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A Mutation Changes Ligand Selectivity and Transmembrane Signaling Preference of the Neurokinin-1 Receptor*

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We studied the biochemical properties of a genetically engineered neurokinin-1 receptor (NK_1R) in which two residues lying on the extracellular edge of the fourth transmembrane domain were replaced by equivalently located elements of the neurokinin-2 receptor (G166C, Y167F NK_1R mutant). The mutation produced two effects. The first is enhancement of the apparent binding affinity for heterologous tachykinins (substance K and neurokinin B) and for N- or C-terminal modified analogues of substance P, but not for substance P itself, its full-length analogues, and several peptide and nonpeptide antagonists. Only two antagonists, as exceptions, were found to exhibit a diminished affinity for the mutant receptor. The second effect is a shift in NK_1R preference for distinct G protein-mediated signaling pathways. NK_1R-mediated phosphoinositide hydrolysis was enhanced both in transiently and permanently transfected cells, while stimulation of cAMP accumulation did not change in transient expression experiments and was reduced in permanently expressing cells.

The effect of the mutation on ligand affinity was not related to any obvious structural commonality, nor to the selectivity for different neurokinin receptors or the agonistic/antagonistic nature of the ligand. However, all ligands responding to the mutation appear to share the ability to induce phosphoinositide signaling more efficiently than cAMP responses when binding to NK_1R. We suggest that the mutation shifts the internal equilibria of different functional forms of NK_1R. A theoretical analysis according to a multistate allosteric model suggests that the link between binding and biological changes can result from altered stability constants of substates in the conformational space of the receptor.

Tachykinins, as several other families of neuropeptides, display a bipartite distribution of topochemical information on their sequence. The C-terminal half of the molecule is a consensus motif tightly conserved among all known hormones from mammalian and nonmammalian species (1, 2) and, conceivably, constitutes a default conditional element to establish binding affinity for any kind of tachykinin receptor subtype (3, 4). The N-terminal part is instead variable even among the three mammalian tachykinin types, and it is believed to contribute interactions establishing selectivity for specific receptor subtypes (3, 4). The interesting question is whether a similar partition in the molecule of the receptor corresponds to such a sharp division between variant and invariant elements in the molecule of the peptide and, if so, to what relative extent do selective and nonselective interactions contribute to the final binding affinity of each peptide hormone and receptor system.

We focused on two amino acids marking the junction between the fourth putative transmembrane domain (TMD)[1] and the second extracellular loop of tachykinin receptors. Replacement of these two residues in the substance P receptor (neurokinin-1 receptor (NK_1R)) with those located at equivalent positions in the substance K receptor sequence produced changes in receptor behavior suggesting a specific role of the C-terminal consensus sequence of the peptide in determining sensitivity to the two-residue transmutation (5). It was thus proposed that the two residues introduced by the mutation may induce in the receptor an improved docking configuration for the consensus sequence of tachykinins (5).

Diverse lines of evidence support the notion that residues located between the C-terminal portion of TMD-4 and the N-terminal half of the second extracellular loop play an important role in recognition and binding of tachykinin ligands. First, the two amino acids targeted by the mutation are part of a stretch of five residues that was found to be shared by NK_1R and a monoclonal antibody specific for the C-terminal sequence of substance P (5). In a comparative study where three-dimensional models of both macromolecules were examined (6), this sequence appears to be involved in forming part of the surface of a putative pocket displaying strikingly similar charge distributions and may thus be involved in binding the C-terminal sequence of substance P. Second, site-directed mutagenesis of residues in human NK_1R closely flanking the two amino acids modified here, such as Gln-165 (7, 8) and Ser-169 (7), results in large changes in the binding affinity for both peptide and nonpeptide ligands. Finally, affinity labeling studies with analogues of substance P bearing photoactivable moieties in the consensus sequence demonstrated alkylation of receptor residues lying in the N-terminal portion of the second extracellular loop and close to the site mutagenized in this study (9–11).

To gain additional understanding of the role of this site of NK_1R, we compared the binding affinities of a variety of peptide and nonpeptide ligands. We show evidence suggesting that the main effect of this mutation is an allosteric effect on the conformational equilibrium of the receptor rather than a change in the local docking interactions between the targeted residues and a specific area of the tachykinin sequence. This conformational shift produces two main effects: (*a*) a change in the binding energy for a chemically heterogeneous group of

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[1] The abbreviations used are: TMD, transmembrane domain; NK_1R, neurokinin-1 receptor; PI, polyphosphoinositide; CHO, Chinese hamster ovary; SP, substance P; NKA, neurokinin A or substance K; NKB, neurokinin B; G protein, guanyl nucleotide-binding regulatory protein; <Glu, pyroglutamic acid.

tachykinin ligands, all of which share the ability to induce polyphosphoinositide (PI) turnover stimulation more efficiently than cAMP accumulation upon activation of NK_1R; and (*b*) an increased ability of the agonist-bound receptor to induce G_q-mediated responses, with no apparent change or even a reduced efficiency in triggering the G_s-mediated pathway.

EXPERIMENTAL PROCEDURES

Ligands and Reagents—Monoiodinated ^{125}I-Bolton-Hunter reagent-labeled substance P (2000 Ci/mmol) was purchased from Amersham Corp. All peptides were purchased from Bachem or American Peptide Co. FK224 (12) and FK888 (13) were provided by Dr. Matsuo (Fujisawa Pharmaceuticals Co.). SR 140333 (14) was supplied by Dr. Edmonds-Alt (Senofi Recherche). Men 10930 (15) was provided by Dr. Manzini (A. Menarini Pharmaceuticals). CP 96345 (16), CP 99994 (17), RP 67580 (18), and CGP 49823 (19) were a gift of Dr. Schwartz (University of Copenhagen). All other reagents were from Sigma.

Mutant and Wild-type NK_1R Genes—The constructs used in this study were rat NK_1R (pCDM8-SPR, kindly donated by Dr. S. Nakanishi) and its G166C,Y167F mutant, prepared by the oligonucleotide-directed M13 method as described previously (5).

Cell Culture—Permanently transfected CHO cells were grown in a mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (1:1) supplemented with 10% fetal calf serum, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 100 μg/ml G418 (Life Technologies, Inc.) at 37 °C in a humidified atmosphere of 5% CO_2. COS-1 cells and C6 glioma cells were grown under identical conditions, except for the growth medium (only Dulbecco's modified Eagle's medium) and the omission of G418.

Transfection of Cells—Wild-type and mutant receptors were transfected in CHO cells using Lipofectin (Life Technologies, Inc.), and stably expressing clones were isolated following selection with 400 μg/ml G418 (Geneticin). For transient expression, COS-1 cells were seeded in 24-well plates and transiently transfected with wild-type or mutant receptor cDNA using a DEAE-dextran/chloroquine procedure as described (20). Gradual levels of receptor expression were obtained by transfecting varying amounts of receptor DNA but maintaining the total mass of transfected DNA (2 μg/well) constant with the addition of empty vector.

Phosphoinositide Turnover and cAMP Assay—To compare the biological activities of mutant NK_1R and the wild-type receptor, we measured phosphoinositide hydrolysis and cAMP accumulation. Identical procedures were used for transient expression experiments or permanently expressing cells, except that in the latter case, the two second messengers were assayed in the same cell extract. Cells were seeded in 24-well plates and allowed to grow to confluence prior to the addition of 2 μCi/ml *myo*-[^{3}H]inositol (80–90 Ci/mmol; DuPont). For transient tranfections in COS cells, the labeled inositol was added 48 h following transfection. After 24 h of incorporation, the medium was removed, and cells were incubated in a buffer containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 20 mM NaHepes, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM EGTA, 11.1 mM D-glucose, and 0.1% bovine serum albumin, pH 7.4. This was supplemented with 10 mM LiCl, 100 μM Ro 20-1724, and various concentrations of substance P or other peptides as required by the experiments. Reactions were conducted for 30 min at 37 °C and arrested by the removal of the supernatant and the addition of 0.5 ml of ice-cold 0.1 N HCl to each well. The plates were placed on ice, and 50 μl of the HCl extract were removed for the determination of cAMP concentration. 1 ml of ice-cold methanol was then added to each well, taking care not to disturb the monolayer, and the resulting mixture was carefully collected for the separation of radiolabeled inositol phosphates. Monolayers were allowed to dry under a hood and then dissolved into 0.5 ml of a solution prepared by mixing 0.8 M NaOH and 5% SDS (1:2). 200 μl of this extract were neutralized with 30 μl of glacial acetic acid, mixed with scintillation mixture (Ultima Flo, Packard Instrument Co.), and counted in a β-counter to determine the levels of the total acid-insoluble inositol incorporated into the cell membranes. The remaining was used for protein determinations.

