Topological analysis of the complex formed between neurokinin A and the NK2 tachykinin receptor

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Abstract
Neurokinin A stimulates physiological responses in the peripheral and central nervous systems upon interacting primarily with the tachykinin NK2 receptor (NK2R). In this study, the structure of NKA bound to the NK2R is characterised by use of fluorescence resonance energy transfer. Four fluorescent NKA analogues with Texas red introduced at amino acid positions 1, 4, 7 and 10 were prepared. When bound to a NK2R carrying enhanced green fluorescent protein at the N-terminus, all peptides reduce green fluorescent protein fluorescence from 10% to 50% due to energy transfer. The derived donor-acceptor distances are 46, 55, 59 and 69 Å for the fluorophore linked to positions 1–10, respectively. The monotonic increase in distance clearly indicates that the peptide adopts an extended structure when bound to its receptor. The present data are used, in combination with rhodopsin structure, fluorescence studies, photoaffinity labelling and site-directed mutagenesis data to design a computer model of the NKA-NK2R complex. We propose that the N-terminus of NKA is exposed and accessible to the extracellular medium. Subsequent amino acids of the NKA peptide become progressively more buried residues up to approximately one-third of the transmembrane-spanning domain.

Keywords: fluorescence resonance energy transfer, G protein coupled receptor structure, peptide conformation, pharmacology


Neurokinin A (NKA) also known as substance K is a decapeptide found in the central and peripheral nervous system (Sternini et al. 1989; Debeljuk et al. 1990). It belongs to the tachykinin peptide family. This widespread group of neuropeptides, found both in invertebrates and vertebrates, also includes substance P (SP) and neurokinin B. All tachykinins share a highly conserved amidated carboxyterminal sequence Phe-X-Gly-Leu-Met-NH₂ (Zhang et al. 2000).

NKA is known to act through the G protein-coupled neurokinin receptors. The NK2 receptor (NK2R) is considered as the endogenous receptor for NKA. There is however a high level of promiscuity among tachykinins and their receptors. Indeed, NKA also binds to the NK3 receptor and acts as a high affinity agonist of NK1 receptor through which it may mediate some of its central effects (Beaujouan et al. 2000).

The NK2R (Sasai and Nakamichi 1989) is mostly expressed in the peripheral nervous system where it is involved in regulation of gastrointestinal and respiratory systems (Van Schoor et al. 1998; Lordal et al. 2001). It is expressed at much lower level in the central nervous system (Saffroy et al. 2003) where it may mediate NKA anxiolytic effects (Griebel 1999). NK2R may also contribute to central...
regulation of pain, as shown with the specific-NK2R antagonist SR48968, which reduces neuronal response in the spinal cord to pressure on knee joints in rat (Neugebauer et al. 1996). Similar hypoalgesic effect towards moderate to intensive pain was obtained by eliminating the ligand by targeted deletion of the preprotachykinin gene A, which encodes both NKA and SP (Zimmer et al. 1998).

Very little information is available about the topology of the NKA-NK2R complex. On one hand, the structure of the NK2R is assumed to adopt a folding similar to that of rhodopsin (Palczewski et al. 2000). On the other hand, the structure of NKA appears to be random in water (Gao and Wong 1999) and contains some helical structure in the C-terminal end when it is in contact with sodium dodecyl sulphate or lipid micelles (Whitehead et al. 1998; Gao and Wong 1999). Structure-activity relationship studies carried out with NKA analogues support that the conserved C-terminal residues are important for high ligand affinity (Regoli et al. 1994; Gembitsky et al. 1999).

So far, the only study that orients part of the ligand towards a specific part of the receptor is based on intermolecular formation of cysteine bridges. It is indeed described that the C-terminal aminocaid of NKA, mutated into a cysteine, forms a cystin bridge with the receptor, when a cysteine is introduced at residue 297 from transmembrane helix (TM) VII (Labrou et al. 2001). Other point mutation experiments combined with 125I-NKA binding point toward residues from the outer part of TMIII, -V, -VI and -VII as aminoaacidic interacting with NKA (Bhogal et al. 1994; Renzetti et al. 1999; Giotitti et al. 2000).

The present study aims at probing the topology of NKA bound to the rat NK2R by measuring intermolecular distances by means of detecting fluorescence resonance energy transfer (FRET) between receptor and ligand. A similar approach aiming at elucidating the structure of a ligand receptor complex has previously been applied to the binding of an antagonist peptide binding the NK2R (Turcatti et al. 1996, 1997). In that study, fluorescent receptors incorporating unnatural amino acids were expressed in Xenopus oocytes. From FRET efficacy determination, distances from the amino acids to tetramethylrhodamine (TmR) linked to the N-terminus of the antagonist hepta-peptide were estimated (Turcatti et al. 1996). This work successfully exploited FRET to demonstrate that peptide antagonists interact with external parts of the hepta-helical domain of the NK2R.

