THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 35, Issue of September 2, pp. 22054–22058, 1994
Printed in U.S.A.

Identification of an Epitope in the Substance P Receptor Important for Recognition of the Common Carboxyl-terminal Tachykinin Sequence*

(Received for publication, Received May 31, 1994, and in revised form, June 28, 1994)

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The substance P receptor and the anti-substance P antibody NC1 share the ability to bind to the COOH terminus of substance P. Sequence analysis identified a direct noninterrupted homology of 5 residues in the two molecules. Replacement of Gly[166] and Tyr[167] in this epitope of the substance P receptor by the corresponding substance K receptor amino acids (Cys and Phe) increases the affinity toward substance P 2-fold and toward substance K and neurokinin B 11- and 21-fold, respectively. A significantly larger effect of the mutation is observed for the hexapeptides of substance P and substance K which show a mutation-induced increase in binding energy of more than 2 kcal/mol. Hence, the NH_2 terminus of substance P and, to a lesser extent, of substance K masks the effect of the mutation. I conclude that the epitope is important for recognition of the common COOH terminus of the tachykinins and for preservation of selectivity. The data furthermore suggest that formation of the peptide-receptor complex occurs through a composite set of interactions which are not adequately described by the two-site/no cooperativity "address-message" model.

The tachykinins constitute a family of biologically active neuropeptides which share the common carboxyl-terminal core Phe-*X*-Gly-Leu-Met-NH_2 (Nakanishi, 1987). The mammalian tachykinins include the three peptides: substance P, substance K, and neurokinin B, all of which elicit their physiological effects by binding to and activating three distinct receptors, termed the substance P (NK_1R),[1] the substance K (NK_2R), and the neurokinin B (NK_3R) receptors. Molecular cloning identified the tachykinin receptors as a subgroup of the guanine nucleotide regulatory protein (G-protein)-coupled heptahelical receptor superfamily (Nakanishi, 1991). Their amino acid sequences are very similar and display an overall homology of approximately 50%.

Although the tachykinins predominantly bind to their corresponding receptor, they do exhibit significant cross-reactivity toward the other tachykinin receptors subtypes (Yokota *et al.*, 1989; Shigemoto *et al.*, 1990). Cross-reactivity is probably due to recognition by each receptor of the fundamental carboxyl-terminal core element of the ligands, which is bound equally well by all three tachykinin receptors (Ingi *et al.*, 1991; Cascieri *et al.*, 1992).

The molecular cloning of the tachykinin receptors has allowed numerous studies to be undertaken to identify the structural basis for the receptors subtype characteristics in discriminating various agonist and antagonist. Two major conclusions emerge from the mutation analysis of the tachykinin receptors. First, transplantation of non-peptide antagonist sensitivity can be achieved without significantly influencing peptide binding, hence demonstrating that different receptor subsites are involved in determining the binding characteristics of peptide and non-peptide ligands (Fong *et al.*, 1992a, 1992b, 1993; Gether *et al.*, 1993a; 1993b; Sachais *et al.*, 1993). Second, these data suggest that many disperse sites throughout the receptor contribute to the generation of ligand selectivity and that the patterns of interactions are distinct for each receptor-ligand pair (Yokota *et al.*, 1992; Fong *et al.*, 1992a, 1992b, 1992c; Gether *et al.*, 1993a, 1993b, 1993c; Huang *et al.*, 1994). However, little data have indicated interaction sites between the receptor and the carboxyl terminus of substance P. Recently, Huang and colleagues (1994) reported that Asn[85] in the second transmembrane domain seems important for binding of the carboxyl-terminal amide-group of substance P.

