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Two Classes of Structurally Different Antagonists Display Similar Species Preference for the Human Tachykinin Neurokinin$_3$ Receptor

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SUMMARY

Two classes of structurally different tachykinin neurokinin$_3$ (NK$_3$) antagonists were used to evaluate species difference in antagonist binding between human and rat NK$_3$ receptors. In competition binding experiments with [^{125}I-MePhe^7]NKB as radioligand, PD 154740, PD 157672, SR 48968, and SR 142801 displayed lower K_i values for the human NK$_3$ receptor (40 ± 4, 12 ± 1, 350 ± 50, and 0.40 ± 0.05 nM, respectively) than for the rat NK$_3$ receptor (2450 ± 130, 288 ± 25, >10,000, and 11.0 ± 0.5 nM, respectively). Data from *in vitro* functional assay showed similar species preference as observed with the competition binding assay. It was shown previously that substitution of only two amino acid residues in the rat receptor to their human counterparts could change the species selectivity of SR 48968, a weak NK$_3$ antagonist. In the double-substituted rat mutant, all three antagonists (PD 154740, PD 157672, and SR 142801) displayed K_i values (76 ± 8, 16 ± 2, and 0.50 ± 0.05 nM, respectively) very similar to the K_i values for the wild-type human NK$_3$ receptor. Thus, in addition to their previously reported effects on SR 48968, these two amino acid residues are responsible for the species selectivity of these three additional NK$_3$ antagonists. Because PD 154740 and PD 157672 are very different structurally from SR 48968 and SR 142801, our results indicate that the two identified residues may be involved in adopting a receptor conformation that favors the binding of NK$_3$ antagonists that display species preference for the human NK$_3$ receptor.

The tachykinin peptides, which include substance P, NKA, and NKB, are widely distributed in the peripheral and central nervous systems and are known to be involved in many important biological functions, including pain transmission, bronchoconstriction, intestinal motility, saliva secretion, and neurogenic inflammation (1). The biological effects of substance P, NKA, and NKB are mediated preferentially through the activation of three distinctive G protein-coupled receptors, NK$_1$, NK$_2$, and NK$_3$, respectively (2, 3).

Species variations in antagonist binding have important implications in drug discovery and have been observed in a variety of receptors belonging to the G protein-coupled receptor family (4, 5). Recent development of potent and selective antagonists for tachykinin receptors also reveals the existence of a wide range of species selectivities among these tachykinin antagonists. Of the three tachykinin receptor subtypes, the NK$_1$ receptor is most well characterized in terms of species difference in antagonist binding. CP 96,345, a nonpeptide NK$_1$ antagonist, is 100-fold more potent for the human NK$_1$ receptor than for the rat NK$_1$ receptor (6). Conversely, RP 67,580, another potent NK$_1$ antagonist, has 5-fold higher affinity for the rat NK$_1$ receptor than for the human NK$_1$ receptor (7). Several NK$_2$ antagonists also have been reported to show species selectivity between NK$_2$ receptors in rabbit and hamster tissues (8, 9). However, due to a lack of potent and selective NK$_3$ antagonists, the species difference in antagonist binding in the NK$_3$ receptor was not well characterized. It has been shown that SR 48968, a potent nonpeptide NK$_2$ antagonist, is also an antagonist for NK$_3$ receptors in humans (IC$_{50}$ of 0.4 μM) and guinea pig (IC$_{50}$ of 0.3 μM) but not in rat (IC$_{50}$ of >10 μM) (10–13). Furthermore, by constructing a series of chimeric receptors between human and rat NK$_3$ receptors and creating site-specific mutants, we identified two amino acid residues (Met-

ABBREVIATIONS: NK, neurokinin; IP, inositol phosphate; PD 154740, {1-[1-(8-hydroxy-octylcarbamoyl)-1-methyl-2-phenyl-ethylcarbamoyl]-2-phenyl-ethyl}-carbamic acid tert-butyl ester; PD 157672, *N*-[(1,1-dimethylethoxy)carbonyl]-L-phenylalanyl-*N*-[7-[(aminocarbonyl)amino]heptyl] methyl-D-phenylalaninamide; SR 48968, (*S*)-*N*-[4-[4-(acetylamino)-4-phenyl-1-piperidinyl]-2-(3,4-dichlorophenyl)butyl]-*N*-methylbenzamide; SR 142801, *N*-(1-{3-[1-benzoyl-3-(3,4-dichloro-phenyl)-piperidin-3-yl]propyl}-4-phenyl-piperidin-4-yl)-*N*-methyl-acetamide; CHO, Chinese hamster ovary; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

