Conformational Compatibility as a Basis of Differential Affinities of Tachykinins for the Neurokinin-1 Receptor

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ABSTRACT: The neurokinin-1 receptor is characterized by sub-nanomolar affinity for substance P and 30−100 nM affinity for other substance P-related peptides, including neurokinin B and septide. We have characterized a neurokinin-1 receptor mutant containing a Y216A substitution in the fifth transmembrane segment. This receptor mutant binds substance P with sub-nanomolar affinity but loses much of its peptide discrimination capability, exhibiting 1−2 nM binding affinity for other tachykinins. Kinetic measurements of ligand binding indicate that the increased binding affinity of neurokinin B and septide for the Y216A mutant compared to the wild-type receptor is due to a 100-fold increase in the association rate constant without appreciable change in the dissociation rate constant. The substantially increased association rate constant for the Y216A mutant suggests that the mutant receptor is probably more flexible in accommodating the approaching peptide molecule. It is proposed that a major determinant of peptide specificity for the wild-type neurokinin-1 receptor is the overall conformational compatibility between the receptor and the ligand, rather than residue-specific interactions with the divergent N-terminal residues of different peptides. Furthermore, the highly conserved nature of Tyr-216 in the G protein coupled receptor family suggests that this residue may also play an important role in the receptor activation process in general.

Understanding the molecular determinants of ligand binding affinity and specificity is especially important in membrane receptor research and the discovery of novel therapeutic agents. While specific interactions are critical in the recognition of small molecules (Drickamer, 1992; Strader et al., 1991), it is less clear whether peptide recognition by related receptor subtypes is also based on the same premise of group-specific recognition. Peptide ligands typically contain 5−30 residues, giving rise to a much higher degree of conformational freedom. In the case of the neurokinin-1 receptor (NK1R),[1] peptides with the C-terminal sequence FXGLM-NH_2 will bind and activate the receptor and downstream effectors through G proteins (Helke et al., 1990; Iversen et al., 1987; Nakanishi, 1991). The undecapeptide substance P (SP) is the most potent endogenous agonist for this receptor while other SP-related peptides are much less potent. Earlier studies have demonstrated that the N-terminal divergent residues of SP can be substituted individually with Ala without affecting the binding affinity (Regoli et al., 1984), suggesting that the N-terminal residues of SP do not contribute directly to high-affinity binding.

More recently, receptor mutagenesis experiments have provided a wealth of information regarding peptide−receptor interactions (Brodbeck et al., 1995; Fong & Strader, 1994; Schwartz, 1994; Strader et al., 1994). These studies have indicated that neurokinins interact with both extracellular and transmembrane residues of the NK1R (Fong et al., 1992a; Gether et al., 1993; Huang et al., 1994a; Yokota et al., 1992). In addition to the NK1R, two other subtypes of neurokinin receptor have been cloned, and all three subtypes share substantial sequence similarity. Because each receptor subtype has its own characteristic amino acid sequence and pharmacological properties, chimeric receptor mutants have been used to test whether the divergent receptor residues interact directly with peptides. These studies and additional results obtained from single-residue mutants suggested that the extracellular divergent receptor residues do not appear to interact directly with divergent residues of peptides (Fong et al., 1992a; Huang et al., 1994b). Most of the receptor residues required for peptide binding are conserved among the three receptor subtypes, and substitution of these residues leads to parallel changes in the affinity for all neurokinins and the C-terminal half analog of SP (Huang et al., 1994a; Werge, 1994). Therefore, receptor mutagenesis indicated that the conserved C-terminal portion of neurokinins interacts with the receptor, whereas the N-terminal half probably helps to maintain the peptide conformation rather than play a direct role in peptide recognition.

Here we report that Tyr-216, which is near the intracellular end of the fifth transmembrane segment and is conserved among most G protein coupled receptors, plays an important structural role in receptor activation and peptide binding specificity. The Y216A mutation abolishes SP-stimulated PI hydrolysis in the transient COS expression system while preserving the high-affinity binding of SP. This receptor mutant is essentially nonselective with respect to many tachykinin peptides and binds many peptides with 1−2 nM affinity. The increased peptide affinity for the Y216A mutant is due to an increased association rate constant, suggesting

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[1] Abbreviations: ^{125}I-SP, [^{125}I-Tyr^8]SP; Ele, eledoisin; ERP, eledoisin-related peptide; NK1R, neurokinin-1 receptor; NK2R, neurokinin-2 receptor; NKA, neurokinin A; NKB, neurokinin B; PI, phosphatidylinositol; septide, [pGlu^6,Pro^9]SP(6−11); SP, substance P; SP-acid, substance P free acid; SPOME, substance P methyl ester.

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that the mutant receptor is more flexible in accommodating various tachykinin peptides. Conformational compatibility between the receptor and ligand therefore appears to be a major component of the peptide recognition process.