With the introduction of the green fluorescent protein (GFP), it is now possible to produce fluorescent G protein-coupled receptors (Vollmer et al. 1999; Kallal and Benovic 2000) in simple expression systems. This allows the detection of receptor interactions with fluorescent ligands, including agonists, by FRET (Vollmer et al. 1999; Lecat et al. 2002) and thus distance measurements in a system where the receptor is still functional and imbedded in the membrane of intact living cells.

Materials and methods

Preparation of GFP-NK2R

The cDNA for expression of the fluorescent NK2R was prepared and transfected into human embryonic kidney 293 (HEK293) cells as described in Vollmer et al. (1999). A cell-line expressing enhanced GFP-NK2R at approximately 1 × 10^6 sites/cell was established by selection with 600 μg/mL hygromycin and kept in culture in growth medium (minimal essential medium with Earls salts, without l-glutamate) supplied with 10% fetal calf serum, 2 mmol/L glutamate, 50 U/mL penicillin and 50 μg/mL streptomycin) at 37°C, 5% CO2. Cells were detached with trypsin-EDTA (0.5 g/L trypsin, 500 μmol/L EDTA, 137 mmol/L NaCl, 5 mmol/L KCl, 5 mmol/L d-glucose and 4 mmol/L NaHCO3, pH 7) and diluted twice a week. The stable cell-lines were used for about 12 weeks.

Starting at the N-terminal does the expressed peptide contain: The signal peptide from chicken α2 subunit of the acetylcholine receptor, the GFP amino acids 3–238, a Leu-Tyr-Lys spacer and the rat NK2R (aa 16–390) (Sasai and Nakanishi 1989).

Preparation of fluorescent NK2R ligands

Four fluorescent NKA analogues with Texas red (Trr) linked to amino acid 1, 4, 7 or 10 in the peptide were prepared from the wild-type NKA and three monocysteine substituted peptides: NKA (His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH2), NKA[D4C], NKA[V7C] and NKA[M10C]. A peptide NK2R antagonist: PhCO-Lys-Ala-β-Trp-β-Pro-Pro-Gly-Leu-Met-NH2, was labelled with TmR on the α-amino group of lysine by reaction with the isothiocyanate derivative of TmR as described previously (Bradshaw et al. 1994). The NKA-wild type was labelled with fluorophore as previously described (Vollmer et al. 1999). For the cysteine containing analogues, peptide was dissolved in dimethylformamide (DMF) to a final concentration of 10 mmol/L and supplied with 10 equivalents of triethylamine in DMF. The reagent Trr CS bromoacetamide in a freshly prepared 30 mmol/L DMF solution was added in two to three steps to finally 0.6–0.9 equivalents. For all five peptides, the progression of the reaction was monitored by reversed-phase HPLC, equipped with C8 column (Zorbax ZSC 25F; Interchim, Montluçon, France) and detection at two wavelengths 219 and 590 nm (Trr) or 540 nm (TmR). Separation was obtained with a 60 min gradient from 100% solvent A (90% H2O with 10% acetonitrile) to 100% solvent B (10% H2O with 90% acetonitrile), both supplied with 0.1% heptfluorobutycric acid for the analytical runs. For isolation of the fluorophore labelled peptide, the reaction was purified in three to five injections. Fractions containing the fluorescent peptide were pooled. The isolated material was reinjected on HPLC with solvent A and B supplemented with trifluoroacetic acid replacing heptfluorobutycric acid, aliquoted and stored at −80°C until use. The purity was verified by analysis of a sample on HPLC and the molecular weight determined by mass-spectroscopic analysis (Matrix Assisted Laser Desorption Ionization Time Of Flight (MALDI TOF)). Experimental molecular weights are as follows: Trr1-NKA: 1865.6 g/mol (expected 1865.3 g/mol); Trr4-NKA: 1852.7 g/mol (expected 1852.5 g/mol); Trr7-NKA: 1868.8 g/mol (expected 1868.1 g/mol); Trr10-NKA: 1837.0 g/mol (expected 1836.1 g/mol) and for the TmR-labelled antagonist (TmR-ant): monoisotopic mass: 1404.60 Da, calculated: 1404.64 Da.
Concentration of labelled peptides were calculated from the absorbance in methanol at 583 nm using molar extinction coefficient, ε = 113 000 cm/mmol/L for TXR labelled-peptides and at 544 nm using ε = 92 000 cm/mmol/L for TmR-ant.