134 and Ala-146) located in the outer portion of the second transmembrane region of the human NK_3 receptor as the key residues responsible for the species difference (14). Substitution of these two amino acid residues in rat to their counterparts in human conveys species selectivity. Recently, two classes of potent but structurally different NK_3 antagonists have been reported (15, 16). In the present study, we compared the species difference in binding affinities as well as in functional activities of these two classes of antagonists in cloned human and rat NK_3 receptors. We also used a double-substituted mutant to characterize amino acid residues responsible for the species selectivity of these two classes of NK_3 antagonists.

Experimental Procedures

Materials. [^{125}I-MePhe^7]NKB (2200 Ci/mmol) was purchased from DuPont-NEN (Boston, MA). [MePhe^7]NKB was purchased from Peninsula Laboratories (Belmont, CA). PD 154740 and PD 157672 were synthesized in-house, whereas SR 48968 and SR 142801 were kindly provided by Dr. X. Emonds-Alt (Sanofi Recherche, France). The construction of CHO cell lines expressing human, rat, and rat double-mutant receptors has been described previously (14).

Cell culture and membrane preparations. CHOK1 cells transfected with indicated tachykinin receptors were seeded in 500-cm^2 plates and cultured in Ham's F-12 nutrient mixture supplemented with 10% fetal bovine serum for 3 days until 90% confluent. Cells were scraped in 20 ml phosphate-buffered saline and 5 mM EDTA and centrifuged at 2000 × *g* for 10 min. The resulting pellet was resuspended in binding buffer (50 mM Tris, pH 7.4, 0.4 mg/ml bovine serum albumin, 0.08 mg/ml bacitracin, 0.004 mg/ml chymostatin, 0.004 mg/ml leupeptin, and 1 μM thiorphan) and incubated for 15 min on ice. The cell suspension was homogenized with a polytron for 30 sec at setting 6, and the homogenate was centrifuged at 20,000 × *g* for 30 min. The membrane pellet was resuspended in ice-cold binding buffer and stored at −80° until needed.

Radioligand binding assay. Agonists and/or antagonists were incubated with 5 μg membrane protein in binding buffer containing 100–300 pM [^{125}I-MePhe^7]NKB in 96-well filter plates (Millipore). Phosphoramidon (25 μM) was included in the radioligand buffer to prevent peptide degradation. The binding assay was incubated for 1 hr at room temperature and terminated by filtration on a vacuum manifold followed by two washes with ice-cold binding buffer. Filter plates were blotted on Whatman filter paper to remove excess liquid and air dried. The dried filter plates were coated with scintillant by submerging the bottoms of filter plates briefly in a melted Meltilex scintillator sheet (Wallac), and the scintillant-coated filter plates were placed into a Wallac 1450 Microbeta scintillation counter for counting radioactivity. K_d and B_{max} values for [MePhe^7]NKB in different NK_3 receptors were estimated from competition binding experiments with the following equations: $K_d = IC_{50} - L$ and $B_{max} = B_0 \cdot IC_{50}/L$, where L is concentration of free radioligand, and B_0 is specific bound radioligand (17). K_i values were determined with the following equation: $K_i = IC_{50}/(1 + L/K_d)$ (18).

Inositol phosphate assay. The formation of IPs was assayed as described previously (19). In brief, 1×10^5 transfected CHO cells were seeded into each well on 12-well plates and preincubated in serum-free F12/Dulbecco's modified Eagle's medium (GIBCO) containing [^{3}H]inositol (1 μCi/ml) for 24 hr. The cells were washed twice with 1 ml of assay buffer (minimum essential medium with 10 mM LiCl, 20 mM HEPES, 1 mg/ml bovine serum albumin) and incubated at 37° for 1 hr with 1 ml assay buffer containing test compound(s). To terminate the incubation, the medium was aspirated, and 0.5 ml of ice-cold 5% trichloroacetic acid was added to each well. Total [^{3}H]IPs were analyzed by applying the trichloroacetic acid extract directly to Dowex anion exchange columns. [^{3}H]Inositol was removed by washing three times with 3.3 ml of 5 mM inositol, and total [^{3}H]IPs were eluted with 4 ml of 1 M ammonium formate in 0.1 M formic acid and determined by counting in a beta liquid scintillation counter with Ready Gel (Beckman) as liquid scintillant.