MATERIALS AND METHODS

All mutations were constructed from the human NK1R by the uracil selection method of site-directed mutagenesis (Bio-Rad, Richmond, CA). All mutated sequences were confirmed by DNA sequencing. All receptors were expressed in COS-7 cells for determination of ligand binding affinity and receptor activation, using 10 μg of plasmid DNA to transfect 10 million cells. The nonpeptide antagonists were synthesized as described previously (Edmonds-Alt et al., 1993; Francis et al., 1994; Hale et al., 1993; Peyronel et al., 1992). All peptide agonists were purchased from Peninsula Laboratories (Belmont, CA). Radiolabeled peptides were obtained from NEN.

The binding affinities of various ligands for the wild-type and mutant receptors were determined from the inhibition of 0.2 nM [^{125}I-Tyr^8]SP (^{125}I-SP) binding to intact transfected COS cells at 4 °C in the presence of varying concentrations of unlabeled ligands (Fong et al., 1992a). IC_{50} values were calculated from these experiments, which represents the ligand concentration at which 50% inhibition of the specific binding of ^{125}I-SP was observed. The receptor expression level (B_{max}) was calculated as described (Swillens, 1992). The formation of total inositol phosphates was measured as previously described (Huang et al., 1994a), in which each well was seeded with 0.7% of the transfection mixture.

The dissociation rate constant (k_2) of ^{125}I-SP at 4 °C was measured first for the wild-type and mutant receptors (Cascieri et al., 1992). The association rate constant (k_1) of ^{125}I-SP at 4 °C was then measured by following the time course of ^{125}I-SP binding (0.2 nM ^{125}I-SP and 0.04 nM receptor). The ratio of bound cpm to total added cpm was plotted as a function of time. Least squares fitting was performed on the data using the differential equation of second-order association:

$$R + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} LR$$

$$d[LR]/dt = k_1[L][R] - k_2[LR]$$

The differential equation was solved numerically. The contribution of the low-affinity state of the NK1R to the binding of ^{125}I-SP is negligible because the concentration of ^{125}I-SP is well below the K_d of the low-affinity state (Mazina et al., 1994) and the dissociation of ^{125}I-SP from the low-affinity state is fast [$T_{1/2}$ = 10 s. (Cascieri et al., 1992)]. The dissociation rate constant (k_2), which has been determined independently (see above), was held constant during curve fitting. The software Scientist (MicroMath, Salt Lake City, UT) was used for fitting differential equations.

The kinetic parameters of unlabeled ligands were determined as described (Sklar et al., 1985). In the coincubation protocol, aliquots of intact cell suspension were added to tubes containing ^{125}I-SP and appropriate concentrations of unlabeled ligand (SP, NKB, septide, or L-703606) and incubated at 4 °C. At various time points, bound radioligand was separated from free radioligand by filtration. The simplest model of ligand binding was used to describe the reaction:

$$R + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} LR \qquad L \text{ is } ^{125}\text{I-SP}$$

$$R + C \underset{k_4}{\overset{k_3}{\rightleftharpoons}} CR \qquad C \text{ is the unlabeled ligand}$$

$$d[R]/dt = k_4[CR] - k_3[R][C] + k_2[LR] - k_1[R][L]$$

$$d[CR]/dt = k_3[R][C] - k_4[CR]$$

$$d[LR]/dt = k_1[L][R] - k_2[LR]$$

With the coincubation protocol, the initial condition of [R] is equal to the total receptor concentration as determined by ^{125}I-SP binding. In the preincubation protocol, cells were preincubated with unlabeled ligand at 4 °C for 2 h. ^{125}I-SP ($^1/_{12}$th of the preincubation volume) was then added, and at various time points the bound radioligand was separated from free radioligand by filtration through GF/C filters. The preincubation protocol is described by the same equations as shown above except that the initial concentration of R has been reduced. In curve fitting, the initial condition of [R] in the preincubation protocol was calculated using the K_i determined in inhibition experiments (see Figure 1A). Each set of data (coincubated or preincubated) was fitted to a system of the three differential equations. Both systems of equations shared the same computer-fitted rate constants (k_3 and k_4) and fixed constants (k_1 and k_2) which have been determined independently, but used different initial conditions of [R], [C], and [CR]. The curve fitting was performed simultaneously so that the computer-fitted k_3 and k_4 would fit both the coincubation data and preincubation data.

