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Two nonpeptide tachykinin antagonists act through epitopes on corresponding segments of the NK_1 and NK_2 receptors

(substance P/neurokinin A/CP96,345/SR48,968)

ULRIK GETHER*, YOSHIFUMI YOKOTA†, XAVIER EMONDS-ALT‡, JEAN-CLAUDE BRELIÈRE‡, JOHN A. LOWE III§, R. MICHAEL SNIDER§, SHIGETADA NAKANISHI¶, AND THUE W. SCHWARTZ*

*Laboratory of Molecular Endocrinology, University Department of Clinical Biochemistry, Rigshospitalet 6321, Copenhagen DK-2100, Denmark; †Institute of Molecular Embryology and Genetics, Kumamoto University Medical School, Kumamoto 862, Japan; ‡Sanofi Recherche, F-34184 Montpellier 04, France; §Department of Exploratory Medicinal Chemistry, Central Research Division, Pfizer Inc., Groton, CT 06340; and ¶Institute for Immunology, Kyoto University, Faculty of Medicine, Kyoto 606, Japan

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ABSTRACT **The molecular mechanism of action for two chemically distinct and highly selective, nonpeptide antagonists, CP-96,345 and SR-48,968, was studied by development of a series of chimeric constructs between their respective target receptors, the NK_1 (substance P) and NK_2 (neurokinin A) receptors. The binding affinities of the natural peptide ligands, substance P and neurokinin A, were not affected by exchanging almost the entire C-terminal half of the NK_1 receptor with the corresponding segment of the NK_2 receptor. In contrast, it was found that transfer from the NK_2 to the NK_1 receptor of a segment corresponding to transmembrane segment VI, the amino-terminal half of transmembrane segment VII, and the connecting extracellular loop 3 completely switched the susceptibility for the nonpeptide antagonists. This chimeric exchange, corresponding to 17 nonconserved residues, conveyed full susceptibility for the NK_2-specific compound SR-48,968 to the previously unresponsive NK_1 receptor—i.e., the K_i value for inhibition of binding of ^{125}I-labeled substance P decreased from >10,000 to 0.97 nM. At the same time the affinity for the NK_1-selective compound CP-96,345 decreased >30-fold. The actual binding site for SR-48,968 was localized to this region of the NK_2 receptor by use of [^{3}H]SR-48,968, which did not bind to the NK_1 receptor but bound with similar high affinities to the wild-type NK_2 receptor and to the chimeric NK_1 receptor with the NK_2 receptor segment incorporated around transmembrane segments VI and VII, K_d = 1.5 nM and 1.0 nM, respectively. Our data indicate that two chemically very different nonpeptide antagonists, CP-96,345 and SR-48,968, act through epitopes located around transmembrane segment VI on their respective target receptors and that at least the nonconserved residues in these epitopes are not important for the binding of the natural peptide ligands, substance P and neurokinin A.**

The number of known neuropeptides in the mammalian nervous system has during the last 20 years increased to around 40 (1, 2). Several or most of these peptides are believed to be of major importance in the regulation of chemical transmission both in the brain and in the periphery (1). Nevertheless, the characterization of the physiological importance of the peptide systems, as well as the development of useful drugs acting on peptide receptors, has been halted by the lack of sufficiently potent and specific antagonists. Until recently only peptide-based antagonists have been available, which in addition to their metabolic instability and low bioavailability, also in general are of low potency (3). However, within the last two years, orally active and potent nonpeptide antagonists for many neuropeptide and peptide hormone receptors have been discovered, mainly through screening of files of chemical compounds (3).

In the tachykinin system, highly potent and specific nonpeptide antagonists have been described both for the NK_1 and for the NK_2 receptors (3–6), for which the natural ligands are the neuropeptides substance P (SP) and neurokinin A (NKA), respectively (7). These nonpeptide compounds have already been very useful in proving the importance of SP in both nociceptive and inflammatory responses (3, 8, 9). For example, nonpeptide NK_1 receptor antagonists, which are orally active, show a potency similar to morphine in some animal pain models (3, 5) and also very efficiently prevent the increased microvascular permeability associated with inflammation (3, 8, 9). This is important, since SP is believed to be involved—for example, in asthma and rheumatoid arthritis—in the neurogenic contribution to the inflammatory process, conceivably precipitated by antidromical stimulation of sensory nerves (10, 11). Thus, nonpeptide SP antagonists may represent a novel class of both analgesics and antiinflammatory drugs (3).