In the present report, I analyze amino acid sequences of the substance P receptor (NK_1R) and the anti-substance P monoclonal antibody (NC1), which share the ability to recognize the carboxyl-terminal fragment of substance P (Ingi *et al.*, 1991; Cascieri *et al.*, 1992; Cuello *et al.*, 1979). I identify a common sequence which in the receptor maps in the carboxyl terminus of the fourth transmembrane segment, while in the antibody it is positioned in the third hypervariable region of the heavy chain (H.CDR.3). Since it has been proposed that binding of the conserved core element of tachykinins, in analogy with the cation amine receptors, involves sequences located in the transmembrane domains (Gether *et al.*, 1993c; Huang *et al.*, 1994) and that the specificity and affinity of the antibody binding sites are governed by the structures of the six hypervariable regions (Jones *et al.*, 1986) of which H.CDR.3 is the more diverse and has the least predictable conformation (Chothia *et al.*, 1989), this consensus sequence might reflect common molecular determinants in the substance P binding sites of the two molecules. Here I show data indicating that this receptor site is directly involved in or controls binding of the carboxyl-terminal tachykinin core.

EXPERIMENTAL PROCEDURES

Peptides—Monoiodinated ^{125}I-labeled Bolton-Hunter substance P (^{125}I-BH-SP) (2000 Ci/mmol) was purchased from Amersham Corp. The penta- and hexapeptides of SK and the chimeric [Ser[6],Val[8]]SP peptide were custom-synthesized by Kem-En-Tek (Copenhagen). Other peptides

* This work was financed through grants from Grosserer L.F. Fogths Foundation (Copenhagen, Denmark) and the European Community (Biotechnology). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: NK_1R, substance P receptor; NK_2R, substance K receptor; NK_3R, neurokinin B receptor; H.CDR.3, third complementary determining region of the heavy chain; SP, substance P; SK, substance K; NKB, neurokinin B; ^{125}I-BH-SP, monoiodinated ^{125}I-Bolton-Hunter substance P.

were obtained from Bachem and Sigma.

Construction of Mutated Receptor—The cDNA encoding the rat substance P receptor (pCDM8-SPR) was generously provided by Dr. S. Nakanishi, Institute of Immunology, Kyoto University, Japan. The NK_1R cDNA was subcloned into the replicative form of M13mp19 DNA. Site-directed mutagenesis was performed using the oligonucleotide-directed *in vitro* mutagenesis system from Amersham Corp. according to the instructions of the supplier. The oligonucleotide used was 5′-TTCCACAATGCTTCTACTCC-3′ purchased from MedProbe. The nucleotide sequence was verified by sequencing using the Sequenase sequencing system from U.S. Biochemical Corp.

Cell Cultures and Transfections—Wild-type and mutant receptors were transiently expressed in COS-7 cells as follows. Cells were plated and grown to 50% confluence in 10-cm tissue culture plates (Falcon) and transfected with plasmid DNA for 6–8 h in serum-free medium using the lipofectin transfection system from Life Technologies, Inc. The cells were shifted into medium supplemented with 10% fetal calf serum, grown for 24 h, and subsequently transferred into 12-well plates (Costar). Transfected cells were assayed after an additional 24–48 h of culturing.

Binding Assay—Characterization of the wild-type and mutant receptor was done using ^{125}I-BH-SP as the radioligand. Transfected cells were incubated for 3 h at 4 °C in the presence of 50 pM ^{125}I-BH-SP and increasing concentrations of cold competing peptide in 0.5 ml of 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM $MnCl_2$, 0.1% bovine serum albumin (fraction V) supplemented with bacitracin (100 μg/ml), leupeptin (5 μg/ml), and chymostatin (10 μg/ml). The incubation medium was removed, and the cells were washed twice in ice-cold binding buffer. The cells were lysed in 1 ml of lysis buffer (3 M acetic acid, 8 M urea, 2% Nonidet P-40) and counted. All samples were done in duplicate. Nonspecific binding was determined as binding in the presence of 3 μM cold substance P.

Data Analysis—Concentration response curves for the competition of tachykinin analogues for the binding of ^{125}I-BH-SP were first analyzed by the computer program ALLFIT (De Lean *et al.*, 1978) to obtain estimates of the IC_{50} and their standard errors. The same competition binding isoterms were next analyzed according to the mass action law using the nonlinear least squares fitting procedure of the computer program LIGAND (Munson and Rodbard, 1980). The equilibrium binding affinity of Bolton-Hunter-iodinated substance P was assumed to be very close to that of substance P in the course of these calculations. The equilibrium affinities (K) of tachykinins analogues were converted into the corresponding standard free energy changes $\Delta G°$ using the equation $\Delta G° = -RT \ln(K)$ (where R is the gas constant and T is the absolute temperature of the binding reaction).