Results

Species difference in binding affinities of NK_3 antagonists. Membranes prepared from CHO cells expressing either cloned human or cloned rat receptors were used to compare the species difference in ligand binding between human and rat NK_3 receptors. With [^{125}I-MePhe^7]NKB as the radioligand in competition binding assay, the apparent K_d values of [MePhe^7]NKB are 4.0 ± 0.4 and 3.2 ± 0.4 nM for the human and rat NK_3 receptors, respectively (Table 1). There is no significant difference in binding affinity of [MePhe^7]NKB between these two species. This is consistent with our previous results in which other NK_3 agonists, including senktide, NKB, and [pro^7]NKB, have been tested and were shown to have little difference in their binding affinities between the two species (11). Contrary to the agonists, two new dipeptide-derived NK_3 antagonists (PD 154740 and PD 157672) (Fig. 1) displayed much lower K_i values for the human NK_3 receptor (40 ± 4 and 13 ± 2 nM, respectively) than for the rat NK_3 receptor (2450 ± 130 and 284 ± 24 nM, respectively) (Table 1). Similarly, two nonpeptide NK_3 antagonists, SR 48968 and SR 142801, also showed much lower K_i values for the human (350 ± 50 and 0.40 ± 0.05 nM, respectively) than for the rat (>10,000 and 11.0 ± 0.5 nM, respectively) NK_3 receptors. Thus, both classes of compounds, although very different in their chemical structures, showed similar species preference for the human NK_3 receptor.

Inhibition of agonist-induced inositol phosphate turnover by NK_3 antagonists. With an *in vitro* functional assay, SR 142801 has been reported to competitively antagonize [MePhe^7]NKB-induced contractions of guinea pig ileum with a pA_2 value of 9.15 (16). To determine whether PD 154740 is also a competitive antagonist, its effects on [MePhe^7]NKB-induced IP turnover in CHO cells expressing human NK_3 receptors were studied. As shown in Fig. 2A, PD 154740 produced a parallel shift to the right of the dose-response curve to [MePhe^7]NKB with no depression of the maximum response. Schild analysis of the data gave a pA_2 value of 7.57 with a slope of −0.95, confirming a competitive action for PD 154740 at the human NK_3 receptor (Fig. 2B). To determine whether the species differences in binding affinity of these antagonists represent their species differences in antagonistic effects, PD 154740 and SR 142801 were ex-

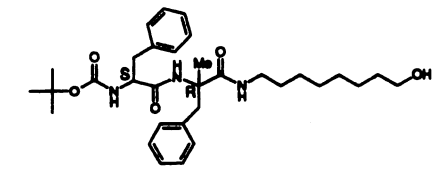
TABLE 1

Comparison of binding affinities of tachykinin agonist and antagonists between human and rat NK_3 receptors

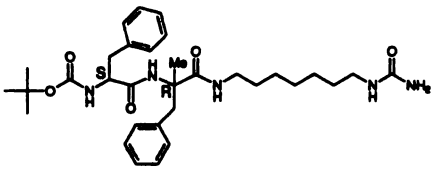
The competition binding experiments were performed as described in Experimental Procedures. K_d and K_i values are given as the mean ± standard error with data obtained from three separated experiments performed in triplicate.

	Human	Rat	Rat/human[a]
	nM		
[MePhe^7]NKB (K_d)	4.0 ± 0.4	3.2 ± 0.4	0.8
PD 154740 (K_i)	40 ± 4	2,450 ± 130	62
PD 157672 (K_i)	13 ± 2	284 ± 24	22
SR 48968 (K_i)	350 ± 50	>10,000	>29
SR 142801 (K_i)	0.40 ± 0.05	11.0 ± 0.5	22

[a] Ratio of affinities.