Nonpeptide compounds are pharmacologically described as competitive antagonists and it is assumed, although no structural evidence is available, that they share binding sites with the natural peptide ligands (3). However, the nonpeptide compounds do not resemble the peptide agonists chemically and their mechanism of action, at a biochemical, molecular level, is not clear. In the present study we map the molecular interaction of two chemically very different and highly specific compounds, CP-96,345 (4) and SR-48,968 (6), to corresponding epitopes on their respective targets, the NK_1 and the NK_2 receptors, by genetic exchange of corresponding segments between these receptors.

EXPERIMENTAL PROCEDURES

Peptide and Nonpeptide Ligands. SP and NKA were purchased from Peninsula Laboratories. CP-96,345, (2*S*,3*S*)-*cis*-(2-diphenylmethyl)-*N*-[(2-methoxyphenyl)methyl]-1-azabicyclo[2.2.2]octan-3-amine, and SR-48,968, (*S*)-*N*-methyl-*N*-[4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)-butyl]benzamide (Fig. 1), were synthesized as described (12, 13).

Construction of Chimeric Receptors. The chimeric receptors between the rat NK_1 (SP) and NK_2 (NKA/substance K) receptor (14, 15) were constructed as described in detail previously, by use of either preexisting or introduced unique restriction sites located at equivalent positions in the receptor cDNAs (16). The restriction sites were introduced by site-directed mutagenesis (16). For construction of chimeric re-

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Abbreviations: SP, substance P; NKA, neurokinin A; [^{125}I]BH-, [^{125}I]Bolton–Hunter reagent-labeled.

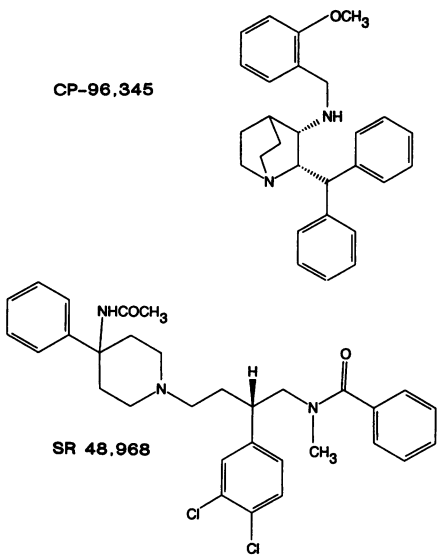

FIG. 1. Structure of the NK_1 (SP) receptor-selective nonpeptide antagonist CP-96,345 (4), and the NK_2 (NKA) receptor-selective nonpeptide antagonist SR-48,968 (6).

ceptors PK4, PK5, PK6, and PK7, restriction sites for *Cla* I, *Hin*dIII, *Nco* I, and *Eco*RV, respectively, were used. These are located in the cDNAs corresponding to the following amino acid positions in the NK_1 receptor: PK4, 129–131; PK5, 194–196; PK6, 249–251; PK7, 276–278. The mutated NK_1 and NK_2 receptor cDNAs were cut with a mixture of *Hin*dIII, *Pst* I, and one of the above-mentioned restriction enzymes. The resulting appropriate fragments of the cDNAs were cloned into the *Hin*dIII–*Pst* I site of the eukaryotic expression vector pCDM8 (17) to generate the chimeric constructs. Chimeric receptor PKP6 [NK_1/NK_2-(251–293)] was constructed by exchanging the 239-bp *Mlu* I–*Msc* I fragment of the NK_1 receptor cDNA in pTEJ-8 (18, 19) with the corresponding fragment excised from the cDNA for chimeric receptor PK6. The structure of the recombinant genes was verified by restriction endonuclease mapping and by DNA sequence analysis (16). The wild-type NK_1 and NK_2 receptors were cloned into the pTEJ-8 eukaryotic expression vector (18, 19).

Transfections and Tissue Culture. The wild-type NK_1 and NK_2 receptors and the chimeric receptors were transiently transfected into COS-7 monkey cells by the calcium phosphate precipitation method as desccribed (18–20).