**Radioligand competition binding**

For competition binding with [3H]-SR48963 as tracer, cells were detached at day 1 with trypsin-EDTA, suspended in growth medium and seeded into 96-well plates, with a density of 15–20 000 GFP-NK2R expressing cells/well. On day 2, the competition binding was performed: The cells were washed once in 100 L ice-cold HBSS, incubated at 37°C for 3 h and then harvested onto a filter plate (Corning, New York, NY, USA). To each well, 25 L of a four time concentrated solution of the NKA analogue or the NK2R antagonist SR48968 (gift from Xavier Emonds-Alt, Sanofi-Synthelabo) in 1 m acetic acid was added into a 96-well plate kept on ice (Corning 3600 polystyrene plate; Corning, New York, NY, USA). To each well, 10 L of a 10 time concentrated solution of the NKA analogue or the NK2R antagonist SR48968 (gift from Xavier Emonds-Alt, Sanofi-Synthelabo) in 1 m acetic acid was added and the reversal in fluorescence intensity of the fluorescent ligand binding, an excess of SR48968 (gift from Xavier Emonds-Alt, Sanofi-Synthelabo) was added and the fluorescence intensity was followed until equilibrium was reached (10 min–1.5 h depending on ligand). To reverse the fluorescent ligand binding, an excess of SR48968 was added and the fluorescence intensity was followed until equilibrium was reached (10 min–1.5 h depending on ligand).

**Table 1** Kᵢ and Kᵣ values. Binding affinities for NKA, TXR-NKA analogues and TmR-ant to the GFNP-NK2R. Kᵢ values refer to radioligand competition binding (Fig. 2). Kᵣ values refer to direct determination of affinities by fluorescence intensities (Fig. 4)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Competition [³H]-SR48968</th>
<th>FRET amplitude</th>
<th>Competition [¹²⁵I]-NKA</th>
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<tbody>
<tr>
<td></td>
<td>Kᵢ (nmol/L) SEM N</td>
<td>Kᵣ (nmol/L) SEM N</td>
<td>Kᵢ (nmol/L) SEM N</td>
</tr>
<tr>
<td>NKA</td>
<td>46.5 15.7 9</td>
<td>–</td>
<td>4.7 3 2</td>
</tr>
<tr>
<td>TXR1-NKA</td>
<td>105.4 10 4</td>
<td>21.5 3.3 4</td>
<td>ND* ND*</td>
</tr>
<tr>
<td>TXR4-NKA</td>
<td>134.6 19.2 4</td>
<td>69.5 46.3 4</td>
<td>16.8 1.5 2</td>
</tr>
<tr>
<td>TXR7-NKA</td>
<td>136.8 30.1 3</td>
<td>143.5 41.3 4</td>
<td>44.5 4.4 2</td>
</tr>
<tr>
<td>TXR10-NKA</td>
<td>328 120 3</td>
<td>184.4 56.4 4</td>
<td>219.7 85.9 2</td>
</tr>
<tr>
<td>TmR-Ant</td>
<td>–</td>
<td>9.6</td>
<td>– 3 3</td>
</tr>
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*Affinity of bodipy derivative of NKA for NK2R was determined in Vollmer et al. (1999) and shown to be equal to that of NKA in competition against [¹²⁵I]-NKA.

**FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; TXR, Texas red; TmR-ant, TmR-antagonist; NKA, neurokinin A.**
The background fluorescence at 510 nm was determined from a sample of non-transfected HEK293 cells diluted to a similar concentration as the suspension of GFP-NK2R expressing cells.

Distance calculation based on FRET efficacy

$E$, the efficiency of energy transfer, and $R_0$, the distance at which energy transfer from the donor (GFP) to the acceptor (TxR or TmR) is half-maximal, were estimated as described previously (Lakey et al. 1991). $R_0$ is given by the equation:

$$R_0 = \left( \frac{1}{K^2} \frac{Q_0}{n^4} \right)^{1/6} \times 9.7 \times 10^3 \, \text{Å},$$

where $K^2$ is taken as 2/3 for freely rotating fluorophores and $n$ as 1.4 in aqueous medium. From absorption spectra in methanol and HEPES, the maximal extinction coefficient in HEPES buffer for TxR was found to be 95 600/cm/mol/L at 594 nm and for TmR 55 500/cm/mol/L at 553 nm. The quantum yield $Q_0 = 0.66$ of GFP is taken from the literature (Heim and Tsien 1996). From the calculated overlap integral for the combined emission of GFP and absorbance of TxR ($J = 3.14 \times 10^{-13}$ cm$^3$/mol/L), the $R_0$ value was estimated to be 49 Å. For GFP and TmR ($J = 2.10 \times 10^{-13}$ cm$^3$/mol/L) and the $R_0$ was estimated to be 53 Å.