RESULTS

Previous studies have shown that Tyr-205 in helix 5 of the NK1R is involved in peptide-induced receptor activation (Huang et al., 1994a). To further investigate the mechanism of receptor activation, Tyr-216 was selected for mutational analysis because it is highly conserved in most G protein coupled receptors and is very close to the third intracellular loop which has been shown to be involved in G protein coupling (Hedin et al., 1993). As shown in Figure 1A and Table 1, the binding affinity of SP for the Y216A mutant was similar to that for the wild-type receptor. However, the Y216A mutant was unresponsive to 10 μM SP in the PI hydrolysis assay (Figure 1B), indicating that the Y216A substitution substantially reduced the activation of phospholipase C. In addition, the ^{125}I-SP binding to the mutant receptor was marginally sensitive to GTPγS (Figure 1C), suggesting that the ability of the Y216A mutant to catalyze the GTP/GDP exchange of G proteins was substantially reduced. The mutant receptor may still bind G protein unproductively (Franke et al., 1990) or may still have a very low level of activity that cannot be detected by the present assay. Further investigation of other substitutions at position 216 indicated that receptors containing Tyr or Phe at position 216 were capable of activating PI hydrolysis and receptors with Trp or Leu were nearly inactive, while Ala or Glu substitution completely abolished the activation in the transient COS expression system (Table 1).

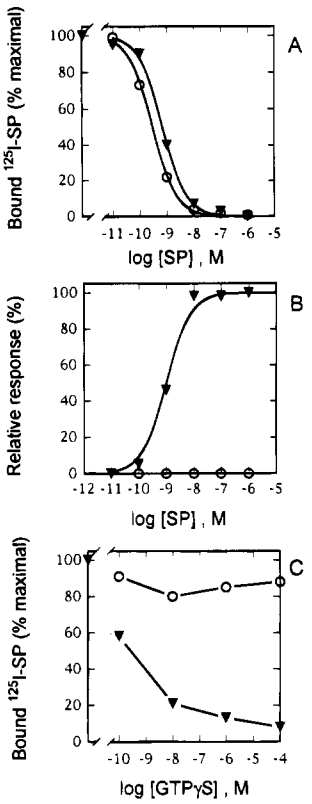

FIGURE 1: Comparison of the wild-type NK1R (closed triangles) and the Y216A mutant (open circles). (A) Inhibition of ^{125}I-SP binding by unlabeled SP. (B) SP-stimulated inositol phosphate accumulation. The data are normalized to the response level of the wild-type receptor in the presence of 1 μM SP. (C) Effect of GTPγS on ^{125}I-SP binding. This experiment was carried out at room temperature.

Unexpectedly, the Y216A mutation produced a dramatic effect on peptide recognition. As shown in Figure 2A, the wild-type receptor displayed a characteristic rank order of potency for SP-related peptides, with SP having an IC_{50} of 0.6 nM and other amidated or esterified peptides having IC_{50} values of 30−100 nM. In contrast, the Y216A mutant was poorly selective, with IC_{50} values of 0.3−2 nM for all amidated or esterified peptides. The affinity of SP-acid was also increased to 107 nM for the Y216A mutant, while SP-acid at 1000 nM did not inhibit the binding of ^{125}I-SP to the wild-type receptor. Contrary to SP-related peptides, non-peptide antagonists bound to the Y216A mutant with either the same or slightly reduced affinity compared to the wild-type NK1R (Figure 2B). Furthermore, glucagon (a peptide unrelated to SP) at concentrations up to 10 μM did not inhibit ^{125}I-SP binding to the Y216A mutant, indicating that only tachykinins exhibit increased affinity for the Y216A mutant.

In addition to the Y216A mutant, the Y216E substitution led to a slight-to-moderate increase in the affinities of NKA, NKB, septide, and SPOME (Table 1). Such an increase in binding affinity was less than that observed for the Y216A mutant. In contrast, the Y216F, Y216W, and Y216L mutants were very similar to the wild-type receptor with reference to the affinities of SP and other peptides tested.

To investigate the mechanistic basis of the increased affinity of the Y216A mutant for peptides such as NKB or septide, association and dissociation rate constants were determined. The rate constants of ^{125}I-SP for both the wild-type receptor and Y216A mutant were determined directly (Figure 3). Although small differences in the rate constants of ^{125}I-SP for the wild-type and mutant receptors were observed, the calculated binding affinities of ^{125}I-SP were similar for both the wild-type receptor and the Y216A mutant (Table 2). To determine the rate constants of unlabeled peptides and to compare the wild-type receptor with mutant receptor directly, an indirect method was used in which the association of ^{125}I-SP was monitored in the presence of unlabeled peptide, with or without prior incubation with the unlabeled peptide (Sklar et al., 1985). The preincubation step changed the initial conditions such that the binding of ^{125}I-SP to receptors preincubated with unlabeled ligand would be heavily dependent on the dissociation rate constant of the unlabeled ligand. Without preincubation, the binding of ^{125}I-SP to the receptor would be less dependent on the dissociation rate constant of the unlabeled ligand. By fitting the two sets of data simultaneously to a system of differential equations, the rate constants for the unlabeled ligand can be estimated. To validate the indirect method, the rate constants of unlabeled SP and unlabeled L-703606 were compared to those of ^{125}I-SP and [^{125}I]-L-703606. As shown in Table 2, the kinetic parameters of SP for the wild-type receptor were very similar to those determined directly for ^{125}I-SP, and the calculated K_d was close to the estimated binding affinity (K_i) determined in equilibrium inhibition assay (Table 2). The dissociation rate constant of L-703606 for the wild-type receptor (Table 2) was also similar to that determined for [^{125}I]-L-703606 (Cascieri et al., 1992).

