The non-peptide substance P (SP) antagonist CP-96,345 plays an important role in pain, neuroimmune interactions and neuromodulation (Helke et al., 1990; Iversen et al., 1987; Maggio, 1988; Nakashishi, 1991; Payan, 1989). It binds to the neurokinin-1 receptor (NK1R) to elicit various biological responses. Antagonists of the NK1R have been proposed for the treatment of diseases involving SP, and several potent and specific NK1R antagonists have recently been described (Garret et al., 1991; Snider et al., 1991). However, subsequent studies have demonstrated that the quinuclidine antagonist CP-96,345 has a higher affinity for the human NK1R than the rat NK1R, whereas the perhydroisoindole antagonist RP67580 shows the reverse species specificity (Beresford et al., 1991; Garret et al., 1991).

Thus, it has been postulated that there may be two subtypes of NK1R within the same species (Watling, 1992). An analogous hypothesis was also proposed for the existence of two NK2 receptor subtypes based on tissue preparations from two species (Patacchini et al., 1991). Understanding the cause of the pharmacological difference observed for species variants of the NK1R will not only clarify the definition of receptor subtypes, but also provide a rational basis for the selection of animal models in drug development.

Molecular cloning and expression of the NK1R from several species have demonstrated that the NK1R belongs to the G protein-coupled receptor family (Fong et al., 1992; Hershey and Krause, 1990; Yokota et al., 1989). Sequence comparison of the human and rat NK1R reveals 22 divergent residues (Fig. 1). It is possible that some of the phylogenetically divergent residues in the human NK1R could interact directly with CP-96,345, thus conferring upon it a higher affinity than that for the rat NK1R. To test whether these residues are directly involved in CP-96,345 and RP67580 binding, divergent residues in the human NK1R were substituted by the rat homologs. Analysis of these mutant receptors revealed that two conservative substitutions within the transmembrane domain are necessary and sufficient to reverse the species selectivity of the two antagonists. The direct involvement of phylogenetically conserved residues in antagonist binding and indirect effects of phylogenetically divergent residues are discussed.

**MATERIALS AND METHODS**

The cDNA clones of the human NK1R and rat NK1R were obtained as described (Fong et al., 1992; Yokota et al., 1989). Chimeric mutants between the human and rat NK1R were constructed by restriction endonuclease cleavage followed by ligation of the appropriate DNA fragments. The Stul site at residue 73 and the BglII site at residue 276 were utilized in constructing the chimeric mutants. Point mutations were constructed from the human NK1R template, and they were introduced by the uracil selection method of site-directed mutagenesis (Bio-Rad). Double or triple mutations were constructed by recombining the appropriate single point mutations. All mutations were confirmed by DNA sequencing.

The wild type and mutant receptors were expressed in COS cells. The (2R,3R)/(2S,3S) racemic mixture of CP-96,345 and the (3R,7aR)/(3S,7aS) racemic mixture of RP67580 were synthesized as described (Low et al., 1992; Peyronel et al., 1992). Racemates of the non-peptide antagonists were used in all experiments.

**RESULTS**

The non-peptide antagonist CP-96,345 has a higher affinity for the human NK1R (IC50 = 0.5 nM) but a lower affinity for the rat NK1R (IC50 = 35 nM). In contrast, the binding affinity of RP67580 for the rat NK1R (IC50 = 4 nM) is 5-fold higher than that for the human NK1R (IC50 = 20 nM; Fig. 2). To
identify regions of the NK1R that contribute to the species selectivity of these two antagonists, chimeric receptors were constructed between the human and rat NK1Rs. Substitution of residues 1-276 or residues 80-276 of the human NK1R resulted in a decreased affinity for CP-96,345 and an increased affinity for RP67580. The binding affinity of RP67580 for both mutants was identical to that of the rat NK1R. However, the binding affinity of CP-96,345 for both mutants was intermediate between those of the human and rat NK1Rs. These data indicate that some of the divergent residues in the region 80-276 are necessary to account for the differential antagonist binding affinities of the NK1R from these two species.

When residues 1-79 and 277-407 or 277-407 alone of the human NK1R were substituted by the rat NK1R sequence (h(1-79, 277-407) and h(277-407) mutants in Fig. 3), the binding affinities of CP-96,345 for both mutants were intermediate between those of the human NK1R and the rat NK1R (Fig. 3). These data rule out any significant contribution from these 6 residues to the species selectivity. The data also indicate that some residues in the 277-407 region are important for the species selectivity. The data also indicate that some residues in the 277-407 region are important for the species selectivity.

FIG. 1. Schematic drawing of the NK1R indicating the sequence positions of the 22 divergent residues. The filled circles and the letters within circles represent the human NK1R residues, while the letters beside circles represent the rat homologs.