PD 154740



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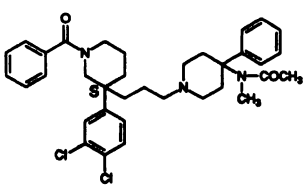
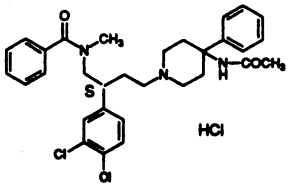


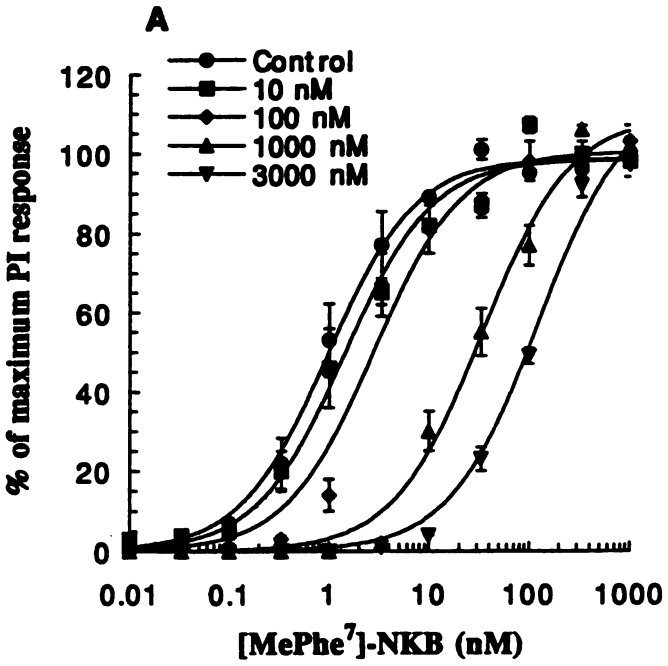
SR 142801



SR 48968

Fig. 1. Structures of tachykinin NK_3 antagonists.

amined for their effectiveness to inhibit [MePhe^7]NKB-induced IP accumulation in CHO cells expressing either human or rat NK_3 receptors. As shown in Fig. 3, [MePhe^7]NKB elicited IP turnover in a dose-dependent manner with similar EC_{50} values for the human (1.3 ± 0.1 nM) and rat (1.2 ± 0.1 nM) receptors, indicating that similar to their binding affinities, there is no species difference in functional responses to the NK_3 agonist, [MePhe^7]NKB. Neither up to 10 μM of either PD 154740 or SR 142801 had any effect on IP turnover in either human or rat NK_3 receptor-transfected CHO cells (data not shown). Because 10 nM of [MePhe^7]NKB induced nearly the maximum IP turnover in both cell lines tested (Fig. 3), this concentration was used to stimulate IP turnover in subsequent inhibitor studies. The concentration of PD 154740 necessary to block 50% of 10 nM [MePhe^7]NKB-induced IP turnover (IC_{50}) in cells containing human NK_3 receptor was 630 ± 50 nM (Fig. 4A). In contrast, concentrations up to 10 μM of PD 154740 failed to show inhibition of [MePhe^7]NKB-induced IP responses in CHO cells expressing the rat NK_3 receptor. This is consistent with data from competition binding experiments (Table 1) that show very low affinity of this compound for the rat NK_3 receptor (K_i = 2450 ± 130 nM). In a parallel experiment, the effectiveness of SR 142801 to inhibit 10 nM [MePhe^7]NKB-induced IP responses were also investigated (Fig. 4). The IC_{50} values were 11 ± 1 and 128 ± 16 nM for the human and rat receptors, respectively. The species difference in IC_{50} values derived from IP turnover experiments between human and rat NK_3 receptors (22-fold) (Fig. 4) is similar to the species difference in K_i values derived from competition binding experiments (12-fold) (Table 1), indicating that for SR 142801, the difference in binding affinities represents the difference in antagonistic effects between the two species.



