Mutations in the Extracellular Amino-terminal Domain of the NK2 Neurokinin Receptor Abolish cAMP Signaling but Preserve Intracellular Calcium Responses*

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By combining real time measurements of agonist binding, by fluorescence resonance energy transfer, and of subsequent responses, we proposed previously that the neurokinin NK2 receptor preexists in equilibrium between three states: inactive, calcium-triggering, and cAMP-producing. Thr24 and Phe26 of the NK2 receptor extracellular domain are considered to interact with neuropeptide agonists based on the reduction of affinity when they are substituted by alanine. Using fluorescence resonance energy transfer, we now quantify the binding kinetics of two Texas Red-modified neurokinin A agonists to the fluorescent wild-type (Y-NK2wt) and the mutant (Y-NK2mut) receptor carrying Thr24 → Ala and Phe26 → Ala mutations. TR1-neurokinin A binds with a fast component and a slow component to the Y-NK2wt receptor and triggers both a calcium and a cAMP response. In contrast, on the mutant receptor, it binds in a single fast step with a lower apparent affinity and activates only the calcium response. Another agonist, TRC4-neurokinin A, binds to both wild-type and mutant receptors in a single fast step, with similar affinities and kinetics and promotes only calcium signaling. Kinetic modeling of ligand binding and receptor interconversions is carried out to analyze phenotypic changes in terms of binding alterations or changes in the transitions between conformational states. We show that the binding and response properties of the Y-NK2mut receptor are best described according to a phenotype where a reduction of the transition between the inactive and the active states occurs.

G-protein-coupled receptors (GPCRs)1 represent the largest family of cell surface receptors and have multiple effects on the activated cell. Despite the variety of molecular types of ligands (hormones, proteins, small peptides, ions, lipids, and sensory stimuli such as odorants, pheromones, and photons), GPCRs share homology in their core domain composed of seven transmembrane α-helices. The conservation of the same overall hydrophobic structure exemplifies the essential role of this structure for the activation process.

GPCR activation promotes GDP to GTP exchange on the α subunit of the heterotrimeric G protein associated with its cytoplasmic loops, thereby initializing the intracellular cascade of signaling events. Among the theoretical models describing the activation mechanism, the two-state allometric model of activation has been proposed, following the discovery of inverse agonists and of constitutive activity for GPCRs (1–4). In this model, a GPCR preexists in equilibrium between an active and an inactive state in the absence of ligand. As such, agonists, higher affinity for the active state having displace the equilibrium toward activation, whereas inverse agonists have higher affinities for the inactive state. Constitutive activity is explained by an intrinsic tendency for a receptor to spontaneously isomerize toward the active conformation. Although the multiple conformational states theory is an attractive model for describing GPCR activation, there is a need for more data correlating the parameters of possible intermediate conversion states with physiological responses.

Indeed, several GPCRs have been reported to mediate multiple signaling pathways through activation of different heterotrimeric G proteins: the receptors for dopamine D1 (G, G, and G (5)), G and G (6)) and D5 (G, and G (7)), the receptors for the parathyroid hormone (G, G, and G (8)), for the corticotropin-releasing hormone (G, G, G, and G (9)), for the melanin-concentrating hormone (G, and G, and maybe G (10)), for the vasoactive intestinal peptide (G, and G (11)), for prostacyclin (G, G, and G (12)), the adenosine A1 receptor (G, and G (13)), the β-adrenergic receptor (G, and G (14)), the muscarinic m receptor (G, and G (15)), the 5-hydroxytryptamine receptor type 4; Bpa, p-benzoyl-l-phenylalanine. (G, G, and G (16)), the endothelin subtype B receptor (G, G, and G (17, 18)). These different couplings suggest that GPCRs may exist in more than a single active state.

We have previously addressed the question of multiple receptor states by using fluorescence resonance energy transfer (FRET) to monitor ligand binding in parallel with cellular responses. A chimeric green fluorescent neurokinin NK2 receptor, EGFP-NK2R was expressed in HEK293 cells. Two of its neuropeptide agonists, the decapetide neurokinin A (NKA) and its truncated form, NKA-(4–10), were covalently linked to the EGFP-NK2R receptor on living cells were monitored as a diminution of fluorescence emission of EGFP due to FRET.

This analysis showed that the NK2 receptor exists in a minimal number of three receptor conformations, denoted R0,
R1, and R2 with intrinsic binding constants specific to each ligand (19). The R0 state corresponded to the inactive state. The R1 state could be populated by the two agonists, was characterized by a rapid association with both ligands, and corresponded to an active conformation triggering calcium responses via heterotrimeric Gq activation. The R2 state was stabilized at equilibrium only by TR1-NKA and was characterized by a slow binding relaxation temporally correlated with a cycle AMP response unique to TR1-NKA. The R2 state was thus assigned to an active conformation triggering cAMP synthesis putatively through Gq activation (20–22).

In the present study, the phenotype of an EYFP chimeric fluorescent NK2 receptor carrying two point mutations located in the extracellular amino-terminal domain Thr24→Ala and Phe26→Ala has been investigated. These substitutions taken independently have been previously shown to lower the apparent affinity of NKA for the receptor (23, 24), suggesting that Thr24 and Phe26 are part of the binding domain for NKA. In addition, the activation process was reported to be unaffected by the substitutions, as measured by the ability of NKA to trigger calcium responses (23). However, given the demonstration that the NK2 receptor exists in several active states (19), we have now reanalyzed intracellular responses of the mutant receptor that we named Y-NK2mut. We find that the Y-NK2mut receptor is unable to activate cAMP production irrespective of the agonist tested. This shows that Thr24 and Phe26 somehow play a role in the activation process. We have studied the kinetics of interaction between the Y-NK2mut receptor and two of its fluorescent agonists (TR1-NKA and TRC4-NKA) by FRET and compared them to those of the wild-type Y-NK2wt receptor. The experimental data are interpreted in the context of the kinetic model previously used to describe the activation of the EGFP-NK2 receptor. We propose that, in fact, the mutant receptor is affected in its capacity to interconvert between the inactive state R0 and the two active states, behaving like a "constitutively inactive" receptor.

**EXPERIMENTAL PROCEDURES**

**Chemicals**

Synthetic peptides were obtained from Bachem or Neosystem. Protease inhibitors were obtained from Sigma and Calbiochem. Fluorescent labels and ion chelators were from Molecular Probes, Inc. (Eugene, OR). SR48968 was a kind gift from Sanofi. [3H]SR48968 was purchased from Amersham Biosciences. Genetica (G418) was from Invitrogen.

**Construction of the Mutant Receptor Chimera and Expression in Stable HEK**

A NotI-Xhol SP-EYFP fragment, in which SP is the signal peptide and EYFP the enhanced yellow fluorescent protein, was generated as described for the SP-EGFP fragment (25) using pEYFP-C3 (Clontech) as a source of EYFP sequence. The wild-type rat NK2 sequence starting at the amino acid 16 was extracted from the pCEP4-chim1 vector as a BamHI fragment (25). The NK2 mutant fragment Xhol-BamHI was amplified by PCR using pCPE4chim1 as a bait with primer 1 (AGCGCTCGAGAATGACCCAGGCTTGTGGACGCACCGTCCATG) (Xhol site underlined) and primer 2 (CGGAGTGATTACGAGCTTGTGGACGCCACCGTATGGGGG) (BamHI site underlined). The mutated nucleotides are in boldface type in primer 1. The NotI-Xhol SP-EYFP fragment was ligated in frame to the Xhol-BamHI NK2 fragment and inserted in the expression vector prEePO (Invitrogen) between the NotI and BamHI sites.