Donor-acceptor separation is given by

$$R = ((E^{-1} \cdot -1)^{1/6} R_0, \text{ where } E, \text{ the maximal energy transfer, given by } E = 1/(F_{DA}/F_{D})), \text{ was determined by measuring specific-donor fluorescence emission in the presence (}F_{DA}\text{) and absence (}F_{D}\text{) of ligand at saturating concentrations (300 mmol/L TxR-NKA, TmR-ant or 1 mmol/L TxR4-NKA, TxR7-NKA and TxR10-NKA). Specific-GFP fluorescence in the absence (}F_{D}\text{) and presence (}F_{DA}\text{) of ligand was determined by subtracting auto-fluorescence of non-transfected cells from total fluorescence of GFP-NK2R expressing cells. In a stable cell line selected with 0.5 mg/mL hygromycin for GFP-fluorescence.}$$

Creation of model

The rat NK2R sequence was aligned to bovine rhodopsin according to the conserved residues in each transmembrane helices and in external and internal loops (Van Rhee et al. 1995). Extensions of the helices were based on their length in rhodopsin. We also did multiple alignments on NK1 and NK2R from rat and human, in order to get structural insights.

The rat NK2R was built by using bovine rhodopsin X-ray structure 1F88 (Palczewski et al. 2000) in Sybyl 6.7 package (Tripos, St Louis, MO, USA) with the biopolymer module. The NK2R model was minimised with a maximum number of iterations of 10 000. All the minimisation procedure was undertaken without charge and in Amber 4.1 force field (Fraternali et al. 1998) with a maximum of 25 simplex steps coupled to a Powell (Fraternali et al. 1998) method, with an energy termination gradient of 0.05 kcal/mol$^{-1}$×Å$^{-1}$.

Putative interaction sites between NKA and NK2R were identified on an external truncated loop NK2R, by Multi-channel function of the Molcad module and taking into account mutagenesis data from NK1 and NK2R, as well as from ligand analogues (Regoli et al. 1990; Bhogal et al. 1994; Li et al. 1995; Turcatti et al. 1997; Holst et al. 1998; Gembitsky et al. 1999; Labrou et al. 1999; Renzetti et al. 1999; Giolitti et al. 2000; Palczewski et al. 2000).

NKA was incrementally (residue by residue) docked into NK2R with the Dock module, starting with the consensus terminal part of the agonist FVGLM according to mutagenesis data and complementary of Molcad lipophilic (Heiden et al. 1993) surfaces of the peptide and the receptor, with the N- and C-terminus residues capped respectively with amide and carboxamide terminal groups. Studies of NKA in micelle environment by NMR (Whitehead et al. 1998) has shown some helical structure in the terminal part of the peptide. However, we could not provide a placement in agreement with the mutagenesis data and the lipophilic complementary surfaces when using helical structure.

A local minimisation was undertaken as described above with the entire NK2R and NKA complex. Following we optimised the placement by using a local molecular dynamic in vacuo around NKA at 300 K, NTV constant during 50 ps, with an acquisition time of 50 fs, and Shake algorithm applied on bonds involving hydrogens (Fraternali et al. 1998). A bath coupling factor of 100 and 2 fs integration step were used. The whole NKA was grown according to interaction sites identified by mutagenesis data.

TxR-labelled NKA analogues were built starting with the NKA docked in NK2R NK2R. The labelled residue was mutated to cysteine, and TxR joined by adding a bond between S and C atoms. TxR was positioned considering channels found with Molcad Multi-channel, directing the fluorophore part towards the extra cellular surface, as it is charged. A local minimisation was then undertaken as described above in the Tripos force field (Fraternali et al. 1998).

Finally GFP was added, using a GFP-linked muscarinic M1 receptor model (Iien et al. 2003) as a starting placement for the GFP relative to NK2R. Only the centroid of the GFP was considered here. In order to find a set of GFP positions compatible with the four FRET distances, a stochastic search was undertaken to find the best position of the centroid (macro written in SPL). GFP was then positioned according to the best centroid placement found and the linker between GFP and NK2R was built ‘manually’ by modifying phi and psi torsions of the linker residues. A final minimisation was applied to the whole systems i.e. GFP-NK2R and the different ligands.