RESULTS

Sequence analysis of NK_1R and NC1 shows the existence of a perfect noninterrupted direct homology of five amino acids. In the receptor it is located at the carboxyl terminus of the fourth transmembranal domain (TM4) (amino acids 166–170) (Yokota *et al.*, 1989), while in the antibody it embraces the first five amino acids in the third CDR of the heavy chain, H.CDR.3 (Piccioli *et al.*, 1991) (Fig. 1). The substance K receptor (NK_2R) displays at position 167–171 (Yokota *et al.*, 1989) a consensus peptide matching three out of the five amino acids (Fig. 1) of the sequence common to NK_1R and antibody. Moreover, when all available mammalian tachykinin receptor sequences are examined, this locus appears to be completely preserved among different species (data not shown).

In an attempt to test the relevance of the consensus sequence as a common molecular determinant in the substance P binding site and the significance of the subtype specific residues in this locus, glycine 166 and tyrosine 167 of NK_1R were replaced by the corresponding amino acids of the NK_2R, *i.e.* cysteine and phenylalanine, respectively.

To assess how the double mutation affected the affinity of the receptor for substance P, both parental (NK_1R^{WT}) and mutant (NK_1R^{CF}) receptors were transiently expressed in COS cells, and their binding properties were examined in a radioreceptor assay based on the competition of tachykinin analogues for the binding of ^{125}I-BH-SP in intact cells. The expression level of NK_1R^{CF} in these experiments was systematically lower than

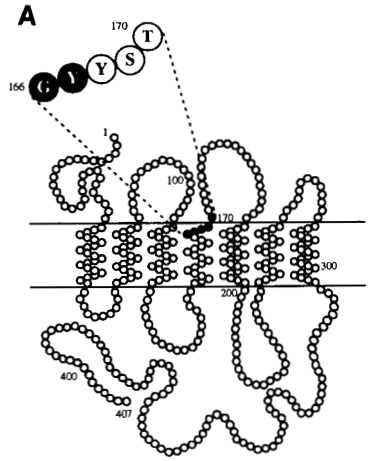

FIG. 1. **Structure and sequence alignment of the substance P receptor.** *A*, schematic model of the substance P receptor indicating the five residues (*filled circles*) shared with the NC1 antibody. The five amino acids are enlarged above the receptor with the 2 residues which differ in the substance K receptor shown in *black*. *B*, the relevant amino acids of the NC1 antibody heavy chain and of the tachykinin receptors for SP and SK are aligned to illustrate the 5-residue homologous sequence shared by the SP receptor and antibody (in *bold*). *Underlined* residues in the receptor and antibody sequences are presumed to be located in the transmembrane segment 4 (Yokota *et al.*, 1989) and the third H-CDR (Piccioli *et al.*, 1990), respectively. Numbering of the sequences is according to Yokota *et al.* (1989) and Piccioli *et al.* (1990).

that of the parental clone, and the reason for this difference is unclear at the moment. Nonetheless, the level of expression of the mutated receptor was sufficient to yield a favorable ratio between specific and nonspecific binding and to allow for an accurate characterization of the transfected protein.

Competition isotherms of substance P, substance K, and neurokinin B for the binding of ^{125}I-BH-SP to the two receptors expressed individually in living COS cells demonstrated that the mutation induces a reduction of IC_{50} for all three tachykinins (data not shown). However, the decrease was greater for substance K (10-fold) and neurokinin B (20-fold) than for substance P (2-fold), suggesting that the two-amino acid replacement enhances receptor affinity for the two peptides to a different extent.

This was confirmed by mass action law analysis of these binding data. The binding constant of substance P was enhanced approximately 2-fold by the mutation, whereas that of substance K was increased 11-fold (Table I). These results support the hypothesis that the identified sequence is involved in binding to the core element of tachykinins, but they also suggest a more complex relationship between recognition of the common core sequence of tachykinin and receptor selectivity.