HEK293 cells, grown in minimal essential medium complemented with 10% fetal calf serum and antibiotics (streptomycin/penicillin), were transfected by calcium phosphate precipitation (26) and selected with 600 μg/ml G418. Stable cell lines were passaged twice a week. New stocks were defrosted every 12 weeks.

**Fluorescence Microscopy**

Cells were grown for 2 days in 24-well plates on 12-mm glass cover slips coated with rat type I collagen. For endosome staining, Texas Red transferrin uptake was done as previously described (27) or cell lines expressing either the Y-NK2wt or the Y-NK2mut receptor were transiently transfected with a pDNA3 plasmid expressing a RbsG(FQ7LI)-myc tagged under a cytomegalovirus promoter. On the day of the experiment, cells were washed twice with PBS, fixed in 4% paraformaldehyde-PBS for 15 min at room temperature and then incubated for 15 min in 50 m M NH4Cl in PBS. Coverslips were mounted onto microscope slides using Mowiol (Calbiochem) and observed either with an inverted microscope (Zeiss Axioplan) equipped with a Hamamatsu CDD camera or with an inverted microscope (Nikon Eclipse TE300) connected to a laser-scanning confocal imaging system (Bio-Rad MRC 1024 ES) using a Plan Apo ×60 1.20 numerical aperture water immersion objective (Nikon).

For epifluorescence measurements, the EYFP was excited at 485 ± 10 nm with a bandpass filter. Emitted fluorescence was detected using a dichroic mirror at 510 nm and a longpass filter of 520 nm. For confocal measurements, excitation was from a 300-milliwatt krypton/argon laser at 10% power. Each stack of two-dimensional images was acquired sequentially in the green channel (FMT2; excitation 488 nm, emission 522 nm) and eventually in the red channel (FMT1; excitation 568 nm, emission 605 nm), before stepping (0.5 μm) the objective in the z axis, typically 10–12 images are taken per cell.

**Immuno precipitations and Immunoblots**

Around 5 million cells were resuspended in 800 μl of cold lysis buffer (in 150 mM NaCl, 1.5 mM MgCl2, 10% glycerol, 5 mM EDTA, 50 mM Hepes, pH 7.5) supplemented with 1 m M dithiothreitol and 10% (vol/vol) EDTA-free protease inhibitor (for 10 mM buffer; Roche Molecular Biochemicals). A postnuclear supernatant was prepared as described previously (27). Immuno precipitations were carried out with a polyclonal rabbit anti-GFP antibody made in our laboratory. The immuno precipitated complex was prepared as described (27) prior to loading on a 12% SDS-PAGE gel. Gels were blot ted onto polyvinylidene difluoride membranes and blots were incubated with a mouse monoclonal anti-GFP antibody (Clontech) at a 1:1000 dilution, followed by incubation with a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences) at a 1:2000 dilution. Detection was achieved using enhanced chemiluminescence (SuperSignal WestNico; PerbioScience).

**Radioligand Binding Experiments**

Radioligand binding assays and quantification of receptor sites per cell were performed as described previously (25). Competition experiments were performed at 4 °C with 1 nM [3H]SR49868 for 3 h, and experimental IC50 values were converted to Kd values using the Cheng and Prusoff relationship (28).

**Synthesis of Fluorescent Analogs**

The peptides NKA (HKTDSFVGLM-NH2), C7-NKA-(4–10) (DSF-CGLMNH2) and C4-NKA (HKTCSFVGHL-MH2) were derivatized with Texas Red as previously described for fluorescent TR1-NKA and TR7-NKA-(4–10) peptides (19).

**Fluorescence Kinetic Measurements**

Adherent cells were rinsed with PBS and dissociated from the culture flask in PBS, 5 mM EDTA buffer, pH 7.4. Unless otherwise stated, cell suspensions were at 107 cells/ml in HEPES–bovine serum albumin buffer (137.5 mM NaCl, 1.25 mM MgCl2, 1.25 mM CaCl2, 5 mM KCl, 5.6 mM glucose, 10 mM HEPES, 0.4 mM Na2HPO4, 1% bovine serum albumin (w/v), pH 7.4) supplemented with protease inhibitors (40 μg/ml bestatin and bacitracin, 20 μg/ml phosphoramidon, 50 μg/ml chymostatin, and 1 μg/ml leupeptin).

All fluorescence measurements on cell suspensions were made on a Fluorolog-2 (SPEX) spectrophotometer equipped with a 450-watt xenon lamp, one double grating excitation set (at 470 nm) and two single grating emission monochromators (time-based set at 530 and 610 nm). Interaction of TR-modified NKA with the cell surface Y-NK2 receptors was monitored as a decrease of EYFP emission at 530 nm due to fluorescence resonance energy transfer toward the acceptor group TR. Data were acquired with a photon-counting photomultiplier (linear up to 107 counts/s) and stored using the DM3000 software provided with the spectrophotometer.

**Screening of Fluorescent Ligands Using the Association Kinetics Measured by FRET**—Cells were placed in a 1-ml cuvette with magnetic stirring and maintained at 21 °C in the thermostatted cuvette handler of the spectrophotometer as previously described (25). Time-based re-
cordings were sampled every 300 ms. Ligand was added to the cuvette at around 20–50 s by stopping the recordings for a short time.

**Kinetic Data Analysis**

Each trace corresponds to the mean of three consecutive recordings. Fitting was carried out with nonlinear regression, by giving to each triplicate a weighting inversely proportional to the corresponding variance. Traces were analyzed with a single exponential, one exponential plus linear, two exponentials and correspond to two of the three states, each state (all of the experimental dissociation traces have been fitted with two exponentials and corresponding to two of the three states, each combination being tested in the modeling), and (iv) intrinsic rates for the transition between two ligand-bound receptor states. The program simulates and represents as a function of time (i) the theoretical binding and dissociation traces for the indicated concentrations of ligand and (ii) the theoretical proportions of ligand-free and ligand-bound receptor states and allows us to derive values for the intrinsic isomerization constants $\Gamma$ between two of the NK2 receptor states.

**RESULTS**

**Construction and Expression of the Fluorescent Y-NK2wt and Y-NK2mut Receptors**

Huang et al. (23) have performed an exhaustive site-directed mutagenesis study of the NK2 receptor to identify residues involved in NKA binding. Important residues were mainly located in the amino-terminal domain, the extracellular loops 1 and 2, and in the second hydrophobic transmembrane helix. We have introduced two point mutations in the extracellular extremity of rat NK2 (substitutions Thr$^{24}\rightarrow$ Ala and Phe$^{26}\rightarrow$ Ala). Y-NK2wt and Y-NK2mut chimeric receptors were generated by joining the carboxyl terminus of the yellow fluorescent protein EYFP to the amino-terminal part starting at residue Leu$^{16}$ of the wild-type and the mutant NK2 (Fig. 1A), as had previously been done for the wild-type NK2 receptor fused to the amino acid preceding the first transmembrane helix according to the recently determined structure of rhodopsin. B, confocal fluorescence images of HEK 293 cells expressing either the Y-NK2wt or the Y-NK2mut constructs taken in the middle of the cells (2.5 µm from each pole in the $z$ axis). C, immunoblot with a monoclonal anti-GFP antibody following immunoprecipitation of each of the chimeric receptors, Y-NK2wt and Y-NK2mut, by a polyclonal anti-GFP antibody. HEK refers to a control experiment performed on non-transfected cells.