Solutions for cell culture were from Invitrogen, Cergy-Pontoise, France; Protease inhibitors from Roche Diagnostics, Meylan, France and ICN Biochemicals, Aurora, Ohio; Fluorescent reagents were from Molecular Probes, Leiden, Netherlands; Solutions for HPLC were from Merck, Darmstadt, Germany; Peptides were from Neosystem, Strasbourg, France.

Results

The efficacy of FRET between two fluorescent groups is dependent on their relative distance. The aim of this study is to make use of this phenomenon to investigate the topology of NKA bound to its cognate NK2R. For this purpose, the NK2R is rendered fluorescent by fusion of its gene to the cDNA encoding GFP, in order to obtain an amino-terminally labelled receptor. This construct was described in a previous work to be fully functional and to exhibit pharmacological properties similar to those of wild-type NK2R (Vollmer et al. 1999). The second fluorophore was introduced to the ligand by preparation of four analogues of NKA labelled with the
fluorophore TxR, prepared by chemically linking the fluorophore to the N-terminal aminogroup of NKA or by derivatizing NKA with cysteine substitutions at position 4, 7 or 10. From the literature, it is known that bulky groups can be introduced at the N-terminal end as well as at position 7 without dramatic changes of the pharmacological properties of the ligand (Turcatti et al. 1995, 1996; Vollmer et al. 1999; Bremer et al. 2000). Analogues modified at positions 4 and 10 were included to evenly distribute the fluorophores on the NKA sequence.

In addition, a TmR labelled peptide antagonist (TmR-ant) structurally unrelated to NKA was prepared for the study. The position of TmR in the complex formed between TmR-ant and the NK2R has previously been characterized using FRET-based distance measurements (Turcatti et al. 1996).

**Distances which can be measured with the GFP-TxR and GFP-TmR FRET approach**

The range of distances that can be determined by using GFP and TxR as a donor acceptor pair is illustrated on Fig. 1. From the overlap between the emission spectrum of GFP and absorption spectrum of TxR in assay buffer (Fig. 1a), the distance at which half maximal efficiency of fluorescence energy transfer is expected to occur, corresponds to 47 Å ($R_0$ value) for freely orienting fluorescent groups. The efficacious range of distances that can be determined with this donor–acceptor pair consequently ranges from approximately 30–75 Å (Fig. 1b). Such distances fall in the range of receptor dimensions. Energy transfer between GFP and TxR is thus expected to be sensitive to distance variations if the fluorophores are moved along NKA sequence.

From the spectral overlap between tetramethyl rhodamin and GFP (not shown) the calculated $R_0$ value was found to 53 Å, allowing for the use of TmR-ant in combination with our fluorescent GFP-NK2R.

To investigate the effect of TxR addition to the ligand on receptor interaction, two approaches were used. They estimate the binding affinities using the classical radioligand competition-binding assay (indirect affinity determination; $K_I$ values) or by using FRET amplitudes to directly derive dissociation constants (KD values).

**Radioligand competition binding**

Figure 2 illustrates the capacity of NKA, the four TxR-NKA analogues and TmR-ant to compete with [3H]-SR48968 for binding to the GFP-NK2R. Binding experiments were carried out on intact HEK293 cells expressing the receptor at about 10^6 sites/cell (Fig. 2).

All four TxR-NKA ligands compete with SR 48968, with variation in binding affinity lower than one order of magnitude, as compared with NKA. The average $K_I$ values presented in Table 1 show that the largest affinity shift is found for TxR10-NKA, which shows an apparent sevenfold drop in affinity for the NK2R. For TxR1-NKA, TxR4-NKA and TxR7-NKA smaller decreases are observed, as manifested by two to threefold reduction of affinities (Table 1). When competition experiments are carried out using [3H]NKA instead, the amplitude of affinity changes is threefold for NKA derivative carrying TxR at position 4, 10-fold for position 7 and 50-fold for position 10 (Table 1). Although the amplitude of affinity changes is larger than that detected with [3H]SR 48968, the rank order of compounds...
binding remains comparable, i.e. moving the fluorophore to more C-terminal positions progressively reduces binding affinity to the NK2R.