Binding Experiments. Monoiodinated [^{125}I]Bolton–Hunter reagent-labeled SP ([^{125}I]BH-SP) and NKA ([^{125}I]BH-NKA) were prepared and purified by HPLC as described (19, 21). [^{3}H]SR-48,968 was prepared by tritiation of the 4-iodobenzoylamide analogue. The transfected COS-7 cells were transferred to 12-well culture plates, 0.5–2.0 × 10^5 cells per well, 1 day after transfection and 24 hr before the binding experiments (19). The number of cells per well was determined by the expression efficiency of the individual plasmid aiming at 5–10% binding of the added radioligand in the competition binding experiments. Binding experiments were performed for 3 hr at 4°C with 50 pM [^{125}I]BH-SP or [^{125}I]BH-NKA or with 0.2 nM [^{3}H]SR-48,968 plus variable amounts of unlabeled peptide or nonpeptide compound in 0.5 ml of 50 mM Tris·HCl, pH 7.4/150 mM NaCl/5 mM $MnCl_2$/0.1% (wt/vol) bovine serum albumin (Sigma) supplemented with protease inhibitors [bacitracin, 100 μg/ml, and chymostatin, 10 μg/ml (both from Sigma)]. All determinations were performed in triplicate, and nonspecific binding was determined in the presence of 1 μM SP or NKA or 10 μM SR-48,968. Specific binding constituted >80% of the total binding. The binding data were analyzed and IC_{50} values were determined by computerized nonlinear regression analysis using INPLOT (GraphPad Software, San Diego). K_d and B_{max} values for binding of radiolabeled SP and NKA to the various receptors were estimated from competition binding experiments with 10–12 different concentrations of the corresponding unlabeled peptide by using the equations $K_d = IC_{50} - L$ and $B_{max} = B_0(IC_{50}/L)$ (L, concentration of free radioligand; B_0, specifically bound radioligand) (22). K_i values were calculated from the equation $K_i = IC_{50}/(1 + L/K_d)$ (23).

RESULTS AND DISCUSSION

Wild-Type NK_1 and NK_2 Receptors. The complementary binding profiles for both the peptide agonists and the nonpeptide antagonists on the NK_1 and NK_2 receptors were confirmed with the cloned rat receptors transiently expressed in COS-7 cells (Table 1 and Fig. 1). SP and CP-96,345 bound with high affinities to the NK_1 receptor and with low affinities to the NK_2 receptor. Conversely, NKA and SR-48,968 bound with low affinities to the NK_1 receptor but with high affinities to the NK_2 receptor (K_i values are given in Table 1). Between the NK_1 and NK_2 receptors the affinities of the nonpeptide antagonists differed by around 4 orders of magnitude, whereas the affinities of the peptide ligands differed by 2–3 orders of magnitude (Fig. 1). To localize the structural elements responsible for these significant differences in binding affinity for both peptide and nonpeptide ligands, we used a series of chimeric NK_1/NK_2 constructs transiently expressed in COS-7 cells. The chimeras were made by gradually exchanging segments from the C-terminal end of the NK_1

Table 1. Binding affinities of the peptide ligands SP and NKA and the nonpeptide antagonists, CP-96,345 and SR-48,968 for the wild-type NK_1 and NK_2 receptors and chimeric constructs

Ligand	K_i, nM (mean ± SEM, n = 3–4)						
	NK_1	PK7	PK6	PKP6	PK5	PK4	NK_2
SP	0.27 ± 0.04	0.34 ± 0.06	0.29 ± 0.12	0.24 ± 0.02	0.14 ± 0.04	5 ± 2	460 ± 160
NKA	33 ± 3	45 ± 10	23 ± 5	16 ± 1	12 ± 2	33 ± 12	0.9 ± 0.2
CP-96,345	8.1 ± 1.2	1.4 ± 0.1	210 ± 40	270 ± 20	700 ± 250	>10,000	>10,000
SR-48,968	>10,000	140 ± 30	0.40 ± 0.08	0.97 ± 0.09	0.43 ± 0.03	1.2 ± 0.2	0.7 ± 0.2