**Conformational Mutation of NK2 Neurokinin Receptor**

![Figure 1](image-url)
Fig. 2. Pharmacological properties and physiological responses of Y-NK2wt and Y-NK2mut receptors activated by NKA and TR1-NKA. A and D, measurement of apparent inhibition constants. Displacement of 1 nM [3H]SR48968 by increasing concentrations of NKA (A) or TR1-NKA (D) on cells expressing the Y-NK2mut (black circle) or the Y-NK2wt receptors (white square) after 3 h at 4 °C. Each point corresponds to the mean value of three independent experiments performed in duplicate. The error bars are the S.D. B and E, calcium responses. The extent of intracellular calcium responses induced at 21 °C by increasing concentrations of NKA (B) or TR1-NKA (E) recorded at 400 nm using the calcium probe indo-1 (excitation 355 nm). Each point corresponds to the mean value of the calcium peak of two independent experiments. The error bars are the S.D. C and F, cAMP responses. The amount of cAMP (pmol/wells) produced at 21 °C after 15-min incubation with various concentrations of NKA (C) or TR1-NKA (F). Each point is the mean value of two experiments performed in duplicate.

Pharmacological Properties and Physiological Responses to NKA of Activated Y-NK2wt and Y-NK2mut Receptors

Equilibrium Binding—The number of receptors expressed at the cell surface were estimated from radiolabeled antagonist [3H]SR48968 binding, which is not sensitive to mutations in the amino-terminal domain (23). For each cell line, we have found ~750,000 Y-NK2wt sites and 1,150,000 Y-NK2mut sites per cell. NKA binding affinities were determined from competition experiments performed at equilibrium using 1 nM [3H]SR48968 (Fig. 2A). The apparent inhibition constants for NKA (K_i ± S.D., n = 3) were 4.5 ± 0.5 nM and 45 ± 8 μM for Y-NK2wt and Y-NK2mut receptors, respectively, indicating an apparent 10,000-fold loss of NKA affinity for the mutant, which is in agreement with published data (23, 24).

Responses—Calcium responses were monitored on cell suspensions using Indo-1 fluorescence variations after agonist addition and quantified as peak response amplitudes. The Y-NK2mut receptor responded to NKA with an EC_{50} of 3.4 ± 0.2 μM (versus 2.2 ± 0.5 nM for the Y-NK2wt receptor) and a maximal response reaching 70% of the maximal Y-NK2wt receptor response for the same agonist (Fig. 2B). Since we and others reported cAMP production following NK2 receptor activation (19, 20, 31), the capacity of Y-NK2mut to initiate cAMP synthesis was investigated. Unexpectedly, the mutant did not induce any cAMP response even at high concentrations of NKA (up to 100 μM) (Fig. 2C). Previously, we have modeled the activation of the NK2 receptor and proposed that its calcium and cAMP responses are mediated by distinct active states R1 and R2, respectively (Fig. 3), which can be detected by ligand binding kinetic determinations (19). We have shown as well, using FRET, that these states exhibit different affinities for two fluorescent agonists (19). Hence, in order to test further this former proposal, the same type of binding kinetic analysis was undertaken on the Y-NK2mut receptor.

Pharmacological Properties, Physiological Responses, and Kinetics of Association of TR1-NKA to the Y-NK2wt and Y-NK2mut Receptors

Equilibrium Binding and Responses—TR1-NKA has a TR fluorophore linked to the amino-terminal extremity of NKA and behaves like NKA on the wild-type green EGFP-NK2 receptor in terms of affinities and responses (19). Pharmacological and functional properties of TR1-NKA association to the Y-NK2wt and Y-NK2mut receptors were investigated. The apparent inhibition constants for TR1-NKA were K_i = 4.9 ± 0.2 nM and K_i = 250 ± 20 nM for Y-NK2wt and Y-NK2mut receptors, respectively (Fig. 2D). Calcium responses to TR1-NKA were followed up to the concentration limit (1 μM). EC_{50} were equal to 0.60 ± 0.1 nM and 180 ± 30 nM for the Y-NK2wt and
The experimental trace for 100 nM TR1-NKA association mainly to the R0 and R1 states of the NK2wt receptor (19).

Fig. 4
its binding to the Y-NK2wt receptor. This is illustrated in both in terms of amplitude and time course as compared with binding of TR1-NKA to the Y-NK2mut surface fluorescent receptors by the fluorescent ligand. The fluorescence at 530 nm reflects the occupancy of the cell thermal equilibration. The decrease of the EYFP-emitted fluorescence shown that TR7-NKA-(4mut) binds to the Y-NK2mut receptor with a better apparent affinity than does nonfluorescent NKA (180-fold) but still with a much lower apparent affinity than to the Y-NK2wt receptor (50-fold). It behaves as a partial agonist in calcium signaling and, like NKA, it does not trigger cAMP production on the Y-NK2mut receptor.

Continuous Monitoring of TR1-NKA Association by FRET—Cells in suspension were placed in a 1-ml cuvette maintained at 21 °C in the cuvette holder of a spectrofluorometer. Ligands were added to the sample 20–50 s after thermal equilibration. The decrease of the EYFP-emitted fluorescence at 530 nm reflects the occupancy of the cell surface fluorescent receptors by the fluorescent ligand. The binding of TR1-NKA to the Y-NK2mut receptor was different both in terms of amplitude and time course as compared with its binding to the Y-NK2wt receptor. This is illustrated in Fig. 4A with 6 and 170 nM TR1-NKA. We have previously shown that TR7-NKA-(4–10) binds with a single fast step, mainly to the R0 and R1 states of the NK2wt receptor (19). The experimental trace for 100 nM TR1-NKA association to the Y-NK2mut receptor could be superimposed on that obtained for 10 nM TR7-NKA-(4–10) binding to the Y-NK2wt receptor (Fig. 4B). This suggests that TR1-NKA may only bind to the R0 and R1 states of the Y-NK2mut receptor. To refine association time courses and determine binding rate constants, we analyzed the kinetics of TR1-NKA association and dissociation on Y-NK2mut and Y-NK2wt receptors by FRET using a stopped-flow apparatus.

Quantitative Analysis of the Kinetics of the Y-NK2mut Receptor/TR1-NKA Interactions

Ligand binding associations and dissociations were analyzed as previously described (19). The rigorous analysis of association kinetics could not be directly performed with an equation based on the model presented in Fig. 3, because the number of unknown parameters associated with this model is too large to allow their confident simultaneous determination. Binding traces were therefore analyzed either as simple bimolecular reactions or as combinations of bimolecular reactions with interconversions, using equations given below. Parameters derived from the following kinetic data analyses were then incorporated in the three-state model from Fig. 3 in a second step (see Figs. 10–12).

TR1-NKA Association Step—The binding traces of TR1-NKA to the Y-NK2mut receptors, respectively (Fig. 2E) with a maximal calcium response of the mutant reaching around 30% of the maximal Y-NK2wt receptor response to the same agonist. TR1-NKA interaction with the Y-NK2wt receptor triggered cAMP synthesis with an EC50 of 32 ± 10 nM. There was no detected cAMP accumulation promoted by TR1-NKA association to the Y-NK2wt receptor (Fig. 2F). In summary, TR1-NKA binds to the Y-NK2mut receptor with a better apparent affinity than does nonfluorescent NKA (180-fold) but still with a much lower apparent affinity than to the Y-NK2wt receptor (50-fold). It behaves as a partial agonist in calcium signaling and, like NKA, it does not trigger cAMP production on the Y-NK2mut receptor.
Under pseudo-first-order conditions, the concentration of free ligand is equal to the concentration of total ligand, and the concentration of RL is deduced from the following differential equation, assuming that at time 0, $[R] = [R_p]$ and $[RL] = 0$.

\[
\frac{d[RL]}{dt} = k_1[R][L] - k_2[RL] \quad \Leftrightarrow \quad \frac{d[RL]}{dt} = k_1[R][L] - (k_1[L] + k_2)[RL]
\]  

(Eq. 3)

This gives then the following,

\[
[RL](t) = a e^{-k_2[L]t} + \frac{k_1[L]}{k_1[L] + k_2} [R]
\]  

(Eq. 4)

where $a$ represents the FRET amplitude.