Although NKB and septide had increased affinity for the Y216A mutant compared to the wild-type receptor, their dissociation rate constants (k_{off}) for the Y216A mutant were similar to those for the wild-type receptor. In contrast, the association rate constants (k_{on}) of both NKB and septide were increased by about 100-fold for the Y216A mutant compared to the wild-type receptor (Table 2, Figure 4). Although the change in the association rate constant for NKB and Septide was large, there were only small changes in the rate constants for the nonpeptide antagonist L-703606. This was consistent with a small change in the binding affinity of L-703606 (Table 1).

DISCUSSION

The molecular basis for ligand selectivity has been extensively studied in many receptor systems. It is generally accepted that specific interactions are critical in determining ligand binding affinity. For example, biogenic amine receptors interact with amines through an Asp residue, and this Asp residue is not present in receptors that do not recognize biogenic amines (Strader et al., 1991). However, it is not yet clear whether the differential binding affinities of similar ligands for a particular receptor and the subtype selectivity are also determined by group-specific interactions (e.g., norepinephrine ≥ epinephrine for the β1 adrenergic receptor *vs* epinephrine > norepinephrine for the β2 adrenergic receptor; SP > NKA > NKB for the NK1R *vs* NKA > NKB > SP for the NK2R). In the case of neurokinin receptors, the divergent N-terminal residues of neurokinins could serve as an address if the NK1R recognizes the divergent residues in SP but not in NKA or NKB. This possibility has been tested using chimeric receptors (Fong et al., 1992a; Gether et al., 1993; Yokota et al., 1992). These studies have concluded that subtype selectivity results from interactions of a particular ligand with multiple residues throughout many

Table 1: Effect of Amino Acid Substitution at Position 216 on Ligand Binding and Receptor Activation[a]

	binding							SP-elicited PI hydrolysis	
	IC_{50} (nM)						B_{max}		maximal
receptor	SP	NKA	NKB	septide	SPOME	L-703606	(fmol/10^5 cells)	EC_{50} (nM)	response (%)[b]
wild type	0.6 ± 0.2	31 ± 5	81 ± 13	58 ± 18	61 ± 40	1.4 ± 5	4.5	1.6	100
Y216F	2.6 ± 0.9	72 ± 27	191 ± 47	177 ± 95	68 ± 27	1.2 ± 0.2	4.2	5	26
Y216W	3.0 ± 1.5	49 ± 9	245 ± 95	217 ± 136	81 ± 59	2.3 ± 0.8	4.1	ND	6
Y216L	1.6 ± 0.8	33 ± 7	255 ± 113	216 ± 51	91 ± 78	0.8 ± 0.5	1.9	ND	12
Y216E	0.2 ± 0.1	5.5 ± 0.3	27 ± 9	17 ± 2	3.3 ± 0.3	4.7 ± 3	0.3	inactive	0
Y216A	0.3 ± 0.1	0.8 ± 0.3	2.2 ± 0.3	1.2 ± 0.1	0.4 ± 0.1	6.2 ± 1.9	0.7	inactive	0

[a] The data represent the average from two to three independent experiments. ND, not determined. [b] Maximal response was measured in the presence of 1 μM SP. All mutants were done in parallel with the wild type, and the mutant data were normalized to that of the wild type. The maximal response of the wild type ranged from 5000 to 15 000 cpm of [^{3}H]inositol phosphates over basal among independent experiments. For each independent experiment, there was no significant difference among the basal levels of [^{3}H]inositol phosphates in untransfected cells and cells transfected with the wild type or mutants. The basal level varied only slightly among independent experiments (4000−7000 cpm of [^{3}H]inositol phosphates).