To investigate this relationship further, the affinities of progressively shorter substance P peptides for the two receptors were determined (Table I). Best fitting equilibrium affinity estimates were converted into the corresponding free binding energies and graphically plotted as a function of the number of amino acid residues extending beyond the common tachykinin core C terminus sequence (Fig. 2). This graph illustrates how the overall change of free energy associated with the equilib-

TABLE I
Equilibrium binding affinities of tachykinin peptides for the wild-type and mutant receptor

Competition binding isotherms of tachykinin peptides in competition for the binding sites labeled by ^{125}I-BH-SP on the wild-type and mutant substance P receptor were analyzed as described under "Experimental Procedures." Best fitting equilibrium affinities computed for each experiment are listed as mean values. All reported data have a standard error lower than 30% of the mean which is not included in the table for clarity. The effect of the mutation is highlighted by taking the ratio of affinities between mutant and wild-type receptors.

Amino acid sequence	Peptide	K		Ratio (CF/WT)	n^a
		NK_1R^{WT}	NK_1R^{CF}		
		M^{-1}			
RPKPQQ FFGLM	SP	3.7×10^8	7.3×10^8	2	5
QQ FFGLM	SP(5–11)	9.4×10^6	1.6×10^8	17	2
Q FFGLM	SP(6–11)	1.5×10^6	5.3×10^7	35	3
FFGLM	SP(7–11)	1.2×10^4	1.8×10^5	14	3
HKTNS FVGLM	SK	5.1×10^6	5.5×10^7	11	4
NS FVGLM	SK(4–10)	1.4×10^5	2.9×10^6	21	2
S FVGLM	SK(5–10)	6.7×10^4	3.8×10^6	57	3

[a] n = number of experiments.

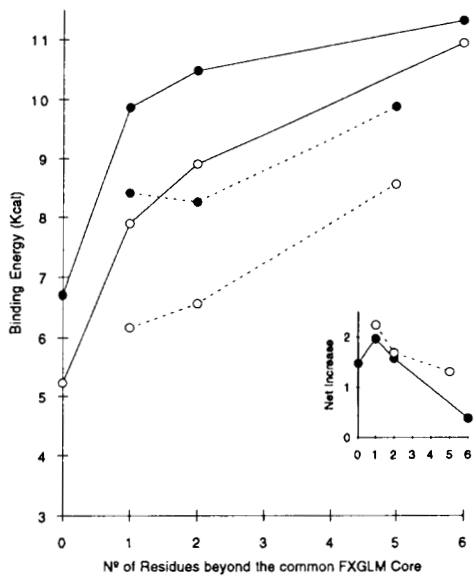

FIG. 2. **Free energy change for tachykinin peptide fragments to the wild-type and mutant substance P receptor.** The standard change of free energy for equilibrium binding of SP peptide fragments (*solid lines*) and SK peptide fragments (*dotted lines*) to the wild-type (*open circles*) and mutant (*closed circles*) substance P receptors are shown as function of peptide length beyond the carboxyl-terminal pentapeptide core sequence. Data were obtained by converting equilibrium affinity constants (Table I) into binding free energy as described in "Experimental Procedures." *Inset*, the net increase in binding energy for each peptide due to the mutation is shown as function of amino-terminal extension of the core sequence.

rium binding reaction of substance P to either wild-type and mutant receptor is build up as we go from the minimally required pentapeptide core element to the full-length 11-residue natural ligand.

Two important indications emerge from such analysis. First, there is a fundamental similarity between wild-type and mutant receptors in the way the full affinity of the binding site is attained as the sequence of the ligand is extended into the amino terminus direction. In both cases, the pentapeptide core element contributes about 50% of the total binding energy, while the remaining 50% is added in a stepwise fashion upon extension of the amino-terminal sequence. Similar results were reported by others (Ingi *et al.*, 1991; Cascieri *et al.*, 1992) for wild-type tachykinin receptors.