The binding assay was performed on intact COS-7 cells transiently transfected with the receptor constructs. [^{125}I]BH-SP was used as radioligand for the NK_1, PK7, PK6, and PK5 receptors, [^{125}I]BH-NKA was used as radioligand for the wild-type NK_2 receptor. B_{max} values (fmol per 10^5 cells) for binding of [^{125}I]BH-SP to the receptor constructs were as follows (mean ± SEM, n = 3–4); wild-type NK_1, 90 ± 30; PK7, 31 ± 11; PK6, 70 ± 34; PK5, 80 ± 35; PK4, 15 ± 8; PKP6, 220 ± 60. The B_{max} value for binding of [^{125}I-BH]NKA to the NK_2 receptor was 70 ± 38 fmol per 10^5 cells. Diagrams of the receptor constructs are shown; filled areas, segments derived from the NK_1 receptor; open areas, segments derived from the NK_2 receptor.

receptor with corresponding segments of the NK_2 receptor (Table 1).

Peptide Binding to Chimeric NK_1/NK_2 Receptors. Nearly half of the C-terminal end of the NK_1 receptor could be exchanged with the corresponding segment of the NK_2 receptor (chimeric receptor PK5) with no decrease in binding affinity for SP and almost no increase in affinity for NKA (Table 1). However, when also transmembrane segment IV and the second extracellular loop of the NK_2 receptor were introduced into the NK_1 receptor, an ≈20-fold decrease in SP affinity was found (PK4 in Table 1). Nevertheless, the affinity for NKA was still similar to its affinity for the wild-type NK_2 receptor (Table 1). In a previous study, membranes prepared from COS cells expressing the PK4 construct did not show specific binding of radiolabeled tachykinins (16). However, in the present study specific binding of radiolabeled SP was detected when the binding assay was performed on intact cells, albeit with an affinity 20 times lower than that for the other constructs, PK5–PK7 (Table 1). Thus, these data indicate that the specific recognition of the two natural peptide ligands, SP and NKA, must be determined by epitopes in the N-terminal half of both the NK_1 and NK_2 receptors in agreement with earlier studies using the chimeric NK_1/NK_2 constructs (16). Analysis of a complete series of NK_1/NK_3 chimeric receptors previously showed that the N-terminal half of the receptor, especially the extracellular N-terminal end, is important for the selective recognition of SP (24). This has also been shown by deletion of the extracellular N terminus of the NK_1 receptor resulting in impaired SP binding (25).

CP-96,345 Binding to Chimeric NK_1/NK_2 Receptors. The results obtained with the PK7 construct showed that the high-affinity binding of CP-96,345 to the NK_1 receptor was not impaired by exchanging the C-terminal part of the NK_1 receptor until the middle of the third extracellular loop with the corresponding part of the NK_2 receptor (Table 1 and Fig. 1). In fact, the affinity of CP-96,345 for PK7 increased from 8.1 nM to 1.4 nM as compared with the wild-type NK_1 receptor. This increase may have been caused by the replacement of the polar Ser^{290} residue with the more hydrophobic leucine residue found in the NK_2 receptor at this position (14, 15). Replacement of Ser^{290} in the rat receptor with an isoleucine residue, as found in the human NK_1 receptor, increases the affinity for CP-96,345 by ≈10-fold (refs. 26 and 27; C. J. Jensen, T.W.S., and U.G., unpublished work).

In contrast to the peptide ligands, the affinity of the nonpeptide antagonist CP-96,345 was reduced >100-fold when we included the N-terminal part of extracellular loop 3 plus transmembrane segment VI from the NK_2 receptor in the chimeric construct (PK6 in Table 1 and Fig. 2). A further 3- to 4-fold decrease in affinity was found when transmembrane segment V was also exchanged (PK5 in Table 1). Finally, when transmembrane segment IV from the NK_2 receptor was included in the chimeric construct, the ability of CP-96,345 to inhibit binding of radiolabeled SP was eliminated (from PK5 to PK4 in Table 1). These data, taken alone, indicate that the specific binding of CP-96,345 to the NK_1 receptor is determined by an epitope mainly located around transmembrane segment VI but possibly also involving epitopes around segments IV and V of the NK_1 receptor. However, our previous studies using NK_1/NK_3 chimeras showed a total loss of CP-96,345 activity in a chimeric construct corresponding to PK5 in which the splice junction was located in the middle of the second extracellular loop instead of at the C terminus of the second extracellular loop (at the N terminus of transmembrane segment V) (28). Thus, the final elimination of CP-96,345 affinity observed in the present study, when going from PK5 to PK4 (Table 1), could be caused by the exchange of residues which are located in the second extracellular loop, close to transmembrane segment V. In conclusion, the present NK_1/NK_2 chimeric constructs confirm our results with the NK_1/NK_3 chimeras (28), which indicated that the specific action of CP-96,345 on the NK_1 receptor is determined by residues located close to the membrane surface in transmembrane segments V and VI.