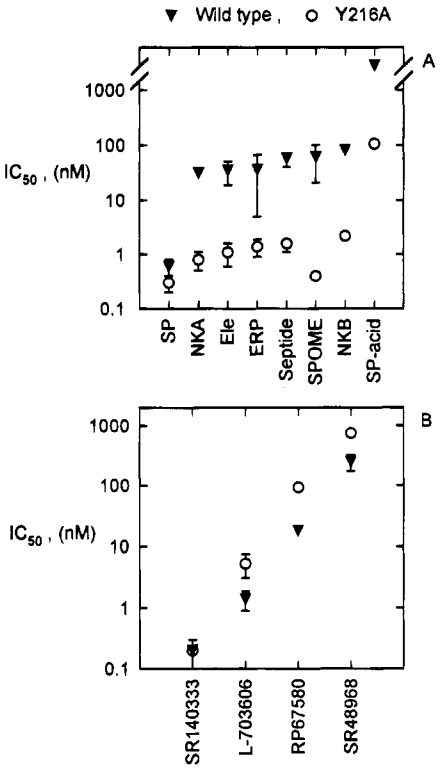

FIGURE 2: Binding affinity of peptide agonists (A) and nonpeptide antagonists (B) for the wild-type human NK1R and the Y216A mutant. The IC_{50} value is derived from the inhibition of ^{125}I-SP binding. SP-acid at 1000 nM did not inhibit the binding of ^{125}I-SP.

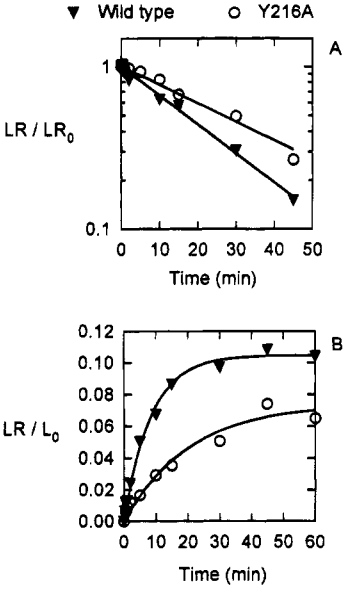

FIGURE 3: Measurement of the dissociation rate constant and association rate constant for ^{125}I-SP. (A) Dissociation of ^{125}I-SP from the wild-type receptor or the Y216A mutant. The ratio of bound ^{125}I-SP (LR) to initially bound ^{125}I-SP (LR_0) is plotted on a common logarithmic scale. The data are fitted to the equation (CPM − CPM[t=∞])/(CPM[t=0] − CPM[t=∞]) = $LR/LR_0 = \exp(-k_2 t)$, in which k_2 is the dissociation rate constant of ^{125}I-SP. (B) Association of ^{125}I-SP with the wild-type receptor or the Y216A mutant. The ratio of bound ^{125}I-SP (LR) to total added ^{125}I-SP (L_0) is plotted as a function of time. The data are fitted to the differential equation $d[LR]/dt = k_1[L][R] - k_2[LR]$, in which L is ^{125}I-SP, k_2 has been determined independently in (A), and k_1 is the computer-fitted association rate constant. The end point of LR/L_0 reflects the amounts of cells used for each receptor.

parts of the receptor. Follow-up studies based on the substitution of a small number of divergent residues indicated that SP-specific interactions are difficult to localize (Huang et al., 1994b). Therefore, the divergent residues in neurokinins do not appear to be an address that would be recognized directly by one receptor subtype but not another.

An alternative explanation for the subtype selectivity is based on the conformational compatibility between ligand and receptor (Huang et al., 1995). Similarly in the case of nonpeptide NK1 antagonists, two divergent receptor residues were found to account for the species selectivity, and the conservative nature of these substitutions suggested a conformational basis for the species selectivity (Fong et al., 1992b). In the present study, we have introduced the Y216A mutation in the inner part of the fifth transmembrane segment, and this substitution changed the peptide selectivity dramatically. The Y216A mutant receptor is able to bind many tachykinin peptides with high affinity, while nontachykinin peptides (such as glucagon) remain inactive. The introduction of an Ala side chain into the mutant receptor would not provide a basis for direct, favorable electrostatic interactions that would explain the increased affinity. Similarly, an unfavorable interaction between the Tyr side chain in the wild type and a bound peptide does not provide a likely explanation for the lower affinities of so many different tachykinin peptides for the wild-type receptor. Furthermore, receptor modeling suggested that Tyr-216 is located almost at the bottom of helix 5, a location unlikely to be reached by a bound peptide (Huang et al., 1994a). Therefore, the Y216A substitution apparently increases the binding affinity for this series of peptides indirectly. Because many antago-