The concentration of radioactive inositol phosphates hydrolyzed was quantified after application of the acid methanolic extract to anion-exchange columns (AG 1-X8, Bio-Rad) and elution into a single fraction as described previously (21). Cyclic AMP levels were determined by radioimmunoassay following acetylation of the sample (22).

Radioreceptor Binding Assays—Binding assays in intact cells were performed as described previously (5) with minor modifications. Cells were seeded in opaque culture plates (Canberra-Packard) and, at confluence, incubated in a reaction buffer with a composition identical to that used for the study of biological activity, supplemented with various concentrations of test peptide and monoiodinated ^{125}I-Bolton-Hunter reagent-labeled substance P (20,000 cpm). Incubations lasted 3 h at 4 °C and were terminated by aspirating the incubation medium and washing the monolayer twice with ice-cold binding buffer. Plates were allowed to drain overnight onto filter paper; then 250 μl of MicroScint (Packard Instrument Co.) were added to each well, and the plates were counted in a Top Count (Canberra-Packard). Binding isotherms of 12 logarithmically spaced concentrations performed in duplicate were routinely used to determine binding parameters.

Data Presentation and Analysis—Phosphoinositide turnover is expressed as the hydrolyzed fraction of total incorporated inositol, calculated as follows: dpm(IP1)/(dpm(Ins) + dpm(IP1)), where dpm(IP1) is the radioactivity determined in the fraction of inositol phosphates eluted from the columns, and dpm(Ins) is that left in the monolayer following the extraction by acidic methanol. The levels of intracellular cAMP are given as pmol/mg of total cell proteins.

Equilibrium binding parameters were computed by analysis of the binding isotherms with the computer program LIGAND (23). Curves for the stimulation of phosphoinositide hydrolysis and cAMP accumulation were fitted using ALLFIT (24) to determine EC_{50}, upper and lower asymptotes, and slopes at midrange. Standard free energy changes were computed as negative natural logarithms of the apparent association binding constants ($1/K_d$) and are therefore expressed in RT units. All the data are means of several independent experiments as indicated.

Simulations of Binding Data According to an Allosteric Receptor Model—To interpret and simulate the change in ligand binding properties induced by the mutation in NK_1Rs, we used a general multistate allosteric model, which is briefly described here. The receptor (R) exists in n freely interconverting states. The concentration at equilibrium of each state (s_i) is given by a stability constant (j) describing the first-order transition from any state $i-1$ to state i. For a sufficient description of this system, microscopic reversibility allows us to consider any state transition as relative to an arbitrarily chosen reference state (s_0). Therefore, for any state i, the corresponding stability constant is given by $j_i = [s_i]/[s_0]$, and the total concentration of the receptor existing in n states is thus as follows (Equation 1).

$$[R]_{\text{total}} = \sum[\text{all states}] = [s_0](1 + \sum_{i=1}^{n} j_i) \qquad \text{(Eq. 1)}$$

If a ligand (H) binds to the receptor, it will perturb its state distribution according to the degree of change that the bound molecule imparts to the stability constants of each state. Thus, in the presence of bound ligand, the stability constant of any state i is given by $bj_i = [Hs_i]/[Hs_0]$, where the factor b indicates the extent to which the bound ligand alters the stability constant of that state with respect to the unbound receptor.

The overall equilibrium binding affinity of a ligand is the result of the binding affinities of all the states (Equation 2),

$$K_{\text{app}} = \frac{\sum[\text{bound states}]}{[H] \times \sum[\text{free states}]} = \frac{[Hs_0](1 + \sum_{i=1}^{n} b_i j_i)}{[H][s_0](1 + \sum_{i=1}^{n} j_i)} = K_0 \frac{(1 + \sum_{i=1}^{n} b_i j_i}{(1 + \sum_{i=1}^{n} j_i)} \qquad \text{(Eq. 2)}$$

where K_0 is the equilibrium affinity for default state s_0. Thus, the allosteric model predicts that experimentally measured binding constants can always be decomposed as the product of a "true" second-order association constant describing the ligand's affinity for the reference state and a factor that includes the first-order stability constants governing the transitions among all possible states within the receptor macromolecule. Hence, any mutation affecting the receptor distribution among states will also affect the apparent binding affinity of a ligand in a manner that depends on the effect that ligand has on the state distribution of the receptor.

To generate experimental binding isotherms representing the concentration of a bound radiolabeled ligand as a function of the total concentration of a second unlabeled ligand, the equilibrium composition of the system must be computed for n number of states and m number of ligands, starting from the equilibrium constants and the total concentrations of receptor and ligands as input parameters. This requires the simultaneous solutions of $m + 1$ equations describing the concentrations of all free species present (Equations 3 and 4).

TABLE I

Dissociation constants of tachykinins for binding to wild-type and G166C, Y167F mutant NK_1Rs

Binding isotherms for tachykinins and nonpeptide ligands in competition for monoiodinated ^{125}I-Bolton-Hunter reagent-labeled SP were generated as described under "Experimental Procedures." Data were analyzed with the computer program LIGAND (23) to calculate binding affinities. Results are presented as nM dissociation constants (K_d) and are means ± SE of the number of experiments (n) indicated. The averages of the ratios between affinities and their standard deviations were computed from the individual values obtained in each experiment and do not always correspond exactly to the ratios of the means of K_d values.

Ligand	K_d ± S.E.		Mutant/wild-type ± S.D.	n
	Wild-type receptor	Mutant receptor		
SP	0.61 ± 0.05	0.54 ± 0.054	1.2 ± 0.38	11
SP-(5–11)[a]	107 ± 13	6.6 ± 2.11	18.9 ± 8.02	2
SP-(6–11)	3098.7 ± 338	80.2 ± 6.12	39.9 ± 12.4	3
SP-(7–11)	93,502.2 ± 12,057	6155.8 ± 962	15.2 ± 0.9	3
SP methyl ester	9.9 ± 1.2	0.78 ± 0.21	13.0 ± 3.0	3
SP free acid	17,097 ± 2028	690.3 ± 153.4	25.0 ± 4.4	3
SP-(1–9)	159,658 ± 22,189	22,958 ± 1000	7.1 ± 1.5	3
Substance K	54.7 ± 5.8	3.68 ± 0.44	14.9 ± 1.4	3
Substance K-(4–10)[a]	7143 ± 1072.5	345 ± 57.5	20.7 ± 2.4	3
Substance K-(5–10)[a]	17,100 ± 5614	266 ± 41.4	58.4 ± 17.35	3
NKB	144 ± 16.9	4.7 ± 0.89	31.2 ± 7.92	3
Septide	2574 ± 264.6	172.7 ± 27.3	14.9 ± 1.27	3
D-Septide	292,620 ± 14,530	27,257 ± 1453	10.8 ± 1.58	3
[Sar^9,Met(O$_2$)^{11}]SP	1.11 ± 0.2	1.95 ± 0.05	0.5 ± 0.09	2
Physalaemin	1.12 ± 0.3	1.2 ± 0.35	0.9 ± 0.09	2
GR 82334	112.23 ± 20	137.1 ± 30.5	0.8 ± 0.05	2
[D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP	1.28 ± 0.2	0.67 ± 0.2	1.9 ± 0.38	2
[D-Arg^6,D-Trp^{7,9}(*N*-Me)Phe^8]SP-(6–11)	1.55 ± 0.4	0.98 ± 0.24	1.6 ± 0.02	3
FK888	246.6 ± 65	292.9 ± 85	0.8 ± 0.03	2
Men 10930	6769.05 ± 1050	9695.4 ± 300	0.7 ± 0.18	2
SR 140333	3.55 ± 1.1	4.97 ± 1.41	0.7 ± 0.07	3
RP 67580	13.74 ± 2.7	19.9 ± 2.91	0.7 ± 0.14	3
CGP 49823	111.45 ± 21.5	129.6 ± 39.5	0.9 ± 0.13	2
CP 99994	236.8 ± 10.0	359.1 ± 49.5	0.7 ± 0.09	2
CP 960345	44.41 ± 6.3	190.6 ± 13.2	0.2 ± 0.06	4
FK224	13.89 ± 1.8	61.4 ± 8.3	0.2 ± 0.02	4