Each experimental binding trace (Fig. 5A) recorded under pseudo-first-order conditions ($[L_p] \geq 10 \times [R_p]$) was fitted separately by replacing Equation 4 into Equation 2. TR1-NKA association and dissociation rate constants $k_1$ and $k_2$, expressed as means ± S.D. of the family of association curves, were found to be equal to $k_1 = 0.8 \pm 0.1 \times 10^8$ M$^{-1}$ s$^{-1}$ and $k_2 = 0.10 \pm 0.01$ s$^{-1}$. The fluorescence intensities relative to the unliganded or the liganded receptor state are $I_0 = 0.93 \pm 0.04$ and $I_1 = 0.75 \pm 0.05$, respectively. The amplitude of the FRET signal increases with ligand concentration up to a plateau value above 300 nM TR1-NKA. The plot of the amplitude of FRET as a function of TR1-NKA concentration (saturation curve) can be fitted with the empirical Hill equation to yield a dissociation constant $K_D = 48 \pm 12$ nM with a Hill coefficient of $1.1 \pm 0.3$ (Fig. 5B).

**TR1-NKA Dissociation**—Dissociation of receptor-ligand complexes obtained at equilibrium with 100 nM TR1-NKA was initiated by rapid mixing with an excess (20 μM) of SR48968 (Fig. 5C). The dissociation relaxation is best represented by a sum of two exponentials (see “Experimental Procedures”) revealing a rapid process representing 50% of the FRET amplitude with a dissociation rate constant $k_{off,a} = 0.30 \pm 0.02$ s$^{-1}$ and a slow dissociation step with a rate constant $k_{off,c} = 0.040 \pm 0.001$ s$^{-1}$. This suggests that TR1-NKA, at equilibrium, is bound to two states of the Y-NK2mut receptor.

**Quantitative Analysis of the Kinetics of the Y-NK2wt Receptor/TR1-NKA Interactions**

Kinetics of TR1-NKA association to the Y-NK2wt receptor were analyzed as previously described (19) according to a bimolecular interaction followed by a rate-limiting isomerization step.

\[ R + L \rightleftharpoons RL \rightleftharpoons R^*L \]

**Scheme 2**

Since the values of $k_2$ and $k_3$ are close to each other (19), we derived an equation for the full scheme rather than two equations describing binding and interconversion separately (19). With $I_0$, $I_1$, and $I_2$, the fluorescence intensities associated with $R$, $RL$, and $R^*L$, respectively, it follows that the measured fluorescence intensity $I$ can be expressed by the following.

\[ I = I_0 + (I_1 - I_0) \frac{[RL]}{[R]} + (I_2 - I_0) \frac{[R^*L]}{[R]} \quad \Leftrightarrow \quad I = I_0 \]

(Eq. 5)

Under pseudo-first-order conditions ($[L] \rightarrow [L_7]$), the concentrations of RL and R*L are deduced from the following differential equations, assuming that at time 0, $[R] = [R_p]$, $[RL] = [R^*L] = 0$,

\[ \frac{d[R]}{dt} = -a[R] + b[R^*L] + \gamma \]

(Eq. 6)

\[ \frac{d[R^*L]}{dt} = a[R] - b[R^*L] \]

(Eq. 7)
with \( \alpha = k_2 + k_3 + (k_3[L_T]) \), \( \alpha' = k_3 \), \( \beta = k_4 - (k_3[L_T]) \), \( \beta' = k_4 \), and \( \gamma = k_1[R_p][L_T] \).

Solution of Equations 6 and 7 yields the following biphasic decay kinetics,

\[
[R_L(t)] = c e^{\alpha t} + c e^{\beta t} + \frac{\beta' \gamma}{\alpha \beta - \alpha' \beta} e^{\beta t} - \frac{\beta' \gamma}{\alpha \beta - \alpha' \beta} e^{\beta t} \quad \text{(Eq. 8)}
\]

\[
[R^*L(t)] = \left( \frac{\alpha + \lambda_3}{\beta} \right) c e^{\alpha t} + \left( \frac{\alpha + \lambda_3}{\beta} \right) c e^{\beta t} + \frac{\alpha' \gamma}{\alpha \beta - \alpha' \beta} e^{\beta t} - \frac{\alpha' \gamma}{\alpha \beta - \alpha' \beta} e^{\beta t} \quad \text{(Eq. 9)}
\]

with

\[
\lambda_{1,2} = -\left( \alpha + \beta' \right) \pm \sqrt{\left( \alpha + \beta' \right)^2 + 4 \alpha' \beta}.
\]

\[
c_1 = \frac{\alpha' \beta' \gamma (\alpha + \lambda_3) - \alpha' \beta \gamma (\alpha + \lambda_3)}{(\alpha \beta - \alpha' \beta)(\lambda_2 - \lambda_3)}
\]

\[
c_2 = \frac{\beta' \gamma (\alpha + \lambda_3) - \alpha' \beta \gamma (\alpha + \lambda_3)}{(\alpha \beta - \alpha' \beta)(\lambda_2 - \lambda_3)}
\]

The experimental binding traces (Fig. 6A) recorded under pseudofirst-order conditions ([L_T] \( \approx 10 \times [R_p] \)) were fitted by substituting Equations 8 and 9 into Equation 5. After fitting each curve separately, the rate constants were determined as means ± S.D. for the family of association curves. The final results are \( k_1 = 1.82 \pm 0.02 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \), \( k_2 = 0.12 \pm 0.04 \text{ s}^{-1} \), \( k_3 = 3.96 \pm 0.04 \times 10^{-2} \text{ s}^{-1} \), and \( k_4 = 6 \pm 4 \times 10^{-3} \text{ s}^{-1} \), where \( k_1 \) and \( k_2 \) are, respectively, the association and the dissociation rate constants for the formation of the RL complex, and \( k_3 \) and \( k_4 \) are the forward and the backward rate constants, respectively, of the equilibrium between RL and R^*L. The relative fluorescence intensities are \( I_1 = 0.58 \pm 0.06 \) for RL and \( I_2 = 0.60 \pm 0.01 \) for R^*L. In a next step, the theoretical receptor site occupancies for R and R^* calculated from Equations 8 and 9 using the above rate constant values were plotted as a function of TR1-NKA concentration (Fig. 6B). This plot can be fitted with the empirical Hill equation to yield for R a dissociation constant equal to 500 ± 90 nM with a Hill coefficient of 1.0 ± 0.1 and for R^* a dissociation constant equal to 1.020 ± 0.044 nM, with a Hill coefficient of 1.000 ± 0.003.

TR1-NKA Dissociation—Dissociation traces obtained by rapid mixing of 20 \( \mu \text{M} \) SR48968 with cells expressing the Y-NK2wt receptor preincubated for 10 min with 100 nM TR1-NKA can be fitted by a two-exponential function (see “Experimental Procedures”) comprising a slow phase with a rate constant \( k_{off b} = 0.0055 \pm 0.0002 \text{ s}^{-1} \) and a rapid phase with a rate constant \( k_{off c} = 0.03 \pm 0.01 \text{ s}^{-1} \). At 10 min, the proportion of the rapid to slow dissociation steps is 22/78% (Fig. 6C).