Table 2: Kinetic Parameters of Ligand Binding[a]

ligand	k_{on} (M^{-1} min^{-1})		k_{off} (min^{-1})		$K_d = k_{off}/k_{on}$ (nM)		K_i (nM)[b]	
	wild type	Y216A	wild type	Y216A	wild type	Y216A	wild type	Y216A
^{125}I-SP	$(3.4 \pm 1.1) \times 10^8$ (9)	$(1.8 \pm 0.5) \times 10^8$ (6)	0.05 ± 0.02 (3)	0.013 ± 0.006 (3)	0.1	0.07	ND	ND
SP	$(4.9 \pm 1.5) \times 10^8$ (2)	ND	0.08 ± 0.03 (2)	ND	0.2	ND	0.4	0.1
NKB	$(4.8 \pm 2.4) \times 10^6$ (3)	$(1.5 \pm 0.5) \times 10^8$ (4)	0.28 ± 0.11 (3)	0.09 ± 0.02 (4)	58	0.6	54	0.7
septide	$(4.7 \pm 1.6) \times 10^6$ (3)	$(4.4 \pm 2.1) \times 10^8$ (4)	0.43 ± 0.19 (3)	0.48 ± 0.24 (4)	91	1	39	0.4
L-703606	$(2.7 \pm 0.3) \times 10^8$ (3)	$(1.7 \pm 0.8) \times 10^8$ (3)	0.12 ± 0.01 (3)	0.25 ± 0.12 (3)	0.4	1.5	0.9	2.1

[a] For ^{125}I-SP, $k_{on} = k_1$, $k_{off} = k_2$. For unlabeled ligands, $k_{on} = k_3$ and $k_{off} = k_4$ (see Materials and Methods for definition). ND, not determined. [b] K_i values were calculated from the IC_{50} values shown in Table 1 which were determined from equilibrium inhibition binding assay (DeBlasi et al., 1989). For SP, $K_{i(SP)} = IC_{50} - [^{125}I\text{-SP}]$. For other ligands, $K_i = IC_{50}/(1 + [^{125}I\text{-SP}]/K_{i(SP)})$.

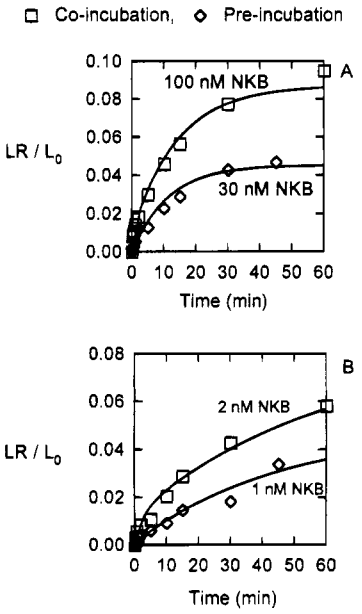

FIGURE 4: Measurement of the association rate constant and dissociation rate constant for NKB by monitoring the association of ^{125}I-SP in the presence of unlabeled ligands. The ratio of bound ^{125}I-SP to total added ^{125}I-SP is plotted as a function of time. The same amount of receptor was used in both the coincubation and preincubation protocols. The data are fitted to a system of differential equations as described in Materials and Methods, in which k_1 and k_2 for ^{125}I-SP have been determined independently in Figure 3 and k_3 and k_4 are the computer-fitted rate constants. (A) Wild-type NK1R. (B) Y216A mutant.

nists have only slightly decreased affinity, the Y216A substitution does not appear to cause a global conformational disruption.

Equilibrium binding studies do not provide information about the processes of association and dissociation of the ligand, which can be critical in determining the mechanistic basis of binding specificity. The lower affinity of NKB or septide for the wild-type NK1R compared to SP can result from either a slower on-rate and/or a faster off-rate. An electrostatic guidance effect (Getzoff et al., 1992) or a conformation sampling process between the receptor and the ligand will certainly affect the on-rate, whereas the noncovalent interactions will determine the off-rate. The unchanged dissociation rate constant of NKB (or septide) for the wild-type receptor and the Y216A mutant is consistent with the interpretation that the specific interactions between NKB and the NK1R are not affected by the mutation, although a different set of specific interactions with exactly the same bond energy cannot be ruled out by the present study. In the Y216A mutant, however, the association rate constants of NKB and septide are increased compared to the wild type, to a level as fast as that of SP. Since it is unlikely for the Y216A mutation to change the electrostatic surface potential of the receptor, the Y216A mutation apparently produces a conformational effect leading to an increase in the association rate constant. One such conformational effect can be that some structural restrictions along the ligand access pathway are removed due to a long-range effect of the mutation. Another possible conformational effect would be increased flexibility of the access pathway (i.e., increased plasticity in an induced-fit mechanism). The interpretation of increased local flexibility as a basis of increased association rate constant is consistent with experimental and theoretical studies of the HIV protease where reduced flap flexibility is correlated with mutations conferring drug resistance (Collins et al., 1995; Gulnik et al., 1995; Nicholson et al., 1995). In addition, the structural effect of the Y216A substitution is very limited and does not change the selectivity of tachykinin over non-tachykinin, because the mutant receptor does not recognize peptide unrelated to SP. As shown in Table 1, the Y216E substitution also causes a slight increase in the affinity of several SP-related peptides, while substitutions with Phe, Trp, or Leu do not. These data suggest that a dramatic change in side-chain volume (Y216A) or hydrophobicity (Y216E) at position 216 may initiate the conformational change.