[a] The dissociation constants of these ligands were obtained in COS cells only (5), but were included in the analysis of Fig. 1 because for all other ligands that were assayed in both cell systems, there was close agreement in the estimates of binding affinity.

$$[s_0] = \frac{[R]_{\text{total}}}{1 + \sum_{i=1}^{n} j_i + \sum_{l=1}^{n} K_{0l}[H_l]_{\text{free}}(1 + \sum_{i=1}^{n} b_{li}j_i)} \quad \text{(Eq. 3)}$$

$$[H_l]_{\text{free}} = \frac{[H_l]_{\text{total}}}{1 + K_{0l}[s_0](1 + \sum_{i=1}^{n} b_{li}j_i)} \quad \text{(Eq. 4)}$$

Solutions were achieved by an iterative numerical procedure directed to minimize, for each ligand (H) present, the implicit function as follows (Equation 5).

$$G(0)_l = [H_l]_{\text{total}} - [H_l]_{\text{free}}(1 + K_{0l}[s_0](1 + \sum_{i=1}^{n} b_{li}j_i)) \quad \text{(Eq. 5)}$$

From free species, the concentrations of all bound species can be computed, and the relation between bound and total ligand can be obtained for each ligand. Binding isotherms thus generated were analyzed by LIGAND (23) to compute the apparent K_d and the corresponding equilibrium affinity ($1/K_d$).

To simulate "allosteric" mutations, we evaluated the effect of changing j parameters either in the absence of any other modification or also in the presence of simultaneous smaller changes in the allosteric b factors of all ligands. To do so, b factors were multiplied by an equally sized matrix of computer-generated random numbers with unitary mean, and a standard deviation equivalent, in energetic units, to one-tenth or one-fifth of the total change that was imposed on the j constants. This introduces scatter in the effect of the mutation that closely resembles that experimentally observed.

RESULTS AND DISCUSSION

Effect of the Mutation on the Binding Affinities of Tachykinin Ligands—Wild-type and mutant NK_1Rs were stably transfected into CHO cells, and a pair of wild-type and mutant CHO clones exhibiting comparable levels of receptor expression were selected for the binding studies presented in Table I. To investigate the pharmacological properties of the mutant receptor, we measured equilibrium binding affinities of an ample selection of structurally diverse tachykinin receptor ligands.

As previously observed in a transient expression system (5), differences between SP and a number of peptide analogues mark the salient feature of the mutation. In fact, while SP affinity is similar for wild-type and mutant receptors, natural tachykinins selective for other types of neurokinin receptors, such as NKA and NKB, and amino-terminal deleted analogues of both SP and NKA displayed enhanced affinity for the mutant (Table I). Based on these data, we suggested that the main effect of the double-residue replacement could be an improved interaction of the receptor for the consensus pentapeptide C-terminal sequence of tachykinins (F*X*GLM). The reason why such improvement was evident for heterologous tachykinins and amino-terminal deleted SP analogues, but not for SP itself, might be explained if we assume that the amino-terminal residues, by providing additional interactions at sites others than those targeted by the mutation, could hinder the improvement in affinity for the consensus sequence generated by the mutation itself.

In this study, however, we identified an additional set of ligands responding with increased affinity to the mutation, which suggests that this "message-recognition" hypothesis is not correct. First, we found that septide and its dextro analogue bearing a proline substitution in the C-terminal consensus sequence display greater affinity for the mutant receptor, just like the unmodified hexapeptide SP-(6–11). Second, three C-terminal modified analogues of SP (the methyl ester, the deamidated analogue, and the carboxy-free version of SP-(1–9))

also displayed significantly greater affinity for the mutant receptor than the wild-type receptor. Thus, responsiveness to the mutation requires neither the presence of an intact tachykinin C-terminal consensus core nor the absence of a SP-like amino-terminal sequence.

One clear consequence of the mutation is a change in selectivity of NK_1R for natural tachykinin, as the ratios of affinities between SP and either NKA or NKB are much larger in the wild-type receptor than in the G166C,Y167F mutant. Because selectivity may depend partly on differences in sequence and partly on the conformational constraints that those differences impose on the common binding elements of the three peptides (25), we considered whether differences in both selectivity and flexibility among ligands might be the major factor conferring sensitivity to the mutation.

However, the comparison of the binding affinities of a wider range of tachykinin peptide analogues (Table I) indicates that the ability to discriminate between mutant and wild-type receptors is related neither to the conformational flexibility of the peptide ligand nor to its selectivity for different receptor subtypes. For example, septide and D-septide, which differ from SP-(6–11) in a constraining proline substitution at position 9 and enhanced NK_1R selectivity, display similar preference for the mutation as their nonconstrained analogues. Also, sarcosine substitution at the same position of the sulfone undecapeptide analogue of SP, although increasing NK_1R selectivity, does not bring enhanced affinity for the mutation. Similarly, physalaemin which does not discriminate between mutant and wild-type receptors, does not acquire selectivity for the mutant when presented as a more rigid conformational version in the neurokinin-1-selective antagonist GR 82334, nor do two spantide-like antagonists, either as a full-length undecapeptide or as a C-terminal hexapeptide analogue, display any important difference in affinity between wild-type and mutant receptors. We also tested senktide, which is a more rigid version of the hexapeptide carrying additional substitutions that establish high selectivity for NK_3Rs and which does not bind to wild-type NK_1R. This peptide did not acquire any apparent affinity for the mutant (no competition at 100 μM for either receptor; data not shown). Taken collectively, these data suggest that conventional structure-activity criteria do not lead to a consistent explanation of why some peptides display enhanced affinity for the mutant receptor, while some others do not.

Table I also illustrates the effect of the mutation on the binding affinities of a representative group of structurally diverse nonpeptide tachykinin antagonists. Most of them showed little difference in K_d between wild-type and mutant receptors, thus behaving just like the agonist SP. But two were exceptions since their binding affinity for the mutant receptor was diminished 5-fold (Table I, last two entries). This suggests that there might be a larger class of tachykinin ligands responding with decreased affinity to the mutation, although we have found only two members thus far. Again, the structural basis for such discrimination remains elusive. There is, in fact, very little apparent structural analogy between CP 96345, a quinuclidine derivative with species selectivity for human and guinea pig NK_1R (16), and FK224, a microbial cyclic peptide that was by serendipity found to be an antagonist of NK_1R (12).

Free Energy Correlations Suggest a Mutation-induced Conformational Change—The finding that no common structural feature satisfactorily predicts the ability of a ligand to detect the change induced by the mutation suggests that the two residues replaced in the receptor may be not primarily involved in direct docking interactions with a particular chemical moiety of the tachykinin sequence, which should be otherwise invariably present in the structure of responding ligands and absent in that of others. Alternatively, they may exert an influence on the entire conformation of the receptor, *i.e.* acting globally, rather than locally, in changing receptor affinity.

We thus propose a different strategy to analyze the data. The suite of ligands examined in this and previous studies can be viewed as a variation across the conformational space of the "tachykinin pharmacophore" fitting the stereochemical constraints of NK_1R. The effect of such variation on binding affinity has been evaluated here for two congeneric forms of the same receptor. Thus, a comparison of binding energy changes due to modification of the ligand with those due to modification of the receptor can provide some insight into the relative extent of the two contributions.