**Ligand affinity determined from FRET efficacy**

The general procedure to measure ligand-receptor interactions is illustrated in Fig. 3. After recording fluorescence of a suspension of cells expressing the GFP-NK2R, the fluorescent NKA derivative was added (Fig. 3a). The interaction of the ligand with the receptor results in a decrease of GFP fluorescence at 510 nm and in an increase of emission at TxR emission wavelength (610 nm). Further addition of an excess (10 μmol/L) of either NKA or SR 48968 reverses the interaction between the fluorescent ligand and the receptor, leading to restoration of initial GFP fluorescence (510 nm) and reduction of TxR fluorescence (610 nm). High concentrations of ligand have been used to ascertain saturation of the receptor sites. Given the high concentrations used, contamination of GFP fluorescence emission by TxR emission was detected beyond 1 μmol/L ligand. Therefore, quantification of receptor sites occupancy was measured, as illustrated in Fig. 3b, by determining the extent of GFP fluorescence recovery after displacement of the fluorescent ligand by a large excess of SR48968. The experimental protocol thus consisted in incubating cells with fluorescent ligand at 21°C for a period necessary to reach equilibrium, (10–25 min, depending on analogue), and then to monitor fluorescent ligand dissociation as shown in Fig. 3b. From the amplitudes of fluorescence variation, the degree of receptor occupancies were determined and plotted as a function of ligand concentration (Fig. 4).

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**Fig. 2** Radioligand competition binding curves for neurokinin A (NKA) wild-type and the four fluorescent NKA-analogues to green fluorescent protein-NK2 receptor. 3H-SR48968 (0.2 nmol/L) binding to human embryonic kidney 293 cells, stably expressing the receptor was displaced by NKA (open triangles), Texas red (TxR1) -NKA (open circles and broken line), TxR4-NKA (open squares), TxR7-NKA (filled circles and broken line) and TxR10-NKA (filled triangles), at 4°C for 3 h.

**Fig. 3** Fluorescence resonance energy transfer between green fluorescent protein-NK2R (GFP-NK2R) and Texas red-neurokinin A (TxR-NKA) analogues. Panel a: Emission spectra (excitation at 470 nm). Solid broken line: human embryonic kidney 293 cells transfected with GFP-NK2R, 2·10⁶ cells/mL corresponding to about 3 nmol/L GFP. Dotted line: GFP-NK2R incubated with 1 μmol/L TxR4-NKA for 20 min. Solid line: Same sample after addition of 10 μmol/L SR48968 and further incubation for 15 min. Thin black line: Fluorescence of a sample of non-transfected cells at identical concentration. Panel b: Example of time-based recording used to determine receptor sites occupancy by TxR7-NKA. Cell samples were incubated with the following concentrations of TxR7-NKA for 15 min: squares: 30 nmol/L; diamonds: 100 nmol/L; triangles: 300 nmol/L; crosses: 1 μmol/L; circles: 3 μmol/L and stars: 10 μmol/L. Five minutes after transferring incubated sample to a cuvette, ligand dissociation was initiated by addition of 10 μmol/L SR48968 and fluorescence emission monitored at 510 nm (excitation wavelength 470 nm). Spectra are normalized according to their final value. For the highest concentrations of ligand, (3 and 10 μmol/L) spectra were corrected for shift in intensity due to the small but measurable fluorescence from TxR at 510 nm prior to normalisation.
TxR1-NKA, TxR4-NKA, TxR7-NKA and TxR10-NKA all bind to a saturable population of SR48968 sensitive binding sites. Their apparent dissociation constants for the GFP-NK2R are presented in Table 1. At 1 μmol/L, they occupy a comparable fraction (95 ± 5%) of receptor sites, and at 10 μmol/L, they all fully saturate the population of receptors. At such concentration, the FRET efficacy for fluorophores randomly distributed in the solution is insignificant and the measurements of FRET for the receptor bound ligand can be performed under equilibrium conditions, with saturating concentrations of the ligand present in the solution. These concentrations are thus used in following experiments to determine efficiencies of fluorescence energy transfer at comparable receptor sites occupancies, namely saturation.

TmR-ant was able to quench the GFP fluorescence, with a Kd equal to 9.6 nmol/L as determined from FRET amplitude for 3 to 300 nmol/L TmR-ant (Fig. 4; Table 1). At 100 nmol/L, the receptor population is close to saturation, which is the concentration used to determine the efficiency in fluorescence energy transfer from GFP to TmR.

**Fig. 4** Direct determination of fluorescent ligands affinity by fluorescence resonance energy transfer (FRET) between green fluorescent protein (GFP) -NK2 receptor and Texas red-neurokinin A (TxR-NKA) analogues. Samples were incubated at 21°C in HEPES buffer with TxR-NKA or TmR-ant until equilibrium was reached. Incubated sample was then transferred to a cuvette and the binding reversed with 10 μmol/L of SR48968. FRET amplitude was monitored as change in GFP fluorescence intensity at 510 nm upon fluorescent ligand dissociation. Panel a: TxR1-NKA, panel b: TxR4-NKA, panel c: TxR7-NKA, panel d: TxR10-NKA and panel e: TmR-ant. The solid line through averaged normalized data from four independent experiments is the best fit with empirical Hill equation.