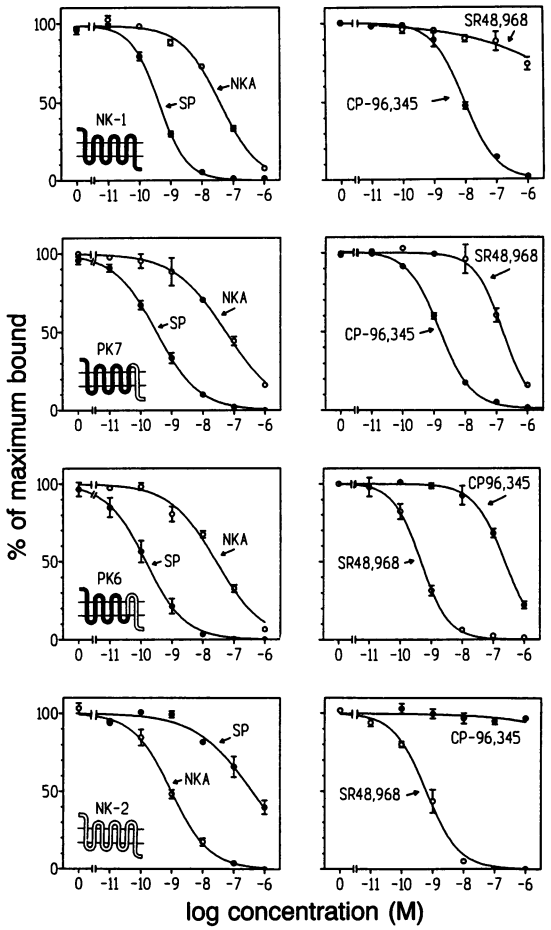

FIG. 2. Identification of domains involved in CP-96,345 and SR-48,968 action by use of NK_1/NK_2 chimeric constructs. (*Left*) Competition binding of the peptide agonists SP (●) and NKA (○) with [^{125}I]BH-SP for (from the top) the wild-type NK_1 receptor, PK7, PK6, and the wild-type NK_2 receptor. (*Right*) Competition binding of the nonpeptide antagonists CP-96,345 (●) and SR-48,968 (○) with [^{125}I]BH-SP for (from the top) the wild-type NK_1 receptor, PK7, PK6, and the wild-type NK_2 receptor. The binding assay was performed on intact COS-7 cells transiently transfected with the receptor constructs. Data are expressed as percentage of maximum specifically bound radioligand (mean ± SEM, n = 3–4). A simplified structure of the receptor is shown for each diagram; filled areas, segments derived from the NK_1 receptor; open areas, segments derived from the NK_2 receptor.

SR-48,968 Binding to Chimeric NK_1/NK_2 Receptors. The affinity for the NK_2-specific nonpeptide antagonist SR-48,968 on the NK_1 receptor was increased from undetectable to a K_i value of 140 nM by incorporating the C-terminal part of the NK_2 receptor up until the middle of the third extracellular loop (PK7 in Table 1). However, just as observed with the NK_1-specific antagonist CP-96,345, the most striking change in the affinity for SR-48,968 occurred between chimeric receptors PK7 and PK6 (Fig. 2). Thus, inclusion of the N-terminal part of the third extracellular loop plus transmembrane segment VI from the NK_2 receptor transferred full susceptibility for SR-48,968; i.e., the K_i increased >300-fold to 0.4 nM, similar to its affinity for the wild-type NK_2 receptor, K_i = 0.7 nM (Table 1 and Fig. 2). To delineate the binding site for SR-48,968 better, we constructed a chimeric

NK_1 receptor in which only residues 251–293 were derived from the NK_2 receptor (see PKP6 in Table 1 and Fig. 3). The affinity of SR-48,968 for this chimera was similar to the affinity of the compound for PK6 and for the wild-type NK_2 receptor as determined by the ability of SR-48,968 to inhibit binding of radiolabeled SP to the receptor constructs (Table 1). Thus, the selective action of SR-48,968 on the NK_2 receptor is apparently determined by nonconserved residues located between residues 251 and 293 of the NK_2 receptor.