To this end, we first scaled the free energy change resulting from variation in ligand structure over that resulting from receptor transmutation. To do so, SP (the highest affinity ligand of the set) was taken as reference, and we plotted the net difference in binding energy between each ligand and SP measured for the wild-type receptor as a function of the net difference due to the mutation. This generated a scattered distribution of values (Fig. 1*A*), among which the points relative to ligands that have increased affinity for the mutation form a well separated cluster. (Similarly, the two ligands responding with decreased affinity to mutation appear to be well separated from the cluster of non-responders, but it is hard to draw any conclusion in the absence of additional points.) Within the responder group, there is no significant correlation ($r = 0.10$, $p = 0.75$) between the two variables, indicating that the extent of binding affinity of a ligand for NK_1Rs does not allow a prediction of how that affinity would change in response to the replacement of the two residues targeted by the mutation. The lack of overlap between responder and non-responder groups on the x axis of the plot suggests that, aside from the ligand-specific effect, a main component of the observed variance is due to the change in the receptor, *i.e.* all members of the responder group appear to differ from all the others primarily by a shift in binding energy caused by the mutation.

To evaluate the magnitude of this shift, we plotted the relation between net binding energies measured for wild-type and mutant receptors (Fig. 1*B*). The data are best described by two distinct and parallel regression lines corresponding to the groups of responders and non-responders, respectively, with slopes not significantly different from unity. Again, the two ligands displaying a significantly reduced affinity for the mutation seem to belong to a third regression line, although the scarcity of points does not allow proper statistical analysis. The unitary slope of such relations indicates that wild-type and mutant receptors react to changes in ligand structure identically. In this sense, the mutation does not alter the fundamental relationship between structural change in the ligand and change in binding energy for NK_1R because that would generate nonparallel lines. However, the line of ligands that respond with increased affinity to the mutation is shifted from that of non-responders by 3 RT units of free energy, indicating that all those ligands have gained, on average, such an extent of binding energy as a result of the change in the receptor. Similarly, by considering a putative line passing through the values of the two antagonists with reduced affinity for the mutant, there may be another subset of ligands displaying an opposite shift with a net loss of 1.5 units of binding energy, although its existence remains speculative.

Although the regression line explains 97% of the variance in the responder group (Fig. 1*B*), the residuals reflect both experimental error and ligand-specific effects of the mutation, and this analysis does not allow us to distinguish among them. We can, nonetheless, conclude that in the mechanism underlying

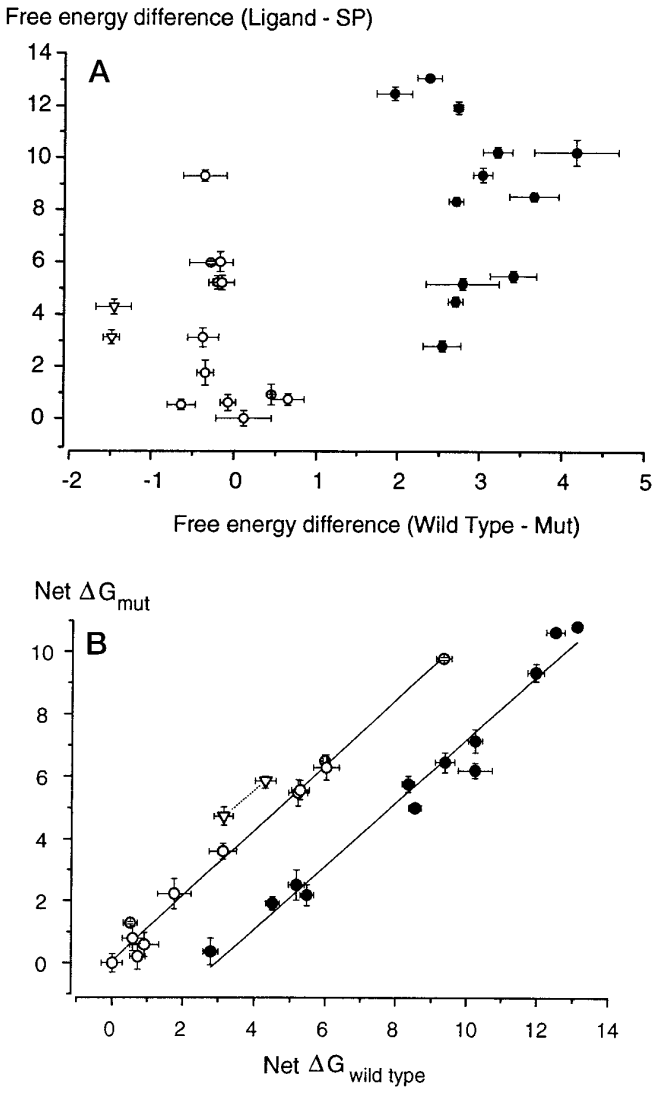

FIG. 1. **Correlation among binding energies of ligands for wild-type and mutant NK$_1$Rs.** *A*, the net differences in free energy changes between each ligand and SP for binding to wild-type NK$_1$R (*ordinate*) are plotted as a function of the net free energy differences between wild-type and mutant receptors for each ligand (*abscissa*). Means ± S.D. (*bars*) were computed after converting into free energy differences (see "Experimental Procedures") the individual affinity values ($1/K_d$) corresponding to the averaged data and the number of experiments reported in Table I. Different symbols were used to mark values corresponding to ligands whose affinities are enhanced (●), unchanged (○), or diminished (▽) by the mutation. *B*, the net differences in binding energy relative to SP (see *A*) measured for the mutant receptor (*Net* ΔG_{mut}; *ordinate*) are plotted *versus* the corresponding values measured for the wild-type receptor (*Net* $\Delta G_{wild\ type}$; *abscissa*). The symbols identify the same groups of values shown in the scatter plot of *A*. The *solid lines* indicate best fitting regression lines fitted to the two sets of values, corresponding to responders (●; slope = 1.02 ± 0.06 and intercept = −2.97 ± 0.51) and non-responders (○; slope = 1.05 ± 0.03 and intercept = 0.078 ± 0.15). The values of the two ligands with decreased affinity for the mutation (▽) were not included in these calculations (*dotted line*). To evaluate whether the improvement in the description of the data given by two separate regression lines compared with a single one traced through all points is statistically significant, we used analysis of variance and *F* statistics according to the extra-sum-of-square principle (37). The critical *F* value was computed from the relation $((SS_1 - SS_2)/(df_1 - df_2)) \times df_2/SS_2$, where SS_1 and SS_2 are the sum of squares of the residual for the simpler two-parameter model (one regression line) and for the more complex four-parameter model (two lines), respectively, while df_1 and df_2 are the corresponding degrees of freedom (number of fitted data minus number of total estimated parameters). The computed value ($F = 69.7$; degrees of freedom = 2, 20) is highly significant ($p < 10^{-6}$) and rejects the hypothesis that a single regression can describe the data equally well. The significance of this test also indirectly validates the assertion that the two groups of values form distinct clusters in the graph of *A*, which can be viewed, in fact, as a way to display the residuals of the regressions in *B*.

the change in apparent affinity of the responders, the constant change affecting the whole set of ligands is much more important than smaller contributions due to changes that act differently upon each ligand.

The constancy of such change in binding energy supports the notion that the main effect of the mutation is on the conformational equilibrium of the receptor itself. It also suggests that the class of ligands that is able to "detect" such change must share a common property despite our inability to detect common features in their chemical structure. The next experiments were designed to evaluate this possibility.

Ligands Responding to the Mutation Share Selectivity in Transmembrane Signaling—Although ligands responding to the mutation are agonists, while antagonists are the majority of non-responders, there is no straightforward relation between mutation-induced change in affinity and intrinsic activity of the ligands. In fact, non-responders also include potent agonists such as SP itself, the [Sar^9]sulfone analogue, and physalaemin. Mutagenesis studies of tachykinin receptors (26–28) suggest that peptide and nonpeptide ligands of NK$_1$R might have only partially overlapping binding sites; therefore, replacement of certain residues might only affect the peptide-selective area of the site. But here we find that the peptide SP and several nonpeptide ligands are equally insensitive to the replacement; therefore, the ability to detect the mutation cannot be ascribed to the peptidergic nature of the ligand.