Previous receptor mutagenesis studies suggested that divergent peptide residues and divergent receptor residues do not contribute to peptide discrimination directly (Fong et al., 1992a; Huang et al., 1995). These studies led to the proposal that the C-terminal conserved residues of the peptide contribute a major portion of the binding energy, and the N-terminal peptide residues probably determine the conformation of the peptide. Thus, a conformational compatibility between the ligand and the receptor can be a major factor in peptide recognition. In other words, the most compatible ligand will be able to maximize specific peptide−receptor interactions (Morton & Mathews, 1995; Perutz et al., 1986). This hypothesis is supported by the current data suggesting that the association step is more important than the dissociation step in determining tachykinin selectivity. The increased association rate constant can be the result of a better fit between the NK1R and tachykinin peptides or increased accessibility for various peptides. The less than 10-fold selectivity of SP over other peptides for the Y216A mutant may be explained by differences in the bond strength between the conserved portion of peptides and a set of receptor residues required for peptide binding (Huang et al., 1994a). Alternatively, the remaining level of selectivity may reflect the contribution of direct interactions between the N-terminal residues of peptides and the receptor.

Studies on the ligand binding site of the NK1R have shown that peptides interact with at least nine residues in both the transmembrane and extracellular domains (Huang et al., 1994a, 1995), and this conclusion is supported by affinity labeling (Li et al., 1995) and the accessibility of bound fluorescent SP (Tota et al., 1994). On the other hand, small molecule antagonists bind to a transmembrane area which overlaps with the peptide binding site (Cascieri et al., 1994; Fong et al., 1994; Gether et al., 1994; Huang et al., 1995). For the Y216A mutant, antagonists tested here do not have increased affinity, suggesting that the structural change in the Y216A mutant is limited to the area specific for peptide binding and does not affect the common region shared by both peptides and antagonists.

In light of the proposed structural effect of the Y216A substitution, it is noteworthy that the functional activity of the Y216A mutant is substantially reduced. Whether the impaired receptor activation is due to the loss of noncovalent interactions within the receptor or arises from the increased receptor flexibility is difficult to distinguish. Other amino acid substitutions at position 216 appear to resemble the wild-type receptor with respect to peptide selectivity but also show greatly reduced activation potency and efficacy (Table 1). These data suggest that the loss of critical intrareceptor interactions may account for the impaired receptor activation observed with these mutants.

In summary, the present study provides an explanation for peptide recognition based on a conformational sampling process. Such a proposal has profound implications for the design of peptidomimetics. These results suggest that peptidomimetics based on the divergent residues of neurokinin peptides may not be useful for the design of subtype selective antagonists and that knowledge of the bound conformation of the peptide may be essential for the success of such an approach. On the other hand, identification of receptor residues that interact directly with the peptide should define the location of the binding site and suggests possible routes of increasing binding affinity and specificity by maximizing interactions with these and nearby residues.

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REFERENCES

Brodbeck, R. M., Sachais, B. S., & Krause, J. E. (1995) *Mol. Pharmacol. 47*, 1065−1071.

Cascieri, M. A., Ber, E., Fong, T. M., Sadowski, S., Bansal, A., Swain, C., Seward, E., Francis, B., Burns, D., & Strader, C. D. (1992) *Mol. Pharmacol. 42*, 458−463.

Cascieri, M. A., Macleod, A. M., Underwood, D., Shiao, L. L., Ber, E., Sadowski, S., Yu, H., Merchant, K. J., Swain, C. J., Strader, C. D., & Fong, T. M. (1994) *J. Biol. Chem. 269*, 6587−6591.

Collins, J. R., Burt, S. K., & Erickson, J. W. (1995) *Nature Struct. Biol. 2*, 334−338.

DeBlasi, A., O'Reilly, K., & Motulsky, H. J. (1989) *Trends Pharmacol. Sci. 10*, 227−229.

Drickamer, K. (1992) *Nature 360*, 183−186.

Edmonds-Alt, X., Doutremepuich, J.-D., Heaulme, M., Neliat, G., Santucci, V., Steinberg, R., Vilain, P., Bichon, D., Ducoux, J.-P., Proietto, V., Van Broeck, D., Soubrie, P., Le Fur, G., & Breliere, J.-C. (1993) *Eur. J. Pharmacol. 250*, 403−413.

Fong, T. M., & Strader, C. D. (1994) *Med. Res. Rev. 14*, 387−399.

Fong, T. M., Huang, R.-R. C., & Strader, C. D. (1992a) *J. Biol. Chem. 267*, 25664−25667.

Fong, T. M., Yu, H., & Strader, C. D. (1992b) *J. Biol. Chem. 267*, 25668−25671.