**Determination of FRET efficacies and estimation of GFP-TxR/TmR separation**

Determination of efficacies of FRET between GFP and bound fluorescent NKA derivatives was carried out by measuring variations of donor (GFP) emission amplitude in the presence and absence of a saturating concentration of ligand. Figure 5 illustrates how variation of GFP fluorescence varies in the presence of 0.3 μmol/L TxR1-NKA or 1 μmol/L of TxR4-NKA, TxR7-NKA or TxR10-NKA (Fig. 5). It is clear from both association and dissociation traces that the amplitude of GFP fluorescence variation is progressively smaller when TxR is moved along NKA from position 1–10. All quantitative measurements of fluorescence variation at 510 nm were carried out on dissociation traces recorded after addition of an excess (10 μmol/L) of SR 48968 (Fig. 5). The resulting maximal fluorescence variation (Table 2) was corrected for cell autofluorescence to determine efficiency (E) of energy transfer and separation between GFP and TxR. The shortest mean distance separating GFP from TxR is found when TxR is positioned at the N-terminal of NKA (46 ± 1 Å). When TxR is linked to position 4, 7 or 10, the corresponding distances are 54 ± 1 Å, 59 ± 1 Å and 68 ± 1 Å, respectively (Table 2).

When 100 nmol/L TmR-ant is present, a concentration at which saturation of receptor sites is reached, 40 ± 1% of the specific-GFP fluorescence is quenched. This corresponds to a distance of 55 ± 1 Å separating the two fluorophores (Table 2).
Discussion

In this study, we investigate the potential structure of the complex formed between NKA and its cognate NK2R. We use FRET to determine distances between GFP grafted to the aminoterminal domain of the receptor and TxA linked to various positions of NKA. The mean distance estimated from efficiency of FRET for each NKA peptide, significantly varies from 46 to 69 Å when TxA is moved stepwise from position 1 to 4, 7 or 10 along the NKA sequence.

These distances are compatible with the dimensions of the receptor-GFP construct. Indeed, according to rhodopsin structure, the size of the receptor itself is approximately 30 Å x 40 Å in the plane of the membrane, and ~80 Å thick. For GFP, the dimensions are in the order of 35 x 25 Å. Further assuming that the N-terminal end of the receptor, may adopt an extended structure, the GFP fluorophore might be up to 100 Å away from the helical bundle of the receptor.

The GFP-TxA distance found for each fluorescent NKA analogue monotonically increases from position 1 (46 Å) to 4 (55 Å), 7 (59 Å) and 10 (68 Å). These data support that, when bound to the receptor, the peptide adopts an extended structure. Indeed, the fluorophore of bound NKA-TxA1 appears to be separated from that of TxA10 by at least 22 Å. For comparison, NKA is roughly 30 Å long in an extended conformation and 10 Å separate the alpha-carbons of residue 1 from 4, 4 from 7 and 7 from 10.

Thus, the measured distances do not support a hairpin conformation of NKA. Also, there is no evidence in favour of a partly helical structure involving residues six to nine as was detected for NKA in the presence of micelles (Whitehead et al. 1998).

Although the measured distances are reasonable in terms of molecular dimensions, they are not sufficient to correctly orientate NKA relative to the NK2R. This is essentially due to the lack of constraints on GFP position relative to the receptor. In order to propose a model of the complex, we include the previously described fluorescent peptide TmR-ant in the study. The position of the fluorophore

Table 2  Distance calculation between fluorescent NK2R ligands and GFP-NK2R

<table>
<thead>
<tr>
<th>Ligand</th>
<th>% Maximal fluorescence extinction</th>
<th></th>
<th></th>
<th></th>
<th>Distance (Å*)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total fluorescence SEM</td>
<td>GFP-specific SEM</td>
<td></td>
<td>SEM</td>
<td>N</td>
</tr>
<tr>
<td>TxA1-NKA</td>
<td>37.8</td>
<td>0.9</td>
<td>49.6</td>
<td>1.3</td>
<td>46</td>
</tr>
<tr>
<td>TxA4-NKA</td>
<td>21.7</td>
<td>0.8</td>
<td>28.6</td>
<td>0.6</td>
<td>54</td>
</tr>
<tr>
<td>TxA7-NKA</td>
<td>15.8</td>
<td>0.9</td>
<td>20.8</td>
<td>0.8</td>
<td>59</td>
</tr>
<tr>
<td>TxA10-NKA</td>
<td>7.9</td>
<td>0.3</td>
<td>9.9</td>
<td>0.3</td>
<td>68</td>
</tr>
<tr>
<td>TmR-ant</td>
<td>28.9</td>
<td>0.9</td>
<td>40.3</td>
<td>0.9</td>
<td>55</td>
</tr>
</tbody>
</table>