There is, however, an intriguing biological property that septide and several other ligands of the responder group appear to share. It is known that the potency of septide in biological assays is much greater than expected from the binding affinity measured in competition for SP (reviewed in Ref. 29). In transfected cells, tachykinin receptors activate at least two different signaling pathways, phosphoinositide turnover and adenylate cyclase, through the intervention of distinct G proteins (30). Recent studies show that in cells expressing NK$_1$Rs, septide, SP-(6–11), NKA, and NKB activate G$_q$-mediated PI turnover with a potency similar to that of SP, despite their low apparent binding affinity. In contrast, the EC$_{50}$ of these peptides for stimulation of intracellular accumulation of cAMP is much lower than that of SP (31). This suggests that ligands responding with increased affinity to the mutation may have in common the ability to activate more efficiently G$_q$- than G$_s$-dependent signaling. We wondered, therefore, whether C-terminal analogues of SP, which like septide respond with increased affinity to the mutation, also exhibit "septide-like" biological properties. To answer this question, we compared, in the two transfected CHO clones used for the binding studies, the relative enhancement of PI turnover and cAMP levels induced by [<Glu^6]SP-(6–11) [2] and SP methyl ester. Both ligands displayed little difference in potency or maximal stimulation in activating PI turnover compared with SP in cells expressing the wild-type receptor (Fig. 2, *top left panel*), but clearly differed in their ability to elicit accumulation of intracellular cAMP (Fig. 2, *bottom left panel*): [<Glu^6]SP-(6–11) displayed a potency 2 orders of magnitude lower than SP in this response, while the methyl ester was not only less potent, but also behaved as a partial agonist, producing 65% of the maximal stimulation observed for SP. In cells expressing mutant recep-

[2] The binding affinities of this peptide for mutant and wild-type receptors are identical to those of SP-(6–11), but the protected amino terminus should confer resistance to aminopeptidase in biological assays carried at 37 °C.

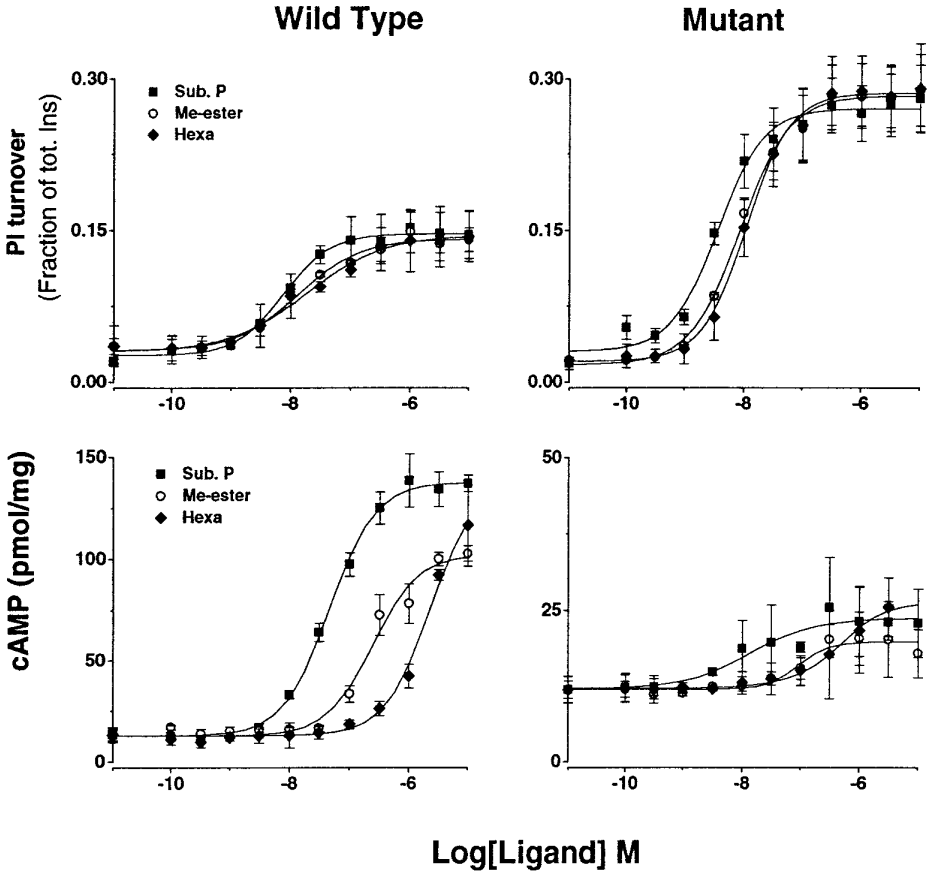

FIG. 2. **Dose responses for PI hydrolysis and cAMP accumulation stimulated by tachykinin ligands in CHO cells expressing wild-type or mutant receptors.** CHO clones expressing similar densities of wild-type (4.32 ± 0.57 pmol/mg) and mutant (3.83 ± 0.64 pmol/mg) receptors ($n = 4$) were seeded in 24-well plates and assayed for PI hydrolysis and cAMP accumulation in response to SP, SP methyl ester (*Me-ester*), and [<Glu^6]SP-(6–11) (*Hexa*) used at the indicated concentrations. The *solid lines* between experimental points were fitted to the data using ALLFIT (24). Plotted data are averages of two independent experiments performed in triplicate, in which wild-type and mutant receptor-expressing cells were tested in parallel and the levels of second messengers were measured in the same cell extract as described under "Experimental Procedures."

tors, the relative potency of the three ligands was not substantially changed (Fig. 2, *right panels*), although the cAMP response measured in this case was small and did not allow reliable comparisons of the concentration-response curves. In fact, the most striking difference between the two transfected clones is that in the mutant-expressing cell, the maximal stimulation of PI turnover is greater, while that of cAMP accumulation is smaller compared with the wild-type receptor (Fig. 2, *right panels*).

Two important conclusions can be drawn from these experiments. First, they indicate that also C-terminal modified analogues of SP share with septide a much better efficiency to activate G_q- rather than G_s-mediated responses. Such a feature may thus be the common denominator of all ligands responding with increased affinity to the mutation. Second, they suggest that the mutation may change the intrinsic ability of the receptor to differentially activate the two G proteins since SP, a ligand that discriminates poorly between the two signaling paths, appears to induce more PI stimulation and less cAMP accumulation in mutant compared with wild-type receptor-expressing cells. This possibility was investigated further.

Differential Ability of Wild-type and Mutant Receptors to Trigger Distinct Signaling Pathways—To study the relation between concentration of expressed receptor and extent of activation of the two different signaling pathways, we first used a transient expression system. In fact, by calibrating the concentration of transfected cDNA for the two receptors, their respective ability to activate second messenger systems can be quantified, despite the lower levels of expression of the mutant. The expression of wild-type NK_1R over a wide range of densities (Fig. 3*A*) did not induce significant changes in PI turnover or intracellular cAMP concentration in the absence of agonist. Similar data were obtained with the mutant, indicating that the mutation does not result in a detectable enhancement of ligand-independent receptor activity. However, in the presence of saturating concentrations of SP, there was a clear-cut difference between mutant and wild-type receptors (Fig. 3*A*). In fact, the unitary increase in PI turnover/pmol of expressed receptor estimated from a linear regression of the data is 0.025 ± 0.0011 and 0.057 ± 0.0033 for wild-type and mutant receptors, respectively. Thus, at equal molar units of expressed receptor, the mutant produces 2-fold more PI turnover stimulation than the wild-type receptor (Fig. 3*A*). In contrast, stimulation of cAMP levels in the same cells did not show a significant difference between mutant and wild-type receptors (Fig. 3*B*). Thus, the mutation can affect the signaling efficiency of NK_1R for only one kind of signal transduction pathway selectively.