In summary, there is no detectable slow relaxation phase of TR1-NKA binding to the Y-NK2mut receptor in contrast to its binding to the Y-NK2wt receptor. This correlates with a lack of a cAMP response. Therefore, the data strengthen the previous interpretation (19) that the slow binding phase represents binding to an active state R2, which induces cAMP production. We next analyzed the binding properties of other agonists to the Y-NK2mut receptor.

**Binding Properties and Responses of TRC4-NKA, an Agonist with Similar Affinities for the Y-NK2wt and the Y-NK2mut Receptors**

Continuous Monitoring and Equilibrium Measurements of TRC4-NKA Association—Other fluorescently labeled analogs of NKA, which carry the TR fluorophore at different positions on the peptide, were screened by FRET at 100 nM by placing the cells in suspension in the 1-ml cuvette maintained at 21 °C in the spectrofluorometer. Among the ligand tested, one peptide, TRC4-NKA, bound to both receptor types with sim-
ilar binding kinetics as opposed to TR1-NKA (Fig. 7A). This agonist carries the TR on a cysteine that substitutes for an aspartate at position 4. At all TRC4-NKA concentrations tested (Fig. 7B, illustration with 1, 10, and 100 nM), the amplitudes of FRET upon binding to the Y-NK2wt and the Y-NK2mut receptors were identical, indicating that TRC4-NKA has the same affinity for both receptors. This was confirmed by competition experiments against [3H]SR48968 giving $K_i$ values of 40 ± 10 and 70 ± 15 nM for the Y-NK2wt and the Y-NK2mut receptors, respectively (Fig. 7C). TRC4-NKA is therefore a ligand that, surprisingly, seems to be unable to discriminate between the mutant and the wild-type receptors in terms of binding properties.

Responses at 21°C—Interestingly, the decrease of EYFP fluorescence due to FRET upon binding of TRC4-NKA to both receptors was fast and reached equilibrium rapidly (Fig. 7A). This suggested that TRC4-NKA might not stabilize the slowly equilibrating R2 state of the NK2 receptor and prompted us to test the responses associated with its binding. TRC4-NKA behaved like a partial agonist as compared with TR1-NKA; TRC4-NKA binding to the Y-NK2wt receptor triggered 75% maximal calcium responses as compared with TR1-NKA binding. The maximal calcium response following TRC4-NKA binding to the Y-NK2mut receptor was only 15% compared with TR1-NKA binding to the Y-NK2wt receptor (Fig. 7D). TRC4-NKA binding to either of the Y-NK2 receptors did not induce any cAMP production (Fig. 7E).

All kinetic analyses described above were performed at 21°C to avoid interference with receptor endocytosis. Hence, all physiological responses were recorded at 21°C as well. However, at this temperature, the Y-NK2mu receptor’s calcium responses to the agonists were reanalyzed at 37°C. Calcium Response at 37°C—As compared with calcium responses recorded at 21°C (Figs. 2, B and E, and 7D), the three agonists elicited larger calcium responses at 37°C (Fig. 8, A–C). Nevertheless, the Y-NK2mut receptor still displayed...
lower maximal calcium responses than the Y-NK2wt receptor. Concomitantly, the EC_{50} values for both receptor types were lower at 37°C than at 21°C. In particular, for the Y-NK2wt receptor, EC_{50} values decreased from 2.2 ± 0.5 to 0.5 ± 0.1 nM for NKA and from 0.6 ± 0.1 to 0.18 ± 0.07 nM for TR1-NKA. For the Y-NK2mut receptor, EC_{50} values decreased from 3.4 ± 0.2 µM to 910 ± 260 nM for NKA and from 180 ± 30 to 130 ± 20 nM for TR1-NKA. At 37°C, TRC4-NKA activated the Y-NK2wt receptor with an EC_{50} of 14 ± 4 nM and the Y-NK2mut receptor with an EC_{50} of 47 ± 10 nM.

cAMP Response at 37°C—NKA and TR1-NKA triggered the synthesis of 3 times more cAMP at 37°C than at 21°C on the Y-NK2wt receptor (Fig. 8D compared with Fig. 2, C and F). Despite the elevation of temperature, NKA and TR1-NKA still failed to evoke any significant cAMP response on the Y-NK2mut receptor (Fig. 8E). Similarly, TRC4-NKA was unable to stimulate any cAMP production mediated by the two receptor types.

Fast kinetic measurement of TRC4-NKA/receptor interactions were carried out next.

Quantitative Analysis of the Kinetics of the Y-NK2wt and the Y-NK2mut Receptor Interactions with TRC4-NKA

TRC4-NKA Association—The binding traces of TRC4-NKA to the Y-NK2mut receptor can be analyzed as single pseudo-first-order relaxations according to a bimolecular interaction best fitted by replacing Equation 4 into Equation 2 derived from Scheme 1 (Fig. 9A). The time courses of TRC4-NKA binding to the Y-NK2mut and to the Y-NK2wt receptors were identical. This is illustrated in Fig. 9B with 100 nM TRC4-NKA binding to the two receptors. For both receptors, fitting of the family of association curves allows determination of the association and dissociation rate constants k_{a1} = 1.0 ± 0.20 × 10^{6} M^{-1} s^{-1} and k_{a2} = 0.067 ± 0.006 s^{-1} and of the relative fluorescence intensities for ligand-free and ligand-bound receptor states, I_{0} = 0.92 ± 0.07 and I_{1} = 0.67 ± 0.05. The amplitude of FRET signal increases with ligand concentration up to a plateau value above 200 nM TRC4-NKA. The plot of the amplitude of FRET as a function of TRC4-NKA concentration (saturation curves) can be fitted with the empirical Hill equation to yield a dissociation constant of K_{D} = 20 ± 3 nM with a Hill coefficient of 1.45 ± 0.45 (Fig. 9C).

TRC4-NKA Dissociation—Dissociation of receptor-ligand complexes, obtained at equilibrium with 100 nM TRC4-NKA and initiated by rapid mixing with 20 µM SR48968 (Fig. 9D), is best fitted with a sum of two exponentials (see "Experimental Procedures"). The rapid process with a rate constant of k_{off,b} = 0.480 ± 0.001 s^{-1} represents 25% of the FRET amplitude versus 75% for the slow dissociation step (k_{off,c} = 0.0420 ± 0.0001 s^{-1} for Y-NK2mut and k_{off,c} = 0.0510 ± 0.0001 s^{-1} for Y-NK2wt).

Summary of the Data Used in the Kinetic Modeling of the Y-NK2wt and Y-NK2mut Receptors

The absence of agonist binding to the cAMP-responsive R2 state in the mutant receptor might be explained by two distinct phenotypes: a “binding defect phenotype” or an “isomerization defect phenotype” as described in Ref. 32 and Fig. 3. The binding defect phenotype results from mutations that selectively alter the intrinsic binding affinities (K) of individual
conformational states for ligands. The isomerization defect phenotype results from mutations that selectively alter the equilibrium constant ($K_{\text{h9003}}$) between two given interconvertible conformations of the receptor. In order to discriminate between the two plausible effects of the mutations, we have listed all the binding parameters determined experimentally (Table I) and used a kinetic model of the NK2 receptor preexisting in equilibrium between three states, the inactive R0 state, the calcium-triggering R1 state, and the cAMP-producing R2 state to simulate the binding traces of the two agonists to each receptor. The physiological response constraints that we took into account in the modeling were the following: (i) TRC4-NKA binding to either of the Y-NK2 receptors triggers no cAMP response; (ii) the Y-NK2mut receptor does not signal via cAMP synthesis; and (iii) TRC4-NKA binding to the Y-NK2mut receptor is similar to its binding to the Y-NK2wt receptor but gives lower calcium signaling.