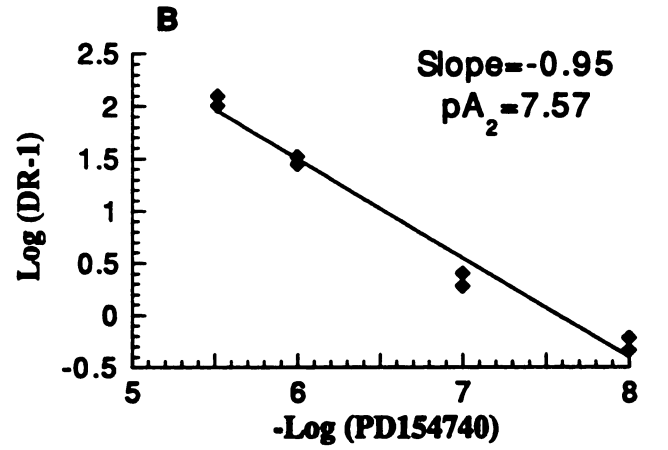


Fig. 2. Competitive antagonism by PD 154740 of [MePhe^7]NKB stimulated IP turnover in CHO cells expressing human NK_3 receptors. A, Dose-response curves for [MePhe^7]NKB-stimulated IP turnover were measured in the absence or presence of 10, 100, 1000, and 3000 nM of PD 154740. Each value represents the mean of two separate experiments performed in triplicate. Error bar represents standard error. B, Schild analysis of dose-response curves for [MePhe^7]NKB-stimulated IP turnover. Each value represents a separate experiment.

Characterization of amino acid residues responsible for species selectivity. We have shown previously that two species-diverged residues (Met-134 and Ala-146) in the second transmembrane region of the human NK_3 receptor is responsible for the species selectivity of SR 48968. To determine whether these two residues are also responsible for the species selectivity of these two classes of more potent and

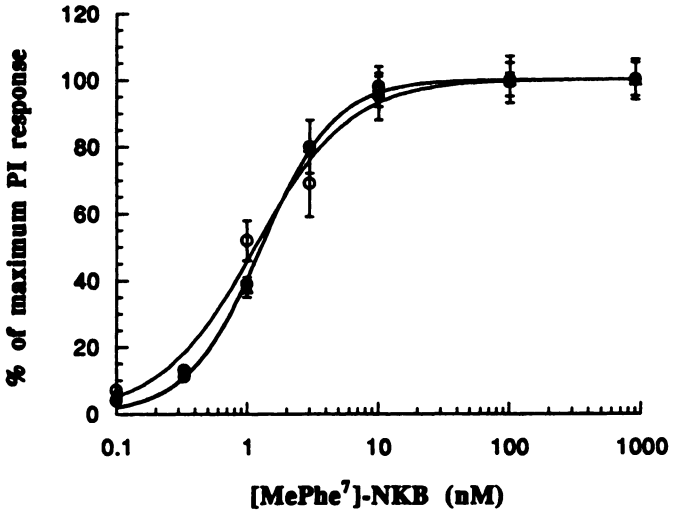


Fig. 3. Comparison of dose-response curves of [MePhe^7]NKB-stimulated IP turnover in CHO cells expressing human (●), rat (○), and rat double mutant (△) NK_3 receptors. The measurements of IP turnover on intact cells were performed as described in Experimental Procedures. Each value is the mean of three separate experiments performed in triplicate. Error bar represents standard error.

selective NK_3 antagonists, the binding affinities of these antagonists were further characterized in a double-substituted rat mutant receptor that has been demonstrated previously to convey the species selectivity of SR 48968. As shown in Fig. 5A, the affinity of PD 154740 for the double-substituted mutant (K_i = 76 ± 8 nM) was only 2-fold lower than its affinity for the human NK_3 receptor (K_i = 40 ± 4 nM) but was 30-fold higher than that for the rat NK_3 receptor (K_i = 2450 ± 130 nM), indicating that these two residues are also responsible for the species difference of this compound. Similarly, the K_i values of PD 157672 and SR 142801 to the mutant receptor (16 ± 2 and 0.50 ± 0.05 nM, respectively) were almost indistinguishable from that to the wild-type human NK_3 receptor (13 ± 2 and 0.40 ± 0.05 nM, respectively) (Fig. 4, B and C). These results indicated that although both classes of NK_3 antagonists are very different in their chemical structures, their species selectivity is determined by the same species-diverged residues (Met-134 and Ala-146) in the human NK_3 receptor.

PD 154740 and SR 142801 were also examined for their effectiveness in blocking the [MePhe^7]NKB-induced IP turnover responses in the double-substituted mutant. As shown in Fig. 3, [MePhe^7]NKB elicited a similar dose-response curve in the double-substituted mutant as that in the human and rat NK_3 receptors with maximum response occurring at ~10 nM, indicating that the mutation did not affect the ability of the mutant receptor to interact with the G protein. The concentration of PD 154740 necessary to block 50% of the maximum IP turnover response (IC_{50}) in the mutant receptor was 800 ± 73 nM (Fig. 4A), which was much closer to its IC_{50} value in the human NK_3 receptor (630 ± 50 nM) than the IC_{50} value in the rat NK_3 receptor (>10 μM). The IC_{50} value of SR 142801 (20 ± 2 nM) for the double-substituted mutant was also similar to that for the human NK_3 receptor (11 ± 1 nM) but very different from that for the rat NK_3 receptor (128 ± 16 nM) (Fig. 4B). Thus, the double-substituted rat mutant was not only similar to the human NK_3 receptor in terms of its binding affinity to these antagonists but also similar to the human NK_3 receptor in terms of its functional responses to these antagonists.