Second, despite the parallelism of the increase of binding energy with the increase in length of peptide sequence, a clear difference exists between mutant and parental receptor, since the mutant starts from a higher energy level and reaches its full binding energy with less amino-terminal extension than does the parental receptor.

A replot of the net increase of binding energy due to the mutation as a function of peptide length (Fig. 2, *inset*) clearly indicates that the effect of the mutation is larger for shorter substance P peptides and apparently reaches a maximum for the COOH-terminal hexapeptide. For this analogue the mutation induces a remarkable net increase of binding energy (2 kcal/mol) compared to that observed for the binding of full-length substance P (0.4 kcal/mol). Therefore the mutation primarily affects recognition by the receptor of the minimal substance P COOH-terminal core element, and the presence of the amino-terminal tail of substance P somehow obstructs the effect of the mutation. This suggests that the mutated amino acids are either part of, or control the conformation of, the binding pocket in which the receptor accommodates the common consensus sequence FXGLM shared by all tachykinin-like peptides.

To further evaluate this hypothesis, peptides corresponding to the COOH-terminal sequence of substance K were examined. Unfortunately, the substance K pentapeptide amide FVGLM was poorly soluble in the reaction buffer, and its binding affinity could not be measured. However, the affinities of hexa- and heptapeptide of the COOH-terminal substance K could be determined and compared with those of the corresponding sequences of substance P (see Table I and Fig. 2). Substance K-derived sequences exhibited a general increase of binding energy as a consequence of the mutation, the extent of which was comparable or even larger than that observed for substance P peptides. Although the mutation-derived increase of binding energy was also greater for the COOH-terminal hexapeptide than for full-length substance K, this difference was much smaller than that observed for the substance P series. Therefore, the amino-terminal extension of substance K does not inhibit the mutation-induced improvement of core binding as it does in substance P.

The data shown above indicate that the two hexapeptides exhibit a comparable enhancement of affinity due to the mutation, while the corresponding full-length peptides do not. To examine whether the amino-terminal sequence of substance P exerts a general detrimental effect on the mutation-induced enhancement of tachykinin hexapeptide binding, a peptide constituted of the carboxyl-terminal hexapeptide of substance K and the amino-terminal substance P pentapeptide was synthesized. The affinity of this hybrid [Ser^6,Val^8]SP peptide for wild-type and mutant receptors was measured. Fig. 3 demonstrates that the chimeric ligand displays a 30-fold mutation induced decrease in IC_{50}. The binding affinity for the parental NK_1R was 1.7×10^7 M^{-1}, and increased to 5.2×10^8 M^{-1} for mutated NK_1R^{CF}. Thus, the effect of the mutation on this chimeric ligand is comparable to or even larger than the effect on authentic substance K despite the presence of the amino-terminal tail of substance P.

DISCUSSION

In an attempt to map in the substance P receptor the sites of interaction with the putative common carboxyl-terminal tachykinin core, I identified a direct noninterrupted sequence common to the carboxyl-terminal ending of the IV transmembrane domain of the receptor and to the H.CDR.3 of the anti-substance P antibody NC1. Since the epitope of this antibody is the carboxyl-terminal half of substance P (*i.e.* the common core), I speculated that this localized homology between the two bind-