Kinetic Modeling of Agonist Binding to the Y-NK2wt Receptor and Responses

With the aim of determining the parameters of ligand binding and receptor state isomerization corresponding to the three-state model described in the legend to Fig. 3, the experimental kinetic parameters (Table I) were tentatively assigned to resting (R0), calcium-coupled (R1), or cAMP-coupled (R2) states using the STOIC program (30). This program generates binding traces and determines the fractional concentrations of each receptor state as a function of time. We first modeled the parameters of the Y-NK2wt receptor, which simultaneously fit the experimental interaction curves of TR1-NKA and TRC4-NKA (Fig. 10, A and B, respectively) and the proportions of inactive and active states as a function of time (Fig. 10, C and D). The set of parameters given in Table II and Fig. 10E corresponds to the combination yielding the best correlation between agonist binding and biological activity. The intrinsic isomerization constant between R0 and R1 is $K_{\text{h9003}} = [R0]/[R1] = 6.7$ and between R1 and R2 is $K_{\text{h11005}} = [R1]/[R2] = 0.63$. The intrinsic affinities $K_{\text{h9003}} = (k_1/k_{\text{off}})$ of Y-NK2mut receptor for each Y-NK2wt receptor state are 376, 16, and 3 nM, respectively (Table II). These isomerization constants and intrinsic affinity values are similar to those determined previously for EGFP-NK2wt (19). TRC4-NKA binding is best simulated when it stabilizes at equilibrium mainly the inactive R0 state and, to a lesser extent, the calcium-triggering R1 state (intrinsic affinity $K_{\text{h9003}} = 54$ nM, $K_{\text{h11005}} = 36$ nM, and $K_{\text{h21032}} = 450$ nM (Fig. 10, E), italic values, and D, red and green lines, respectively). This is in agreement with the partial agonism observed experimentally at 21°C for the calcium re-
sponse of TRC4-NKA as compared with TR1-NKA (Fig. 7D versus Fig. 2E).

Kinetic Modelings of Agonist Binding to the Y-NK2mut Receptor and Responses

Fitting of the Experimental Binding Traces—In order to model a binding defect phenotype, the intrinsic receptor equilibrium constants between states were kept unchanged compared with those simulated for the Y-NK2wt receptor ($10^6 = 6.7$ and $10^6 = 0.63$), and different affinities for the ligands were tested. The parameters used to simulate the best fit for TR1-NKA (Fig. 11A) and for TRC4-NKA (Fig. 11B) are summarized in Table II and Fig. 11E. They were obtained using the association rate constant $k_1$ determined experimentally and by varying the dissociation rate constant $k_{off}$ of each receptor state. It was possible to fit the binding traces of TR1-NKA and TRC4-NKA to the Y-NK2mut receptor (Fig. 11, A and B, respectively) using $k_{off}$ values similar to those measured in the ligand dissociation experiments at equilibrium (compare Tables I and II).

In order to model an isomerization defect phenotype, the intrinsic affinities of TR1-NKA and of TRC4-NKA for the three states of the mutant receptor were chosen to be very similar to the ones described for the wild-type receptor (Table II and Fig. 12E). However, calcium responses triggered by TR1-NKA as well as TRC4-NKA binding to the Y-NK2mut receptor are best described by the isomerization defect phenotype. Indeed, TR1-NKA, experimentally, triggers less calcium signaling upon activating the Y-NK2mut receptor than upon activating the Y-NK2wt receptor. In the isomerization defect model, less of the Y-NK2mut R1 state than of the Y-NK2wt R1 state is indeed occupied at a saturating concentration of TR1-NKA (Fig. 12C compared with Fig. 10C, green lines), whereas the contrary is observed in the binding defect model (Fig. 11C compared with Fig. 10C, green lines). Experimentally, TRC4-NKA also triggers lower calcium responses on the Y-NK2mut than on the Y-NK2wt receptor. In the isomerization defect model, TRC4-NKA, despite the same intrinsic affinities for the wild-type and the mutant NK2, stabilizes, indeed, less Y-NK2mut R1 states than Y-NK2wt R1 states at a saturating concentration of ligand (Fig. 12D compared with Fig. 10D, green lines), whereas in the binding defect model, TRC4-NKA behaves like an inverse agonist stabilizing only the R0 state, thus producing no calcium at all (Fig. 11D, red line).

DISCUSSION

In the present study, two fluorescently labeled NK2 receptor types, the wild-type Y-NK2wt receptor and the mutant

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**Table I**

Summary of experimental rate constants determined from fluorescence experiments

| Y-NK2wt | Kinetic step | Value of the rate constant | Determination |
|---------|-------------|----------------------------|--------------|
| TR1-NKA | $k_1$ | $k_1 = 1.82 \pm 0.02 \times 10^7$ $\text{s}^{-1}$ | Fitting TR1-NKA association kinetics with eq.(5) |
|         | $k_2$ | $k_2 = 0.12 \pm 0.04$ $\text{s}^{-1}$ | |
|         | $k_3$ | $k_3 = 0.0396 \pm 0.0004$ $\text{s}^{-1}$ | |
|         | $k_4$ | $k_4 = 0.0006 \pm 0.0004$ $\text{s}^{-1}$ | |
|         | $k_{off}$ | $k_{off} = 0.03 \pm 0.01$ $\text{s}^{-1}$ | Fitting TR1-NKA dissociation kinetics at equilibrium |
|         | $k_{off}$ | $k_{off} = 0.0005 \pm 0.0002$ $\text{s}^{-1}$ | |

| Y-NK2mut | Kinetic step | Value of the rate constant | Determination |
|---------|-------------|----------------------------|--------------|
| TR1-NKA | $k_1$ | $k_1 = 0.8 \pm 0.1 \times 10^6$ $\text{s}^{-1}$ | Fitting TR1-NKA association kinetics with eq.(2) |
|         | $k_2$ | $k_2 = 0.10 \pm 0.01$ $\text{s}^{-1}$ | |
|         | $k_{off}$ | $k_{off} = 0.30 \pm 0.02$ $\text{s}^{-1}$ | Fitting TR1-NKA dissociation kinetics at equilibrium |
|         | $k_{off}$ | $k_{off} = 0.040 \pm 0.001$ $\text{s}^{-1}$ | |
| TRC4-NKA | $k_1$ | $k_1 = 1.01 \pm 0.22 \times 10^6$ $\text{s}^{-1}$ | Fitting TRC4-NKA association kinetics with eq.(2) |
|         | $k_2$ | $k_2 = 0.067 \pm 0.006$ $\text{s}^{-1}$ | |
|         | $k_{off}$ | $k_{off} = 0.480 \pm 0.002$ $\text{s}^{-1}$ | Fitting TRC4-NKA dissociation kinetics at equilibrium |
|         | $k_{off}$ | $k_{off} = 0.0420 \pm 0.0001$ $\text{s}^{-1}$ | |
Y-NK2mut, have been first compared with respect to their interaction with the fluorescent agonist neurokinin A, TR1-NKA. As such, it is a complementary analysis supporting the prior estimates of the TR1-NKA intrinsic affinities for the NK2 receptor, together with TR7-NKA-(4–10), which does not trigger cAMP response upon binding to the wild-type NK2 receptor.