Binding of [^{3}H]SR-48,968. The binding epitope of SR-48,968 was more directly identified by use of the radiolabeled nonpeptide antagonist itself. [^{3}H]SR-48,968 bound to COS-7 cells expressing the wild-type NK_2 receptor with a K_d value of 1.5 ± 0.4 nM (mean ± SEM, n = 4) (Fig. 3), whereas no specific, high-affinity binding of [^{3}H]SR-48,968 was observed with cells expressing the wild-type NK_1 receptor (data not shown). However, [^{3}H]SR-48,968 bound to COS-7 cells expressing the PKP6 construct, with a K_d value of 1.0 ± 0.1 nM (n = 4) (Fig. 3). Thus, exchange of the 17 residues which differ between the NK_1 and NK_2 receptors around transmembrane segments VI and VII and the connecting extracellular loop conveyed full high-affinity binding to the NK_1 receptor for SR-48,968 (Fig. 3). This indicates that in the NK_2 receptor, SR-48,968 is bound by an epitope located around segments VI and VII. This binding epitope for SR-48,968 on the NK_2 receptor appears to partially overlap with that for the NK_1-specific compound CP-96,345, as the affinity of CP-96,345 was reduced by >30-fold in PKP6 as compared with the NK_1 receptor (Table 1). In other words, by exchanging 17 nonconserved residues in the NK_1 receptor we have swapped the specificity of the receptor in its ability to bind nonpeptide antagonists, but in respect to peptide binding the chimeric receptor is essentially still an NK_1-type receptor, binding SP with high affinity and NKA with low affinity (Table 1).

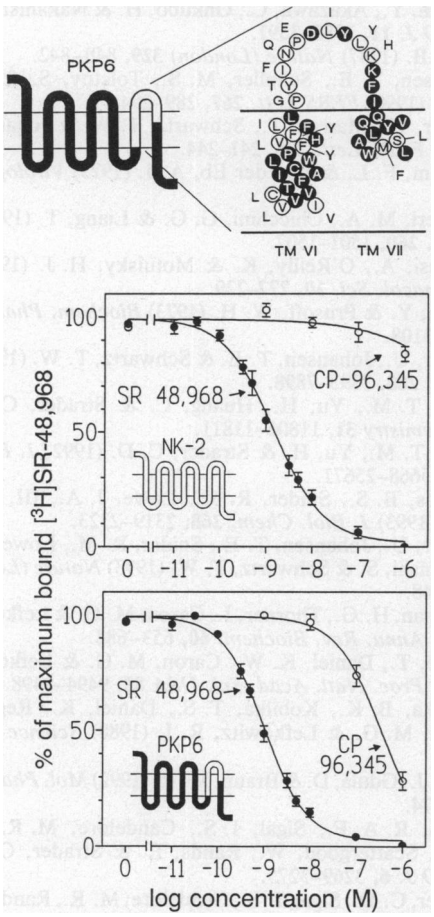

FIG. 3. Transfer of full NK_2-like affinity for SR-48,968 to the previously SR-48,968-unresponsive NK_1 receptor through construction of chimeric receptor PKP6 [NK_1/NK_2-(251–293)]. (*Top*) Structure of chimeric receptor PKP6 and amino acid sequence of the segment around transmembrane domain (TM) VI and TM VII in the NK_1 receptor that was exchanged with the corresponding segment from the NK_2 receptor. Filled circles indicate amino acid residues that are conserved between the NK_1 and NK_2 receptors. Open circles indicate nonconserved residues that are specific for the NK_1 receptor and that were exchanged with the corresponding residues in the NK_2 receptor. The corresponding NK_2 residues are shown next to the open circles. (*Middle* and *Bottom*) Competition binding of SR-48,968 (●) and CP-96,345 (○) with [^{3}H]SR-48,968 for the wild-type NK_2 receptor (*Middle*) and for PKP6 (*Bottom*). Data are expressed as percentage of maximum specifically bound radioligand (mean ± SEM, n = 4). The binding assay was performed on intact COS-7 cells transiently transfected with the receptor constructs.