*Ångström

Total: Directly measured FRET intensity as the difference in fluorescence intensity at 510 nm for GFP-NK2R expressing cells in equilibrium with saturating concentrations of TxA-NKA or TmR-ant and the fluorescence intensity after dissociation with 10 μmol/L SR48968 relative to the fluorescence from the GFP-NK2R cells without ligand. GFP-specific (equal to E in distance calculation). Total intensity corrected for non-specific fluorescence corresponding to non-transfected cells. Distance: Distance between GFP and TxA when ligand is receptor bound, calculated from the E-values in second column. The values are average for values found in at least 5 independent experiments.

FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; TxA, Texas red; TmR-ant, TmR-antagonist; NKA, neurokinin A.
relative to the receptor has been well established, in the bound form of the (Turcatti et al. 1996, 1997) and the distance determined to GFP delimits the statistically most significant position of GFP relative to the receptor. In the previous study, TmR-ant peptide was used as an energy acceptor of the nitro benzoxa-diazole (NBD) fluorescent group. The NBD group was incorporated at different positions in six NK2R mutants and pairwise distances separating TmR from NBD were determined. The fluorophore was found to protrude between the TM5 and -6 at the interface between lipid and solvent with the peptide most likely inserted between transmembrane helices 3, 5, 6 and 7. In our work, we determined that the distance from GFP to TmR is 55 Å.

The three dimensional model of the NK2R is based on rhodopsin structure (Palczewski et al. 2000) (Fig. 6). After generating the NK2R model, NKA is docked into the protein taking into account appropriate hydrophobic interactions and space (see materials and methods), and the possibility to link the TxR fluorophore on the selected positions 1, 4, 7 and 10. Additional pieces of information which are used to constrain the model are NKA analogue binding studies, mutagenesis data from NK2R (Bhogal et al. 1994; Renzetti et al. 1999; Bremer et al. 2000; Giolitti et al. 2000), as well as mutagenesis and photoaffinity labelling data obtained on the closely related NK1-receptor (Li et al. 1995; Turcatti et al. 1997; Holst et al. 1998). The resulting NK2R model correctly accounts for all but one inter-aminoacid distances proposed by Turcatti and colleagues (Turcatti et al. 1996, 1997), supporting that the NK2R structure fits into the rhodopsin template. Second, the model accounts for the capacity of the receptor, bearing a cysteine residue at position 297 (TM7), to establish covalent bonds with NKA containing a cysteine at position 10 (Labrou et al. 2001). In our modelling of the NKA-NK2R complex, we find it possible to place fluorophores outside the helical bundle in such a way that all five measured distances to GFP fit with experimentally determined values.

In the modelled NKA-NK2R complex, NKA is rather linear with the N-terminal part situated close to the receptor surface, in the vicinity of residues belonging to the extracellular loop 2 and N-terminus of the receptor. NKA residue 4 is in the vicinity of extracellular loop 2 and of the top of TM7. Residue 7 is located close to receptor residues Ile114, Met117, Val182, Tyr266 and Phe270. The C-terminal amino acid of NKA is more deeply buried in the receptor structure, close to residues Met117, Tyr266, Trp263, Phe293 and Met297. This position is similar to the site where retinal forms a Schiff base with lysine 296 in rhodopsin, about one-third down in the helical structure of the receptor (Palczewski et al. 2000).

The finding that the C-terminus of NKA is not located at the surface of the NK2R may seem contradictory with experimental data obtained with NK1 receptor showing that the C-terminus of SP is exposed to the extracellular medium (Turcatti et al. 1997). Although the introduction of a fluorophore at the C-terminus of NKA may result in a reorientation of the C-terminal end of NKA, it is well documented that C-terminal parts of NKA and SP do not
contribute identically to signalling on their respective receptors (Regoli et al. 1994).

Altogether, the proposed position of NKA is in good agreement with site-directed mutagenesis data showing that agonists and the competitive antagonist SR 48968 binding pocket is surrounded by residues from the 253–295 domain mainly with amino acid 289 (Gether et al. 1993a,b; Huang et al. 1995; Labrou et al. 2001) within the receptor monomer. The present experimental determination of bound ligand structure thus proves useful to decipher areas of the receptor protein that contribute to the binding pocket of natural ligands. It should bring valuable information to understand ligand selectivity and possibly to set up efficient structure-assisted drug design approaches.

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References


Cheng Y.-C. and Prussoff W. H. (1973) Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 per cent inhibition (IC50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099-3108.