To get more insight into the phenotype of the mutant, our kinetic model for activation of the Y-NK2wt receptor was used as a starting point. TR1-NKA binding kinetics on the Y-NK2mut receptor and subsequent physiological responses are better fitted with an isomerization defect rather than with an affinity defect phenotype. The study of TRC4-NKA binding kinetics gave important corroborating data; TRC4-NKA was found to interact with the same apparent affinity on the mutant or the wild-type NK2 receptor and with the same kinetics. This could be explained by the proposal of Labrou et al. (24), who have predicted from their model of NKA interaction with the NK2 receptor that the Asp$^9$ of NKA would interact directly with Thr$^{24}$ and Phe$^{26}$ of the receptor. However, our analysis leads us to a different conclusion. First, despite exhibiting the same affinities for the wild-type and mutant receptors, TRC4-NKA promotes less calcium signaling from the Y-NK2mut receptor. Second, the association kinetics of TRC4-NKA with the Y-NK2mut receptor and the subsequent physiological responses are best fit with an isomerization defect model. Finally, the isomerization defect model for TRC4-NKA binding to the Y-NK2mut receptor was developed using the same equilibrium constants between states in the absence of ligand than the one used in the isomerization defect model for TR1-NKA binding; $\Gamma_0 = 27$ and $\Gamma_0 = 6$. Consequently, it is possible to describe the phenotype of the Y-NK2mut receptor as an isomerization defect mutant using the two agonists. The Y-NK2mut receptor could be called “constitutively inactive” mutant, since, in the absence of ligand, more than 95% of the receptor is in the R0 state as opposed to 70% of the Y-NK2wt receptor.

The Cysteine 4 Substitution in the NK2 Peptide—The cysteine substitution of Asp$^9$ of the NKA analogs (Tyr$^{-}$NKA and Tyr$^{9}$-NKA) was described to reduce 23-fold the affinity for the human NK2 receptor compared with NKA (33). By competition experiments with $[^{3}H]$SR48968, we have determined a 9-fold loss of affinity of TRC4-NKA for the Y-NK2wt receptor compared with NKA or TR1-NKA. TRC4-NKA behaves as a partial agonist of calcium responses on the Y-NK2wt receptor. As a new finding, TRC4-NKA is the second ligand identified, together with TR7-NKA-(4–10), which does not trigger cAMP synthesis. The expression of Y-NK2wt receptors, with Thr24 and Phe26 have previously been proposed to be essential for the high affinity binding of the neuropeptide NKA by two independent groups (23, 24). Huang et al. (23) argued that Thr$^{24}$ and Phe$^{26}$ have no influence on the activation mechanism. We have now clearly established that activation of the Y-NK2mut receptor by the tested agonists is, in fact, affected, since this receptor is unable to trigger cAMP synthesis and that, overall, it does not trigger as much maximal calcium response as the Y-NK2wt receptor.

Fig. 10. Three-state model simulating the experimental binding kinetics of TR1-NKA and TRC4-NKA to the Y-NK2wt receptor. Simulations were carried out using experimentally defined data given in Table I. A and B, simulated kinetics of ligand association as compared with experimental data. Derived kinetic parameters were estimated by manual fitting of TR1-NKA (A) and TRC4-NKA (B) interaction traces in black with the simulation in blue. C and D, simulated kinetics of the proportions of each receptor states. The parameters best fitting the binding data (Table II) were further tested for the corresponding simulated proportions of receptor states (with and without 300 nM ligand (solid and dotted lines, respectively)) as a function of time (red, R0; green, R1; blue, R2) for TR1-NKA (C) and for TRC4-NKA (D).

E, summary of the parameters describing TR1-NKA (underlined) and TRC4-NKA (italic type) binding to the Y-NK2wt receptor states. $\Gamma_0$, the isomerization constants of the ligand-bound states. $\Gamma_0$ values are identical to the previously published ones of the EGFP-NK2 construct (the intrinsic rate for the R0-R1 transition is 4.5 s$^{-1}$; the rate for the R1-R0 transition is 30 s$^{-1}$, the rate for the R1-R2 transition is 0.042 s$^{-1}$, and the rate for the R2-R1 transition is 0.025 s$^{-1}$). and K2 = 3 nM/4 nM) and of the receptor equilibrium constants between states in the absence of ligand: $\Gamma_0 = 6.7$ and $\Gamma_0 = 0.6$. As discussed in our previous work (19), interconversion of the R0 state toward the R1 state occurs at a rate of 4.5 s$^{-1}$ in the Y-NK2wt receptor and of 2.25 s$^{-1}$ in the Y-NK2mut receptor (present work). These rates are faster than the observed rates of agonist binding (experimentally determined $K_{app}(k_1L + k_2)$ values below 2 s$^{-1}$). Therefore, binding to R0 or to R1 could not be kinetically resolved.

Conformational Mutation of NK2 Neurokinin Receptor

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model explain the variation of apparent affinities of the mutant receptor for NKA, TR1-NKA, and TRC4-NKA; the more partial the agonist is for the Y-NK2wt receptor, the better overall affinity it has for the Y-NK2mut receptor and the less calcium it triggers.

**Putative Amino Acids of the Amino-terminal Domain of Neurokinin Receptors Implicated in the Interaction with NKA and Substance P**—Photoaffinity labeling is a useful complementary tool in identifying structural domains of receptors involved in ligand binding. N-Biotin-[Tyr1-Cys9]NKA and N-biotin-[Tyr1-Cys10]NKA were used to demonstrate the interaction of NKA with Met297 of the NK2R receptor (24). But no other report of NKA interaction with NK2R exists. Studies of the interaction of Substance P (SP), a neurokinin that shares a common carboxyl-terminal sequence with NKA (Phe-X-Gly-Leu-Met), with its main receptor NK1, have given more insight into neurokinin interaction with the amino-terminal domain of their receptors. Alanine substitutions of Asn24 and Phe26 in the NK1 receptor, residues equivalent to Thr24 and Phe26 of the receptor NK2, lower the apparent affinity of SP (34, 35). However, weighing against a direct interaction of Asn24 and Phe26 with SP are photoaffinity labeling experiments using 125I-D-[Tyr0-Bpa3]SP. This photoprobe was first shown to covalently attach to a segment extending from residues 1 to 21 of the NK1 receptor (36). Later, the segment was further restricted to residues 11–21 (37).

Our conclusion is that Thr24 and Phe26 of the NK2 receptor are not likely to take part in a direct interaction with NKA. More importantly, in our model, the substitutions do not appear to modify the intrinsic affinities of each NK2 receptor state for the two agonists, TR1-NKA and TRC4-NKA, arguing that there is no abolition of other critical contact sites between the receptor and the neuropeptide. Thr24 and Phe26 could rather affect the interaction of the amino-terminal domain of the receptor with the seven-transmembrane core of the protein, thereby interfering with the conformational transition required for activation. This would explain why Huang et al. (23) observed an apparent abolition of the high affinity state of the NK2 receptor not only for NKA but also for SP and the neurokinin B.

### Maximal Amplitudes of FRET

The maximal amplitude of energy transfer is reached when all of the fluorescent receptors at the cell surface are occupied by the fluorescent ligand. With the total absence of a slow relaxation phase, the second most striking feature of TR1-NKA binding to the Y-NK2mut receptor, as opposed to the Y-NK2wt receptor, is the diminution of the maximal amplitude of FRET, 15–20% versus 40–50% of fluorescence, respectively.