To substantiate this finding in a more "physiological" expression system, we strived to prepare a number of stably transfected cell lines differing in the number of expressed mutant or wild-type receptors. Using Chinese hamster ovary cells, we obtained two series of clones with a reasonably wide range of wild-type or mutant receptor expression, which were analyzed for both phosphoinositide turnover and accumulation of cAMP in response to SP. The maximal stimulation of PI turnover in these cells was related to receptor density (Fig. 4*A*), but did not show a linear trend as observed in COS cells. Nonetheless, also in this case, equal levels of PI stimulation occurred at lower expression of the mutant receptor compared with the wild-type receptor. Instead, the mutant was substantially impaired in mediating stimulation of cAMP accumulation (Fig. 4*B*). Direct assessment of adenylate cyclase activity in membrane prepared from two selected clones expressing either the wild-type or mutant receptor confirmed that little G_s-mediated stimulation of enzymatic activity can be seen by the mutant receptor in this cell system (data not shown).

Additional studies were carried out in transfected C6 cells, a glioma line that expresses endogenous bombesin and β-adre-

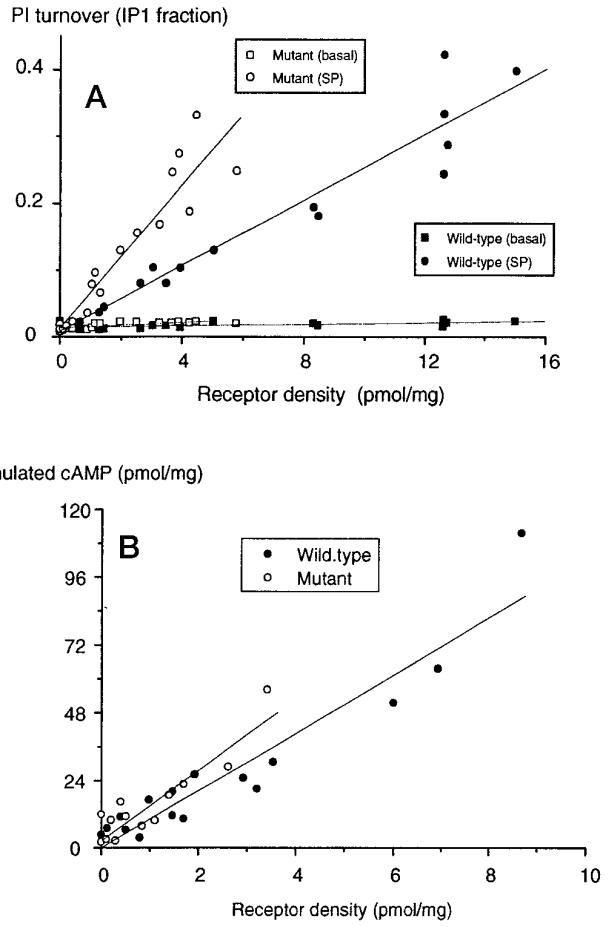

FIG. 3. **Biological activity of mutant and wild-type neurokinin-1 expressed in COS-1 cells.** COS-1 cells grown in 24-well plates (0.2×10^6 cells/well) were transfected using the DEAE-dextran procedure with cDNA encoding wild-type and mutant NK_1Rs. cDNA concentrations were ranged between 0.01 and 0.4 μg/well for the wild-type receptor and 0.05 and 2 μg/well for the mutant. The total concentration of DNA was maintained constant at 2 μg/well by the addition of empty vector. For both PI turnover and intracellular cAMP accumulation, cells were assayed following 72 h of expression in the presence or absence of 10 μM SP. The stimulation of PI turnover (*A*) is given as the fraction of counts in IP1 calculated as described under "Experimental Procedures." Stimulated cAMP (*B*) is the net difference in intracellular cAMP between the presence and absence of SP since there was no effect of increasing receptor expression on basal levels of cAMP. Although the same concentrations of cDNA were used in all experiments, the actual levels of expressed receptor varied between different experiments. Therefore, the two plots display an overlay of several individual experiments ($n = 3$ in *A* and *B*), in which mutant and wild-type receptors were compared side-by-side. Each data point is the mean of duplicate determinations of either IP1 or cAMP. The level of expression was measured in each experiment by transfecting in parallel cells that were seeded in 24-well plates with the same concentrations of cDNA and that were used to generate binding isotherms for SP using 12 log-spaced concentrations in duplicates as described under "Experimental Procedures." The maximal binding capacity was computed using LIGAND (23). To assess the significance of the difference between mutant and wild-type receptors, the data were fitted by linear regressions assuming zero intercepts (*solid lines*). The calculated slopes (lower-upper 95% confidence limits) are as follows: *A*, 0.0254 (0.0231–0.0277) for the wild-type receptor and 0.0573 (0.0504–0.0642) for the mutant; *B*, 10.4 (9.1–12) for the wild-type receptor and 14.2 (11.4–16.8) for the mutant. The difference in response between receptors is significant for PI turnover, but not for cAMP accumulation.

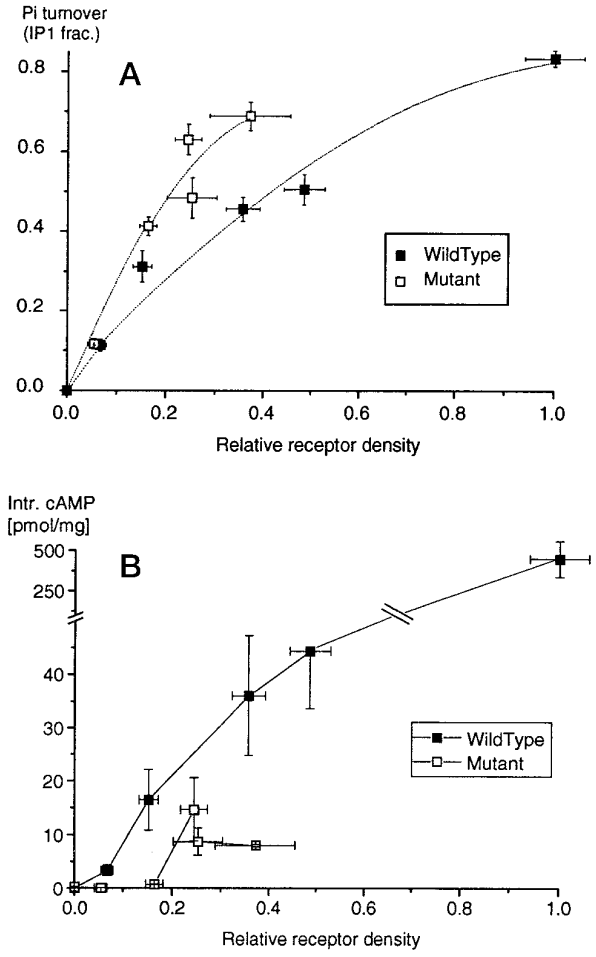

FIG. 4. **Relation between mutant and wild-type NK_1R densities and biological responses in CHO cells.** CHO cell clones expressing different levels of mutant or wild-type receptors were assayed for PI turnover stimulation (*A*) and intracellular cAMP accumulation (*B*) in the presence or absence of 10 μM SP. The two second messengers were measured in the same cell extract as described under "Experimental Procedures"; thus, both panels display the same experiments, and in both, the data are plotted as net responses following subtraction of the activities in the absence of SP. Response data are the means ± S.E. of six independent experiments, in which all clones were tested in parallel. The receptor concentrations in each clone were normalized to that measured in the highest expressing cell line (8 ± 0.48 pmol/mg of cell protein) and are means ± S.E. of three independent determinations, in which all clones were measured in parallel.

nergic receptors, coupled to G_q and G_s, respectively, but not tachykinin receptors. However, only a few mutant-expressing clones were obtained, which displayed receptor densities far below those measured in wild-type receptor-expressing cells. Thus, to compare the best mutant-expressing clone with the wild-type receptor-expressing cells, we divided the extent of maximal PI turnover stimulation in the presence of SP by the molar concentration of receptors measured on the cell surface in parallel experiments. Again, the mutant receptor exhibited an enhanced ability to activate phosphoinositide hydrolysis (maximal stimulation in fractional PI units/pmol of receptor was 0.025 ± 0.003 in the wild-type receptor and 0.034 ± 0.004 in the mutant receptor). There was no accumulation of intracellular cAMP in response to SP agonists in transfected glioma cells expressing either the wild-type or mutant receptor, although all the clones displayed large and comparable increases in cAMP concentrations in response to isoproterenol, similar to those measured in nontransfected C6 cells.