Fong, T. M., Yu, H., Cascieri, M. A., Underwood, D., Swain, C. J., & Strader, C. D. (1994) *J. Biol. Chem. 269*, 14957−14961.

Francis, B. E., Swain, C., Sabin, V., & Burns, H. D. (1994) *Appl. Radiat. Isot. 45*, 97−103.

Franke, R. R., Konig, B., Sakmar, T. P., Khorana, H. G., & Hofmann, K. P. (1990) *Science 250*, 123−125.

Gether, U., Johansen, T. E., & Schwartz, T. W. (1993) *J. Biol. Chem. 268*, 7893−7898.

Gether, U., Nilsson, L., Lowe, J. A., III, & Schwartz, T. W. (1994) *J. Biol. Chem. 269*, 23959−23964.

Getzoff, E. D., Cabelli, D. E., Fisher, C. L., Parge, H. E., Viezzoli, M. S., Banci, L., & Hallewell, R. A. (1992) *Nature 358*, 347−351.

Gulnik, S. V., Suvorov, L. I., Liu, B., Yu, B., Anderson, B., Mitsuya, H., & Erikson, J. W. (1995) *Biochemistry 34*, 9282−9287.

Hale, J. J., Finke, P. E., & MacCoss, M. (1993) *Bioorg. Med. Chem. Lett. 3*, 319−322.

Hedin, K. E., Duerson, K., & Clapham, D. E. (1993) *Cell. Signalling 5*, 505−518.

Helke, C. J., Krause, J. E., Mantyh, P. W., Couture, R., & Bannon, M. J. (1990) *FASEB J. 4*, 1606−1615.

Huang, R. R. C., Yu, H., Strader, C. D., & Fong, T. M. (1994a) *Biochemistry 33*, 3007−3013.

Huang, R. R. C., Yu, H., Strader, C. D., & Fong, T. M. (1994b) *Mol. Pharmacol. 45*, 690−695.

Huang, R. R. C., Vicario, P. P., Strader, C. D., & Fong, T. M. (1995) *Biochemistry 34*, 10048−10055.

Iversen, L. L., Watling, K. J., McKnight, A. T., Williams, B. J., & Lee, C. M. (1987) in *Topics in Medicinal Chemistry* (Leeming, P. R., Ed.) pp 1−9, Royal Society of Chemistry, London.

Li, Y.-M., Marnerakis, M., Stimson, E. R., & Maggio, J. E. (1995) *J. Biol. Chem. 270*, 1213−1220.

Mazina, K. E., Strader, C. D., & Fong, T. M. (1994) *J. Recept. Res. 14*, 63−73.

Morton, A., & Mathews, B. W. (1995) *Biochemistry 34*, 8576−8588.

Nakanishi, S. (1991) *Annu. Rev. Neurosci. 14*, 123−136.

Nicholson, L. K., Yamazaki, T., Torchia, D. A., Grzesiek, S., Bax, A., Stahl, S. J., Kaufman, J. D., Wingfield, P. T., Lam, P. Y. S., Jadhav, P. K., Hodge, C. N., Domaille, P. J., & Chang, C.-H. (1995) *Nature Struct. Biol. 2*, 274−280.

Perutz, M. F., Fermi, G., Abraham, D. J., Poyart, C., & Bursaux, E. (1986) *J. Am. Chem. Soc. 108*, 1064−1078.

Peyronel, J.-F., Truchon, A., Moutonnier, C., & Garret, C. (1992) *Bioorg. Med. Chem. Lett. 2*, 37−40.

Regoli, D., Escher, E., & Mizrahi, J. (1984) *Pharmacology 28*, 301−320.

Schwartz, T. W. (1994) *Curr. Opin. Biotechnol. 5*, 434−444.

Sklar, L. A., Sayre, J., McNeil, V. M., & Finney, D. A. (1985) *Mol. Pharmacol. 28*, 323−330.

Strader, C. D., Gaffney, T., Sugg, E. E., Canderlore, M. R., Keys, R., Patcher, A. A., & Dixon, R. A. F. (1991) *J. Biol. Chem. 266*, 5−8.

Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., & Dixon, R. A. F. (1994) *Annu. Rev. Biochem. 63*, 101−132.

Swillens, S. (1992) *Trends Pharmacol. Sci. 13*, 430−434.

Tota, M. R., Daniel, S., Sirotina, A., Mazina, K. E., Fong, T. M., Longmore, J., & Strader, C. D. (1994) *Biochemistry 33*, 13079−13086.

Werge, T. M. (1994) *J. Biol. Chem. 269*, 22054−22058.

Yokota, Y., Akazawa, C., Ohkubo, H., & Nakanishi, S. (1992) *EMBO J. 11*, 3585−3591.

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