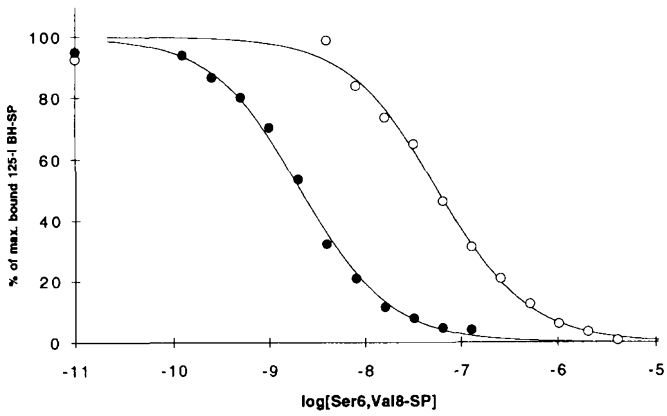

FIG. 3. **Competition binding profiles for [Ser^6,Val^8]SP on the wild-type (*open circles*) and mutant (*closed circles*) substance P receptor.** Data are expressed as percent of bound ^{125}I-BH-SP to each receptor in the absence of cold competitor. Each pair of curves was obtained from one experiment in which the two receptors were expressed individually on living COS cells and it is representative of three similar experiments done in duplicate. Specific bound/total (B/T) in the absence of cold competitor was approximately 40% for the wild-type and 20% for the mutant receptor, while nonspecific B/T was less than 4%.

ing proteins may not be serendipitous, but constitutes an unusual example of antibody mimicry. That is, being both antibody and receptor directed toward binding an identical pentapeptide sequence, they might share common structural elements in the region of their binding sites, despite the vastly different nature and function of the two proteins. Thus, the antibody CDR could constitute a template on which to find an "image" of relevant binding motifs that ought to be shared by all subtypes of tachykinin receptors.

This common sequence of NK_1R was absolutely conserved across species, but it showed only 2 invariant residues across the three tachykinin receptor subtypes. A rational strategy to address this hypothesis was, therefore, to generate a NK_1R mutant chimera in which the 2 variant residues of the NK_1R sequence were replaced with those corresponding to the sequence of NK_2R.

Two key biochemical properties of the mutant NK_1R support the idea that the targeted residues control primarily the ability of the receptor to recognize common tachykinin molecular determinants. First, the mutation enhanced the affinity of NK_1R, albeit to a different extent, for all prototypic members of the family of mammalian tachykinins. This is exactly what it may be expected for a mutation that alters the molecular sites involved in recognition of the common binding element of tachykinins. Second, the mutation enhances the affinity of the receptor for carboxyl-terminal hexa- and pentapeptide much more than it does for that of the full-length peptide, indicating that its effect is greater the closer the ligand conforms to the common tachykinin sequence.

These biochemical features, however, also raise a number of questions on the mechanism of interaction between tachykinins and their receptors and on current models explaining the generation of molecular diversity in subtypes of peptide receptors. The first question is whether the data presented here fit to a prevalent view of tachykinin peptide-receptor interactions which is based and the address-message model of peptide hormones action (Schwyzer, 1977; Yokota *et al.*, 1992; Fong *et al.*, 1992a; Gether *et al.*, 1993c). According to this view, the COOH-terminal half (the "message") interacts with subsites of the receptor that are common to all tachykinin receptors, while the NH_2-terminal half of the sequence (the "address") establishes contacts with additional subsites that differ among different receptor subtypes and provide the molecular basis for selectivity.

The data presented here, however, do not support such a model. They indicate that the mutation does not induce a constant increase of binding energy for all fragments of the same peptide family, but that the net enhancement is largest for corelike ligands and smallest for full-size peptides. In contrast, the address-message model would predict that if a favorable change of the receptor results in increased binding energy for the common message peptide, such a net enhancement should remain a constant base line and be additive to the increase of binding energy due to the extra interactions established by the amino-terminal tail. Further, the observation that the affinity of [Ser^6,Val^8]SP, a hybrid molecule carrying the message region of substance K and the address part of substance P, is enhanced by the mutation as much as the affinity of the substance K hexapeptide is, clearly indicates that it is not possible to transfer the "inhibitory" effect of the amino-terminal tail of substance P to the carboxyl terminus of substance K simply by tethering the two sequences into a single molecule. Hence, the receptor peptide interactions seem inadequately described by a "simple" two-site/no cooperativity address-message model. Two alternative hypothesis should therefore be considered.

One is that the formation of the peptide-receptor complex occurs via an induced-fit mechanism assisted by conformational flexibility in the structure of both binding partners. Hence, as the consensus core is extended with amino-terminal residues, while the overall affinity of peptide becomes greater due to the larger total number of interacting subsites, the net contribution of binding energy due to the interactions of the consensus carboxyl terminus becomes progressively smaller because of the negative heterotropic effect transmitted by the binding of the amino-terminal residues to the receptor.