In conclusion, these experiments indicate that NK_1R-mediated stimulation of PI turnover is potentiated by the mutation in three different cell systems. The effect of the mutation on cAMP responses is less clear: there is no effect in COS cells; there is marked impairment in CHO cells; and it cannot be evaluated in C6 cells, where NK_1Rs do not appear to control cAMP levels. Whether these differences across cell lines reflect

a divergent degree of cross-talk between the two signaling pathways or differences in stoichiometry between the transfected receptor and the pool of endogenous G α-subunits remains to be investigated. We cannot draw any further conclusions at the moment, except for an obvious consideration of the importance of the surrogate expression host cell type in the study of recombinant receptors.

The core message of such experiments, however, is that the mutation induces a small but definite shift in receptor preference for the two signaling pathways and, consequently, for the G proteins G_q and G_s, which are involved in these two modes of signal transduction. This implies that the chemical nature of the pair of residues targeted by the mutation can influence either the respective affinities of the receptor for different G proteins or the interconversion of the receptor into divergent "active" forms that have different specificity for distinct G proteins. In either case, the effect is clearly allosteric in nature because the mutated residues, given their location, cannot form direct contact sites for the G protein in the cytosolic region of the receptor.

Allosteric Linkage between Change in Apparent Affinity of the Ligands and Shift in Signaling Property of the Receptor—There is a phenomenological link between the effects of the mutation on the binding and signaling properties of the receptor: the mutation-induced enhancement of apparent affinity is only "detectable" by agonists that preferentially trigger G_q-mediated responses, and correspondingly, the mutant receptor appears to produce more efficient G_q-mediated signaling even when it is activated by the relatively "transducer-unselective" agonist SP.

The interesting question is whether this link can be interpreted on a more mechanistic and quantitative basis. A ternary complex model describing the interaction between the receptor and two distinct G proteins may be a potential tool to interpret the shift in apparent affinities observed in this study. But it is conceptually difficult to apply such model to the binding data presented here, which were generated in intact cells incubated in isotonic buffer at 0 °C. In fact, at the intracellular concentrations of guanine nucleotides (close to the mM range), this model predicts that the fraction of bound receptor in the G protein-coupled form is negligible at equilibrium, and the measured affinity reflects that of the free receptor state (32). Indeed, the binding isotherms of SP were always consistent with a single class of sites in this study (data not shown).

However, the allosteric concept of ligand-receptor interactions predicts that macroscopic binding affinities are apparent also in the absence of the stabilizing effect of the G protein. Even a monomeric receptor with a single binding site for ligands can be thought of as existing in equilibrium among a large number of different tautomeric states, in each of which the binding affinity of that site will be different (33). The introduction of a ligand in this system shifts the equilibrium toward those conformations that have the highest affinity for that ligand. Therefore, any macroscopic binding constant can always be interpreted as the aggregate of two energetic components: one due to the second-order association process taking place between the two reacting partners and the other resulting from the displacement that the process itself imposes on the intramolecular equilibria within the receptor (34).

Accordingly, even if a mutation does not directly alter "docking" residues within the ligand-binding region of the receptor, it can nonetheless modify "allosterically" the binding affinity of a group of ligands through two distinct mechanisms: (*a*) by changing the stability of the state (or subset of states) that is commonly induced by that group of ligands or (*b*) changing the extent to which each ligand alters the stability of those states. The first will produce an equal shift to the affinities of all those ligands; the second affects the affinity of each ligand differently. More realistically, we should think that any mutation may always produce changes by a combination of both effects, but if the change in stability constants of the states is proportionally greater than those affecting the allosteric factors of each ligand, we shall detect a constant shift in the analysis of free energy correlations presented in this study.

To illustrate this by a simplified numerical example, we simulated an allosteric receptor existing in a minimum of four interconverting states (s_0 through s_3), interacting with four groups of ligands, each consisting of 50 distinct members. Within each group, ligands differ in affinity for the reference state (s_0), but share the ability to stabilize preferentially the same receptor state.

To generate the mutation, we applied a net overall free energy change of 4.5 *RT* units unequally distributed over the stability constants of the four states so that the constant of s_3 was enhanced by the equivalent of 3 *RT* free energy units, that of s_1 was diminished by 1.5, while the others remained unaltered (Fig. 5, *upper panels*). The mutation was simulated in two ways. First, we only allowed a change in stability constants (*j* parameters), and then we also allowed smaller random changes in the allosteric factors of each ligand (*b* parameters). The results of theoretical modeling are plotted in Fig. 5, exactly as it was done for the experimental data of Fig. 1. Two points are clear from such simulations.

First, a mutation altering the stability constants of discrete states of the receptor can allosterically change the apparent affinities of all ligands that stabilize those targeted states. If we introduced scatter in the data by allowing the mutation to also change randomly the allosteric factors of ligands, the binding energies of each class of such ligands formed well delimited clusters in plots of wild-type receptor binding energy as a function of net mutation-induced change (Fig. 5, *left panels*). In contrast, the binding energies of ligands inducing states that were not targeted by the mutation could not be discriminated at all.

Second, the magnitude of change in the stability constants of the targeted states is faithfully reflected in the extent of displacement it imposes on the relation between binding energies for mutant and wild-type receptors. In this simulated example (Fig. 5, *right panels*), the clusters of binding energies corresponding to ligands selective for s_1 and s_3 (the stability of which is diminished and enhanced, respectively, by the mutation) generated regression lines with unitary slopes that are shifted 1.5 units to the left and 3 units to the right, respectively, from those of all the other ligands.

According to such a model, the effect of the G166C,Y167F mutation in NK_1R can be interpreted as resulting from the enhancement of the intrinsic stability of a state (or collection of substates) that is induced by septide-like ligands and heterologous tachykinins, but not by SP, its full-length analogues, or various antagonists. The same mechanism may also explain the linkage between the change in signaling properties of the receptor and the shift in affinities for ligands sharing increased selectivity for PI signaling if we imagine that the set of functional states induced by septide-like ligands (and favored by the mutation) may preferentially signal via G_q.

CONCLUSION

In summary, it was shown previously (5) that replacement in NK_1R of two NK_2R residues located near the extracellular edge of TMD-4 reduces the selectivity of the receptor. We show in this study that the mechanism of increase in affinity for heterologous tachykinins (which underlies such change in selectivity) is not conceivably the result of "local" interactions be-