FIG. 2. Inhibition of ['251]BHSP binding to the human or rat NK1R by CP-96,345 or RP67580. Each point is the average of duplicate measurements. The data shown are representative of at least two independent experiments.

FIG. 3. Comparison of antagonist binding affinities for the wild type human NK1R (human WT), mutants of the human NK1R, and the wild type rat NK1R (rat WT). The human NK1R mutants are designated by h, followed by a number in parentheses representing the amino acid residues in the human NK1R that were substituted by the rat homologs. All the receptors in the figure are divided into four groups (separated by dashed vertical lines), and all the receptors in one particular group have very similar antagonist binding affinities. The first group from left includes mutant human NK1Rs that have similar affinities as the wild type human NK1R. The second group from left includes mutant human NK1Rs containing the I290S substitution. The third group from left does not contain the I290S substitution. The fourth group from left includes mutant human NK1Rs that have similar affinities as the wild type rat NK1R. Mean ± S.E. from at least two independent experiments are shown.

mediate between those of the human and rat NK1Rs. The similarity of binding affinities of these two mutants again argues against a role for the divergent residues in the 1-79 region in determining species selectivity. The data also indicate that some residues in the 277-407 region are important for the species selectivity.

The h(290) mutant in which the isoleucine-290 of the human NK1R was substituted by the rat homolog serine also exhibited properties similar to those of the h(277-407) mutant. Moreover, when residues 1-290 of the human NK1R were substituted by the rat NK1R sequence (the h(1-290) mutant), the binding affinities of both antagonists were identical to those of the rat NK1R (Fig. 3). These data rule out any contribution from the C-terminal tail of the NK1R to the species selectivity of antagonists, and indicate that residue 290 is critical in this respect. Therefore, some of the residues in the region of 80-276 as well as residue 290 are necessary and sufficient to explain the differential antagonist binding properties of the human and rat NK1Rs.

Within the 80-276 region, there are 8 divergent residues between the human and rat NK1R. Combinations of point mutations at positions 80, 152, 190, 191, 195, and 266 with the h(290) mutant yielded mutant receptors with binding affinities similar to those of the h(277-407) mutant. Moreover, when residues 1-290 of the human NK1R were substituted by the rat NK1R sequence (the h(1-290) mutant), the binding affinities of both antagonists were identical to those of the rat NK1R (Fig. 3). These data rule out any significant contribution from these 6 residues to the species selectivity of antagonist binding. In contrast, the binding affinities of both antagonists for the rat NK1R can be reproduced in the double mutant h(116, 290) in which valine-116 and isoleucine-290 of the human NK1R were substituted by the rat homologs leucine and serine (Fig. 3).
Therefore, residues 116 and 290 are necessary and sufficient to account for the species selectivity of CP-96,345 and RP67580 binding to the NK1R. However, the antagonist binding affinities of the single mutant h(116) were very similar to those of the wild type human receptor, indicating a cooperative effect of substitutions at residues 116 and 290 on the binding properties of NK1R.

The endogenous agonist SP exhibited a 3-fold difference in binding affinity for the human and rat NK1Rs. For all the mutants reported here, the binding affinities for SP fell within this 3-fold range (Fig. 4), indicating that there is no large scale structural rearrangement of the receptor due to the mutations.

**DISCUSSION**

The present study was designed to elucidate the molecular basis of the observed species selectivity of antagonists for the NK1R. To test whether divergent residues at different positions could lead to the observed species selectivity of the two antagonists, chimeric and point mutants of the human NK1Rs were analyzed. These results demonstrate that the N-terminal sequence (1–54), the intracellular C-terminal tail (291–407), and the divergent extracellular residues (190, 191, 195) do not contribute to the species selectivity of antagonist binding. In the transmembrane region, 2 divergent residues at positions 80 and 152 possess chemical characteristics (see Fig. 1) that might be expected to contribute to additional binding energy for CP-96,345 in the human NK1R or for RP57680 in the rat NK1R. However, substitution of either of these 2 residues in the human NK1R does not enhance the binding affinity of RP67580 nor reduce the affinity of CP-96,345. In contrast, substitution of valine-116 and isoleucine-290 of the human NK1R by the rat homologs leucine and serine resulted in a mutant human receptor where both CP-96,345 and RP67580 exhibited binding affinities very similar to those of the wild type rat NK1R (Fig. 3). Because the two antagonists change their affinities in opposite directions in this double mutant, the effect is specific and does not seem to be a result of abnormal folding of the receptor. In addition, the single mutant h(116) does not significantly change the antagonist affinities, whereas the single mutant h(290) binds the antagonists with affinities that are intermediate between the rat and human NK1Rs. Therefore, the 2 residues at positions 116 and 290 are both necessary and sufficient to account for the species selectivity of the two antagonists.

