, R80M-213, PO Box 2000, Rahway, NJ 07065.