An alternative hypothesis is that the effect of the amino-terminal extension of tachykinin is not transmitted via the receptor sequence, but intramolecularly within the tachykinin peptide itself, and consists in constraining the degree of freedom of the carboxyl-terminal consensus sequence. Thus, if a mutation improves the configuration of the docking points in the receptor for the carboxyl-terminal sequence of tachykinins, its positive effect would be larger when this sequence is presented to the receptor as a flexible short peptidic fragment than when it is as a conformationally constrained part of a longer peptide. Evidence for a restricting effect of the amino-terminal tail on the conformation of the carboxyl-terminal half of tachykinins comes from structure-activity studies of conformationally limited synthetic peptide analogues. An impressive outcome of those studies was the synthesis of shorter peptide fragments of tachykinins displaying affinities and selectivities comparable or even greater than those of their natural full-length counterparts (Regoli *et al.*, 1988; Ingi *et al.*, 1991; Cascieri *et al.*, 1992).

The results presented here cannot obviously indicate an unambiguous discrimination between the two mechanisms discussed above. However, the analysis of [Ser^6,Val^8]SP hybrid suggests that the induced-fit mechanism might not be an adequate interpretation of the data, because the inhibitory effect on the docking pocket of the receptor for the consensus core exerted by binding of the amino-terminal tail of substance P should be transmitted regardless of the type of COOH-terminal sequence entering that pocket. Instead, these results might better be explained by a tail-mediated constraining mechanism on the consensus COOH-terminal half, and they also suggest that a high degree of selectivity exists for the combinations of cores and tails among tachykinins.

The importance of the residues identified in this study is consistent with recent mutagenesis analysis of chimeric tachykinin receptors (Yokota *et al.*, 1992; Fong *et al.*, 1992a; Gether

et al., 1993a, 1993b, 1993c), which have indicated that the fourth transmembrane segment, among other receptor sequences, is important for receptor recognition and selectivity, particularly toward conformationally constraint carboxyl-terminal peptides.

A second question is with regard to the origin of molecular diversity among tachykinins and the structural basis of receptor-ligand selectivity. The data presented here show that a discrete replacement of only two amino acids between a very localized locus of substance P and substance K receptors has a dramatic effect on the ability of the substance P receptor to discriminate among tachykinin ligands. The mutation caused a reduction, not a change, of selectivity. In fact, a surprising outcome of this study is that the transplantation of amino acidic residues belonging to the sequence of NK_2R into this locus of NK_1R resulted in a new sort of pan-reactive tachykinin receptor subtype rather than in a receptor with a switch of specificity from the substance P to substance K. One possible implication of these data is that selectivity in this family of receptors was achieved through evolution of a complex balance of positive and repulsive interactions with both the variant and invariant regions of the tachykinin molecule, rather than by constructing new interactions with the variant part of the molecule while maintaining rigid the anchoring configuration of the invariant part.

As a final consideration, it is important to emphasize that these results do not exclude that the homology between NK_1R and the CDR of the NC1 antibody highlighted here might be the result of chance. It will be important to construct similar mutations in the sequence of the other neurokinin receptor subtypes and in the CDR of the antibody to further test the relevance of the homology region. Regardless of whether it was chance or necessity that inspired this study, the present data underline the importance of a short stretch of residues located at the junction between the fourth transmembrane and the second extracellular domain of the substance P receptor. This locus had not been identified in previous studies, but its location and the mutagenesis results presented here suggest that it may play a crucial role both in the recognition of the consensus sequence of tachykinins and in the preservation of receptor selectivity.

Acknowledgments—I am particular grateful to Dr. Tommaso Costa (Istituto Superiore di Sanità, Italy) for hospitality, support and discussions, for careful reading and criticism of the manuscript, and for providing the computer software utilized in this study. I am grateful to Prof. P. Calissano for laboratory space and facilities during my visit to his laboratory. I am grateful to Dr. S. Nakanishi for providing the cDNA for the substance P receptor.

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