We do not think that the diminution of energy transfer amplitude is due to a partial occupancy of the cell surface Y-NK2mut receptor by TR1-NKA, since (i) in the various model attempts that we have performed, no set of fitted data could account for TR1-NKA occupying only a fraction of the Y-NK2mut receptors at equilibrium and (ii) specific [3H]SR48968 binding to the Y-NK2wt and Y-NK2mut receptors was fully displaced by NKA and TRC4-NKA and almost fully displaced by TR1-NKA (due to concentration limitation). We do not think either that the diminution of energy transfer ampli-

### Table II

**Summary of the values used in the modeling**

R (0–1-2) indicates R0, R1, and R2. $k_{off}$ values similar to the experimentally determined ones are denoted with an asterisk. Y-NK2mut values changed and compared with those determined for the Y-NK2wt, in order to model one of the phenotypes, are in boldface type and underlined.

| R0-TR1-NKA → R0 + TR1-NKA | R1-TR1-NKA → R1 + TR1-NKA | R2-TR1-NKA → R2 + TR1-NKA | R0-TRC4-NKA → R0 + TRC4-NKA | R1-TRC4-NKA → R1 + TRC4-NKA | R2-TRC4-NKA → R2 + TRC4-NKA |
|----------------------------|----------------------------|----------------------------|----------------------------|----------------------------|----------------------------|
| koff0 0.06* (K0 = 54 nM)   | koff1 0.04* (K1 = 36 nM)   | koff2 0.05* (K2 = 446 nM)  | koff0 0.06* (K0 = 54 nM)   | koff1 0.04* (K1 = 36 nM)   | koff2 0.05* (K2 = 446 nM)  |

| R0-TR1-NKA → R1 0-TR1-NKA | R1-TR1-NKA → R1 0-TR1-NKA | R2-TR1-NKA → R2 0-TR1-NKA |
|----------------------------|----------------------------|----------------------------|
| 0.63                       | 0.63                       | 0.63                       |

| R0-TRC4-NKA → R1 0-TRC4-NKA | R1-TRC4-NKA → R1 0-TRC4-NKA | R2-TRC4-NKA → R2 0-TRC4-NKA |
|----------------------------|----------------------------|----------------------------|
| k1 = 1.86 x 10^6 M^-1 s^-1 | k1 = 1.12 x 10^6 M^-1 s^-1 | k1 = 1.12 x 10^6 M^-1 s^-1 |

| Y-NK2wt | Binding-defect Phenotype | Isomerization-defect Phenotype |
|---------|--------------------------|-------------------------------|
| 6.7     | 6.7                      | 27                            |

| $\Gamma_0$ | R0 ↔ R1 | $\Gamma_0$ | R0 ↔ R1 |
|------------|---------|------------|---------|
| 0.63       | 0.63    | 0.63       | 0.63    |

$\text{R} (0–1-2)$ indicates $\text{R}_0$, $\text{R}_1$, and $\text{R}_2$. $k_{\text{off}}$ values similar to the experimentally determined ones are denoted with an asterisk. Y-NK2mut values changed and compared with those determined for the Y-NK2wt, in order to model one of the phenotypes, are in boldface type and underlined.
Fig. 11. Three-state model simulating a binding defect phenotype of the Y-NK2*mut receptor. In this model, the same isomerization constants for the wild-type and the mutant receptor were used (Table II). A and B, simulated kinetics of ligand association as compared with experimental data. Manual fitting of the interaction traces (black) with the simulation (blue) of TR1-NKA (A) or TRC4-NKA (B) binding to the Y-NK2*mut receptor. C and D, simulated kinetics of the proportions of each receptor states. The parameters fitting best the binding data were further tested for the corresponding simulated proportions of receptor states (with and without 300 nm ligand (solid and dotted lines, respectively) as a function of time (red, R0; green, R1; blue, R2) for TR1-NKA (C) and for TRC4-NKA (D). E, summary of the parameters describing TR1-NKA (underlined) and TRC4-NKA (italic type) binding to the Y-NK2*mut receptor states (Table II).

mplitude is due to an excess of fluorescent mutant receptors localized in intracellular compartments, which might be inaccessible to the extracellular ligand but which could contribute to the total fluorescence measured. From co-localization experiments of Y-NK2*wt and Y-NK2*mut receptors with the endosomal markers Texas Red transferrin and Rab5 in the absence of ligand, the intracellular localization of the Y-NK2*mut receptor, principally in the endocytic pathway, was not more pronounced than for Y-NK2*wt receptors (data not shown). In addition, there is the same diminution of total fluorescence for cells expressing either Y-NK2*wt or Y-NK2*mut receptors, upon incubation with saturating TRC4-NKA concentrations, suggesting that comparable amounts of receptors are present at the cell surface in the two cell lines.

Alternatively, since FRET is dependent upon the distance between the donor/acceptor pair of fluorophores, the distance between EYFP and the Texas Red could be smaller in the TR1-NKA-Y-NK2*wt than in the TR1-NKA-Y-NK2*mut complexes, whereas this distance would be identical in the TRC4-NKA-Y-NK2*wt and the TRC4-NKA-Y-NK2*mut complexes. If this is the case, the following two possibilities arise. (i) TRC4-NKA, which at equilibrium stabilizes mainly R0 and weakly R1 states, gives a maximal amplitude of FRET of 25–30% on cells expressing either the wild-type or the mutant receptor. TR1-NKA stabilizes only the R0 and R1 forms of the Y-NK2*wt receptor and gives a maximal efficiency of 15–20%, whereas it stabilizes mainly the R2 state of the Y-NK2*wt receptor, giving

Fig. 12. Three-state model simulating an isomerization defect phenotype of the Y-NK2*mut receptor. In this second model, the isomerization constants between the states have been changed, but the affinities of each ligand for the mutant and the wild-type receptor are similar (Table II). The legend is as described in the legend to Fig. 11. The intrinsic rate for the R0-R1 transition is $2.25 \text{s}^{-1}$, the rate for the R1-R0 transition is $60 \text{s}^{-1}$, the rate for the R1-R2 transition is $0.0004 \text{s}^{-1}$, and the rate for the R2-R1 transition is $0.0025 \text{s}^{-1}$ (Table II).

45–50% of FRET at the maximum. Taken together, these data suggest that the distance separating the EYFP and the Texas Red is higher in the R0 and R1 states (giving a maximum of 20–30% of FRET) than in the R2 state (giving a maximum of 45–50% of FRET). In a photolabeling affinity study of NK1 with SP, Pellegrini et al. (38) have indeed discussed the possibility that the amino-terminal domain of NK1 might fold over the central core of the receptor, inhibiting the dissociation of SP, thereby producing the high affinity state. (ii) Knowing that the defect of the Y-NK2*mut receptor is at the level of the transition between states rather than in the affinity for ligand, one may propose an alternative explanation: the substitutions Thr$^{24} \rightarrow$ Ala and Phe$^{26} \rightarrow$ Ala could affect the structure of the amino-terminal domain of the Y-NK2*mut receptor such that it changes not only the isomerization constants of the receptor but that the mean EYFP position in the structure is changed as well. In addition, TR1-NKA and TRC4-NKA would be bound to the Y-NK2*mut receptor in such a way that the distance between EYFP and Texas Red would be affected when the fluorophore is on the first amino acid, but not when it is on the fourth amino acid of the neuropeptide. It would be interesting to test this hypothesis on a three-dimensional structural model of NK1 bound to the NK2 receptor.

Implications for NKA Interactions with the NK1 Receptor—SP interacts preferentially with the NK1 receptor, but NKA displays high affinity binding for both NK1 and NK2 receptors (39). Based on homologous binding experiments, it was proposed that the NK1 receptor might exist in two different forms, with similar affinities for SP but with high and low affinities for NKA (40). On the other hand, upon activating the NK1 receptor, SP can trigger both calcium and cAMP synthesis, but NKA triggers mainly calcium signaling. Finally, a mutant of the NK1 receptor, carrying a cysteine substitution of
Gly\textsuperscript{166}, eliminates the discrepancy between the low and the high binding constant of NKA (40). It would be interesting, therefore, to test by FRET the kinetics of interactions of TR-NKA with fluorescent wild-type or mutant NK1(Gly\textsuperscript{166} → Cys) receptors and of a Texas Red-modified SP with the fluorescent NK1(Asn\textsuperscript{18} → Ala, Phe\textsuperscript{26} → Ala) receptor.

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