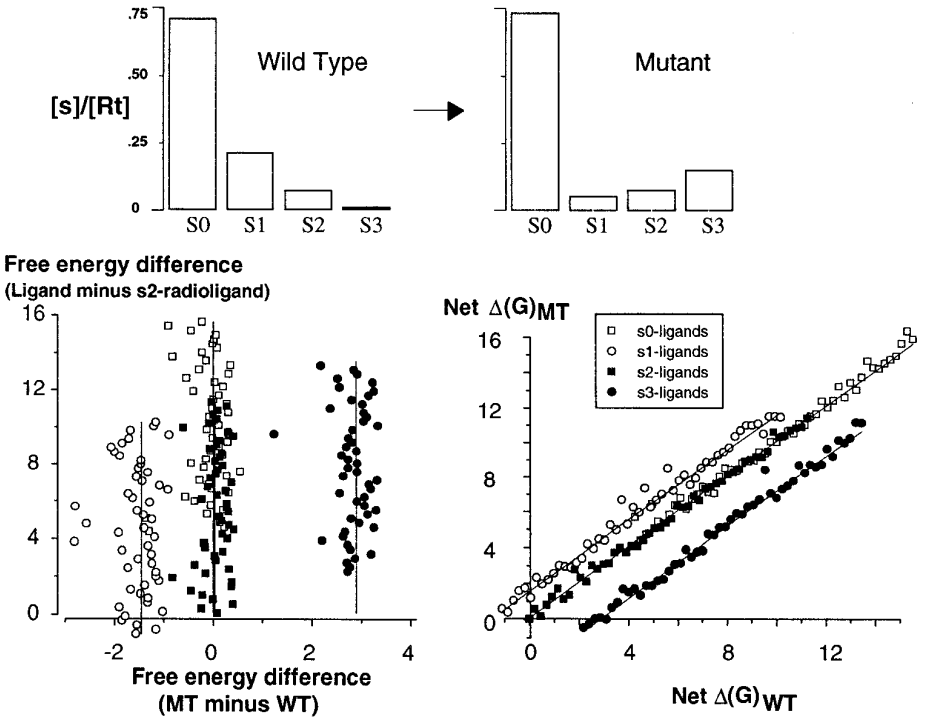

FIG. 5. **Simulations according to an allosteric receptor model of a mutation altering stability constants of substates.** Simulations were generated for a receptor undergoing transitions among four states (s_0 through s_3) as explained in the theoretical model described under "Experimental Procedures." The stability constants j_1, j_2, and j_3 in the wild-type receptor are 0.3, 0.1, and 0.01, respectively, and were assumed to be changed to 0.067, 0.1, and 0.201 by the mutation (in free energy terms, this corresponds to a total change of 4.5 *RT* units, with an increase of 3 units for j_3, a decrease of 1.5 units for j_1, and no change in j_2). The consequence of the mutation on the equilibrium concentration of the four states is shown in the *top panels*, where states, as a fraction of the total concentration of the receptor (*Rt*) in the absence of ligand, are shown in histogram form before and after the mutation. Four hypothetical classes of ligands, each consisting of 50 members, are defined by their state preference (*i.e.* within each class, ligands differ in the equilibrium binding affinity for the default state s_0 (K_0), which was varied over 3 orders of magnitude, but all share the highest *b* value for the same state). Accordingly, they are marked as *s0-*, *s1-*, *s2-*, and *s3-ligands*, respectively. The binding isotherms for both wild-type (*WT*) and mutant (*MT*) receptors of the entire panel of 200 ligands were simulated as described under "Experimental Procedures," assuming that the binding of each ligand was measured in competition for a fixed "tracer" concentration of the highest affinity member of the s_2-selective class of ligands (*s2-radioligand*). Apparent binding affinities were obtained by fitting (23) the simulated curves, and the results are plotted (*bottom panels*) as the experimental results of Fig. 1. The mutation was simulated either as a pure change in the *j* parameters only, which generates error-free changes (*solid lines*), or as joint changes in parameters *j* and *b*, where the latter were assumed to affect all ligands randomly and were produced as explained under "Experimental Procedures." This generates noisy data that are displayed with different symbols for each class of ligands as indicated. Although not shown here, an identical kind of scatter could also be produced by applying an equivalent degree of simulated percentage error to the final affinity estimates generated by mutations that affect *j* only. This strengthens the idea that the error around the regression lines of Fig. 1*B* includes both ligand-specific effects of the mutation and experimental error in an inextricable manner (see also the discussion of the data of Fig. 1*B* under "Results and Discussion").

tween the transmutated side chains and specific structural elements of the responding ligands. It consists instead of a broader conformational change that improves the apparent affinity of transducer-selective septide-like tachykinins and also shifts the transducer preference of the receptor itself.

This deduction is supported by three kinds of experimental evidence. First, the analysis of the binding energies of 26 ligands for wild-type and mutant receptors indicates that within the modifications due to the mutation, the constant shift in apparent affinity affecting an entire class of structural heterogeneous tachykinins bears much greater weight than specific changes affecting differently the affinity of each individual ligand. Second, C-terminal modified analogues of SP exhibit the same transducer selectivity as the other members of the group of ligands responding with increased affinity to the mutation, suggesting that this property is shared by all those ligands. Third, the relation between receptor density and biological response evaluated in three different mammalian expression systems indicates that G166C,Y167F NK$_1$R triggers PI signaling more efficiently than the wild-type receptor upon activation by SP.

We interpret these findings by proposing that the G166C,Y167F mutation may enhance the intrinsic stability of a functional substate of NK$_1$R that is preferentially induced upon binding by septide-like agonists, but not by SP-like agonists or nonpeptide ligands. Simulations based on a multistate allosteric receptor model indicate that if a mutation alters individual stability constants of receptor states to a greater extent than the allosteric effect of each ligand, the pattern of change in apparent affinities closely matches that observed in this study. The model therefore supports this interpretation and also provides theoretical background to the analysis of free energy correlations proposed in this paper. Such an approach may be generally useful in the study of site-directed mutagenesis of G protein-coupled receptors and the structure-activity relation of ligand-receptor interactions.

A mutation resulting in selective enhancement of affinity similar to that reported here was described recently for human NK$_1$R (35). Alanine replacement of the highly conserved Tyr-216 located near the cytosolic end of TMD-5 increases the affinity for the heterologous tachykinins NKA and NKB, septide, and C-terminal modified analogues of SP, but not for nonpeptide antagonists and SP itself (35). The increase in binding affinity was attributed to a conformational change in the receptor rather than to a direct interaction between the bound ligands and the mutated side chain (which is very unlikely given the location of the targeted residue in that case). Unlike the G166C,Y167F mutation, the Y216A mutation abol-

ishes PI turnover stimulation, although the possibility that cAMP responsiveness may have been left unchanged was not investigated (35). Considering that TMD-4 and TMD-5 are tethered via a common extracellular loop but are connected to distinct cytosolic segments of the sequence, the finding that different mutations located at opposite edges of the two helices induce similar conformation-mediated changes in ligand binding but divergent effects on receptor activation is provocative. It suggests that the effect on binding of the two mutations may be mediated by a common mechanism, such as, for example, long-range influences producing in both cases a favorable orientation of the second extracellular loop, whereas the differences in receptor activity may reflect the diverse roles that the two intracellular loops play in the process of conversion of the receptor into the active form and in the formation of a docking configuration for the intervening G proteins. Additional studies based on the construction of double-mutant cycles (36) may be useful to evaluate whether there is additivity or cooperativity between perturbations imposed in these two distinct sites of the NK_1R transmembrane bundle.

One implication of the results of both studies (this study and Ref. 35) pertains to the mechanism underlying the selectivity of the receptor for different endogenous ligands. As proposed previously (5, 25, 26, 35), the different affinities of tachykinin receptors for their natural ligands may be the result of a selection of optimal matching conformations between common structures in both ligands and receptors, rather than the product of specific local interactions between the variant "address" regions of the peptides and complementary divergent residues in the sequence of the receptors. If selectivity depends on ligand-induced selection of distinct receptor conformers, any mutation that alter the tautomeric equilibria between receptor conformations must also change selectivity. The results presented here confirm this prediction and use the concept of allosteric transition (33) to address this notion on a more theoretical and quantitative basis. In addition, we show here an uncommon example of how receptor mutagenesis can help to identify functional differences among agonists (31) that cannot be easily rationalized by classical pharmacological theories of drug efficacy. Our theoretical analysis indicates that mutations affecting the state distribution of the receptor can dissect discrete clusters within the variation in binding energies of ligands, just like ligands select discrete states within the conformational space of the receptor.

A second implication of this study concerns the mechanism of G protein promiscuity in seven-transmembrane receptors. Here we show that two residues located near the extracellular part of the receptor (the side chains of which are therefore not likely to provide docking sites in the cytosolic binding region for the G protein) are nonetheless important in determining G protein preference since their replacement produces a small but detectable shift toward G_q-mediated PI signaling. This suggests that also G protein selectivity may depend on a conformation-driven selection mechanism as discussed above for the ligands. It could also explain how septide-like agonists, although sharing the same binding site of SP on the receptor, can trigger signals that differ distinctively in transducer selectivity.

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