Discussion

We previously characterized several rat/human chimeric and rat mutant receptors and showed that two amino acid residues (Met-134 and Ala-146) located in the second transmembrane region of the human NK_3 receptor are responsible for the species difference in binding affinities of the NK_3 antagonist SR 48968 (14). These two residues have been demonstrated to be involved in species-selective binding of two classes of structurally different more potent and selective NK_3 antagonists. Our present data further strengthen the idea that these two residues may be involved in adopting the receptor into a conformation that favors the binding of antagonists that display species preference for the human NK_3 receptor. As more structurally different NK_3 antagonists become available, it will be interesting to determine whether the same two residues also play a role in determining the species selectivity of antagonists that display species preference for the rat NK_3 receptor. Similar to our findings, common species-diverged residues responsible for high affinity binding to chemically distinct antagonists were also reported for the NK_1 receptor (20). Combined substitution of Leu-116 and Ser-290 to their human counterparts showed the rat NK_1 receptor to have human-like affinities for CP 96345 and FK 888, two chemically distinct NK_1 antagonists. Thus, the existence of a species-specific receptor conformation that favors the binding of more than one class of antagonist may be a general phenomena for receptors in the G protein-coupled receptor family. However, as a word of caution, it should not be assumed that exactly the same set of residues will be responsible for the species selectivity of all antagonists. For example, Val-97 (instead of Leu-116) and Ser-290 are primarily responsible for the species-selective high affinity binding of WIN 51708 to the rat NK_1 receptor (25). As reported previously (6, 7, 14), data in mutagenesis experiments do not allow us to distinguish between direct involvement of these residues in ligand binding and indirect effects of these residues in changing the local conformation of the receptor, which in turn may create a more favorable environment for other species-conserved residue(s) to interact with NK_3 antagonists. In either case, local conformation around these two amino acid residues must play an important role in determining binding affinities of NK_3 antagonists. Based on the facts that human NK_1 and NK_2 receptors both contain the same Met and Ala residues in their corresponding positions and that neither of them binds to these two classes of NK_3 antagonists with high affinities (15, 16), it appears to suggest that these two residues are necessary but not sufficient to confer high affinity binding to NK_3 antagonists.

Although both classes of NK_3 antagonists recognize the same species-specific receptor conformation, it is likely that the way they interact with the NK_3 receptors may not be exactly the same. For example, in the human NK_3 receptor, the species-selective amino acid residues may be the major factor in determining the affinity of PD 154740 because in the rat NK_3 receptor, which lacks these two species-selective residues, the binding affinity of PD 154740 is greatly reduced (K_i = 2450 ± 130 nM). Conversely, for SR 142801, additional amino acid residues in the NK_3 receptor may be involved.