Nonpeptide Antagonists Versus Peptide Agonist. Our studies suggest that the mechanism of action for the nonpeptide antagonists, which are *not* chemically related to their corresponding peptide agonists, may differ from that of the antagonists for the small, classical transmitters. Mutational studies in both adrenergic and muscarinic receptors have demonstrated that residues within transmembrane segments VI and VII also are important for the selective recognition of antagonists in these systems (29–33). However, binding of these antagonists also involves interactions with residues located several angstroms below the surface of the membrane in other transmembrane helices (29, 34). These interactions are often shared with the corresponding *agonists* (34), which is not surprising since these antagonists in most cases are chemically related to the agonists. Previously, by using smaller chimeric substitutions, we have demonstrated that CP-96,345 acts through epitopes located closer to the surface of the membrane than the epitopes identified for antagonists of the small, classical transmitters (28). Further, we have in both the present and previous mutational studies identified a long series of substitutions which severely impair or even eliminate the action of the nonpeptide antagonists without affecting the binding of the corresponding peptide agonists (Table 1) (28). Recently, both in the NK_1 and in the cholecystokinin systems, other mutations have been identified that impair nonpeptide antagonist binding without affecting peptide agonist binding (35, 36). Thus, in contrast to the antagonists for small classical messengers, which partly share binding site with the corresponding agonists, it appears that the binding epitope for the nonpeptide antagonists, at least to a major degree, is not shared with the natural peptide agonists. This concept is indirectly supported by the fact that during evolution amino acid substitutions have occurred in these receptors, which are neutral with respect to peptide binding, but which have led to sometimes substantial differences in the binding affinity of the nonpeptide antagonists among different species (26, 27).

Is there any spatial overlap in peptide and nonpeptide antagonist binding site? There could in fact be a larger overlap between these sites than our mutations tend to suggest. This study and previous studies have found that the C-terminal half of the tachykinin receptors can be interchanged without effect on the binding of SP and NKA (16, 24, 28). One interpretation could be that this is the region of the receptors which is activated by the common C-terminal, amidated part of the tachykinins, the "message sequence" (16). Since this is the area of the receptor which binds the nonpeptide antagonists, the peptide and the nonpeptide compound could share interaction points on *conserved* residues, which would not be identified by our present approach. Nevertheless, recently even a conserved residue in the middle of the identified binding epitope was shown to be important for CP-96,345 binding but not for SP binding (36).

We cannot completely exclude the possibility that the chimeric exchanges between the NK_1 and NK_2 receptors may also cause indirect effects—e.g., by affecting the packing of the transmembrane helices. However, such indirect effects should generally be expected to be disruptive for binding. We emphasize that in the key experiments of the present study, although we observed a decrease in the affinity of the NK_1 nonpeptide antagonist CP-96,345, the affinity for the peptide ligands were not affected and, importantly, full affinity for the NK_2-specific compound SR-48,968 was gained. This suggests a direct interaction between the nonpeptide compound and specific amino acid side chains in the identified domain.

Mechanism of Action of Nonpeptide Antagonists. It is surprising that both nonpeptide antagonists, CP-96,345 and SR-48,968, appear to act through epitopes which are located within the same region of their respective target receptors. This similarity in mechanism of action is especially interesting since the compounds chemically are very different and since they, with a high degree of specificity, inhibit two different albeit homologous receptors. The location of the target epitope around transmembrane segment VI suggests a common, general mechanism of action for at least a class of nonpeptide antagonists for peptide receptors. The intracellular loop connecting transmembrane segments V and VI, and especially the part just below segment VI, plays a crucial role in the specific G-protein coupling, signal transduction, and allosteric induction of the high-affinity state for this whole class of receptors (29). Thus, the molecular mechanism of action for nonpeptide antagonists, which bind with high affinity to transmembrane segment VI and surrounding elements of the receptor, may be related to perturbation of the overall, functional structure required for agonist binding and receptor activation. It should therefore be interesting to investigate whether nonpeptide antagonists in general act through epitopes located in the same general area of their target receptors. Recently, it was shown that nonpeptide antagonists for cholecystokinin receptors have at least one interaction point in transmembrane segment VI (35).

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