The observation that the affinity of RP67580 for the rat NK1R is higher than that for the human receptor could be the result of a favorable interaction with residues 116 and 290 in the rat receptor or an unfavorable contact (such as repulsive force or steric hindrance) with the 2 residues in the human receptor (and vice versa for CP-96, 345). Comparison of affinity measurements on several mutants, however, argues against a direct interaction of residues 116 and 290 with the antagonists and supports an indirect effect of substituting the 2 residues on antagonist binding for the following reasons. First, substitution of residue 116 alone does not significantly affect the antagonist binding affinities. Thus, either residue 116 does not interact directly with the antagonists or the direct interaction is too weak to be measurable. Second, the potential hydrogen bonding capability of serine 290 in the rat NK1R does not explain the higher affinity of RP67580 for the rat NK1R because the h(1–276), h(80–276), and h(1–290) mutants have the same affinity for RP67580 as the wild type rat NK1R. In other words, serine 290 is not absolutely required for the higher affinity binding of RP67580. This result also indicates that steric hindrance of isoleucine 290 in the human NK1R does not explain the reduced affinity of RP67580 for the human NK1R. Third, binding energy calculations suggest that van der Waals interactions involving hydrocarbon side chains (such as isoleucine) usually contribute less than 1 kcal/mol of binding energy, which is equivalent to a less than 5-fold change in binding affinity (Andrews, 1986). In the present study, substitution of isoleucine-290 in the human receptor by serine resulted in a 10-fold reduction in the affinity of CP-96,345, arguing against a direct van der Waals interaction between isoleucine 290 and CP-96,345. Fourth, an unfavorable direct contact between CP-96,345 and serine 290 can not explain the reduced affinity of CP-96,345 for the rat NK1R because the affinity of CP-96,345 for the h(290) mutant is similar to that for the h(80–276) and h(1–276) mutants which do not contain serine 290. Taken together, all the present data suggest that residues 116 and 290 do not interact directly with the two antagonists.

The results from the h(116), h(290), and h(116, 290) mutants also indicate a cooperative effect on the binding properties of the NK1R when both residues 116 and 290 are changed. Such a cooperative effect is consistent with a localized conformational change as a result of substituting the 2 residues. Similarly, both the h(116, 290) mutant and the h(80–276) mutant reproduce the affinity of RP67580 for the rat NK1R, suggesting that different combinations of amino acid substitutions can lead to the same enhanced affinity for RP67580. Thus, we propose that the phylogenetically divergent residues affect the local helical packing of the receptor (Chothia et al., 1981), and some of the phylogenetically conserved residues must interact directly with the antagonists. Such a local change in helical packing can affect the 3-dimensional positions of neighboring residues that are important for antagonist binding.

The discovery of non-peptide antagonists (such as CP-96,345, RP67580, and the heterosteroid antagonists reported by Appell et al. (1992)) and the observation of species differences in antagonist binding affinity for these three classes of antagonists have prompted the speculation that multiple subtypes of NK1R may exist in the same species (Watling, 1992). Based on the present data and all the cDNA and genomic cloning data, however, it seems appropriate to classify the human NK1R and rat NK1R as species variants of the same receptor subtype rather than as two different NK1R subtypes. There is precedent in other systems for two species variants of a protein that give rise to some functional differences. For
instance, two species homologs of the serotonin-1B receptor share 96% sequence identity in the transmembrane domain, but they exhibit different pharmacological profiles (Hartig et al., 1992). Substitution of one phylogenetically divergent residue in the glycine receptor affects the binding of agonists and antagonists (Kuhse et al., 1990). Examination of the x-ray structures of clam hemoglobin and human hemoglobin has demonstrated that structural divergence can explain the observed differences in cooperative oxygen binding (Royer et al., 1990).

In summary, the binding sites for CP-96,345 and RP67580 in the NK1R are not identical because substituting residues 80–276 in the human NK1R by the rat homologs can reproduce the binding affinity of RP67580, but not CP-96,345, for the rat NK1R. Furthermore, the present results demonstrate a role for residues 116 and 290 of the NK1R as determinants of the differential affinities of CP-96,345 and RP67580 for the NK1R from different species. This conclusion is consistent with the observation that the guinea pig NK1R, which contains the same residues in the transmembrane domain as the human NK1R, has binding affinities for both CP-96,345 and RP67580 similar to those of the human NK1R (Beresford et al., 1991; Garret et al., 1991; Gorbulev et al., 1992). Similarly, the mouse NK1R contains the same residues in the transmembrane domain as the rat NK1R except for residue 266, and both the mouse and rat NK1Rs bind CP-96,345 with the same affinity (Beresford et al., 1991; Sundelin et al., 1992). All the present data are consistent with a model in which the residues at 116 and 290 influence the geometry of the antagonist binding pocket. These data further demonstrate that a minor sequence divergence among variants of the same receptor can lead to pronounced pharmacological differences.

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