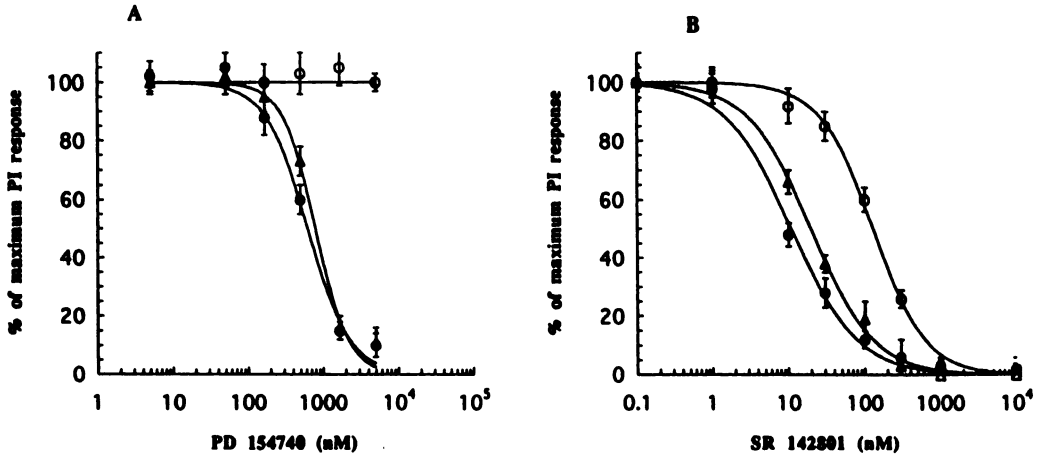


Fig. 4. A, Effects of PD 154740 on IP turnover elicited by [MePhe^7]NKB in CHO cells expressing human (●), rat (○), and rat double mutant (△) NK_3 receptors. B, Effects of SR 142801 on IP turnover elicited by [MePhe^7]NKB in CHO cells expressing human (●), rat (○), and rat double mutant (△) NK_3 receptors. The measurements of IP turnover on intact cells were performed as described in Experimental Procedures. The values are presented as percentage of maximal effect obtained with 10 nM of [MePhe^7]NKB. Each value is the mean of three separate experiments performed in triplicate. Error bar represents standard error.

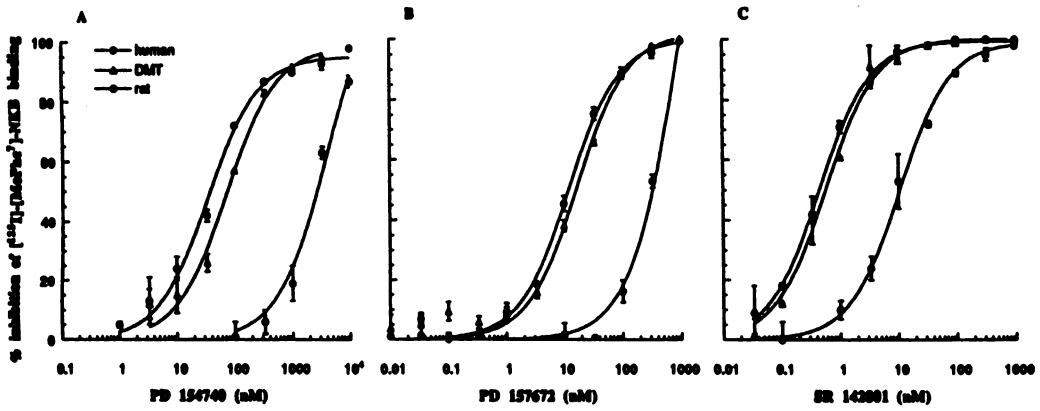


Fig. 5. A, Displacement of [^{125}I-MePhe^7]NKB binding by PD 154740 at human (●), rat (○), and rat double mutant (△) NK_3 receptors. B, Displacement of [^{125}I-MePhe^7]NKB binding by PD 157672 at human (●), rat (○), and rat double mutant (△) NK_3 receptors. C, Displacement of [^{125}I-MePhe^7]NKB binding by SR 142801 at human (●), rat (○), and rat double mutant (△) NK_3 receptors. The competition binding experiments were performed as described in Experimental Procedures. Each value represents the mean of three separate experiments performed in triplicate. Error bar represents standard error of the mean.

The rat NK_3 receptor, which lacks the species-selective residues, still binds to SR 142801 with high affinity (K_i = 11.0 ± 0.5 nM), indicating that other amino acid residues, which may be common in both human and rat NK_3 receptors, are also involved in determining the binding affinity of SR 142801. Recent mutagenesis data on the NK_1 receptor also support the existence of additional species-conserved residues involved in antagonist binding. Two species-specific amino acid residues (Val-116 and isoleucine-290) within the transmembrane regions of the human NK_1 receptor have been shown to be responsible for species-selective binding of the nonpeptide NK_1 antagonist CP-96,345 (20, 23). Although other species-conserved residues (Glu-165, His-197, His-265, and Tyr-287) may also contribute to the high affinity binding of various nonpeptide NK_1 antagonists (21–24). By making chimeric constructs between rat NK_1 and NK_3 receptors and between human NK_1 and NK_3 receptors, it should be possible to identify any additional amino acid residues involved in high affinity binding of SR 142801 to the rat and human NK_3 receptors.

Acknowledgments

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