Involvement of the Second Extracellular Loop (E2) of the Neurokinin-1 Receptor in the Binding of Substance P

PHOTOAFFINITY LABELING AND MODELING STUDIES

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Substance P (SP) interacts with the neurokinin-1 (NK-1) G-protein-coupled receptor, which has been cloned in several species. In the present study, the domains of the NK-1 receptor involved in the binding of SP and SP-(7–11) C-terminal fragment have been analyzed using two peptide analogs containing the photocrosslinker amino acid para-benzoylphenyalanine ((p-Bz)Phe) in position 8 of their sequence. This study was carried out with [BAPA-Lys9,(p-Bz)Phe8]-Pro9,Met(O2)11]SP-(7–11) and [BAPA10,(p-Bz)Phe8]SP on both rat and human NK-1 receptors expressed in CHO cells. Combined tryptin and endo-GluC enzymatic complete digestions and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry analysis led to the identification of the same domain of covalent interaction, 173TMP177, for the two photoactivatable peptides. Further digestion of this fragment with carboxypeptidase Y led to the identification of 173TMP175 in the second extracellular loop (E2) of the NK-1 receptor as the site of covalent attachment. Models of the conformation of this E2 loop in the human NK-1 receptor were generated using two different strategies, one based on homology with bovine rhodopsin and the other based on the solution conformation preferences of a synthetic peptide corresponding to the E2 loop.

Among peptides of the tachykinin family, substance P (SP) is implicated in many physiological and pathophysiological processes including transmission of pain and inflammation but also depression, epilepsy, and angiogenesis (1–3). These biological effects are mediated via a G-protein-coupled receptor, the NK-1 receptor, which has been cloned in several species (see Ref. 4 for a review). Binding experiments with SP analogs in tissues and in cells transfected with the NK-1 receptor have shown that two types of non-stoichiometric binding sites with distinct pharmacological profiles are associated with the NK-1 receptor (5–12). In CHO cells, Bmax values for the two binding sites were found to be 6000 fmol/mg proteins and 800 fmol/mg proteins for the major binding site (labeled with [3H]Pro9]SP) and the minor binding site (labeled with [3H]propionyl-Met(O2)11]SP-(7–11), respectively (7). With the same clone it has been shown that in the presence of choler toxin, the tachykinin NK-1 receptor is uncoupled to G-proteins (7). However, Bmax values remain unchanged for the two radioligands (7). Differences in the Bmax values were observed as well with a CHO clone expressing a lower level of NK-1 receptors in membrane homogenates prepared from the clone with the highest expression and in rat submandibular glands (7). Furthermore, the two binding sites internalize differently as observed with radiolabeled peptides (12). Altogether these results suggest that the differences in Bmax values for the two types of ligands cannot be explained by effects secondary to G-protein interactions. With a plethora of SP analogs in CHO cells expressing high levels of the human NK-1 receptor, the binding affinity for the more abundant binding site could be correlated to the potency to accumulate cAMP, whereas the binding affinity for the less abundant site could be correlated to the potency to activate inositol phosphate production (8). In the CHO clone used herein, the more and less abundant binding sites represent 85 and 15% of the total population of receptors (6 pmol/mg of proteins), respectively (7). Substance P binds the two binding sites with high affinity, whereas some C-terminal fragment analogs of substance P and some substance P(1–11) analogs, as well as the endogenous tachykinin NK-2 ligand neurokinin A, bind only the less abundant one (7–12). Although several structure-activity relationship studies have been carried out, little is known about the differences in the molecular recognition of these two binding sites (8–12). Photoaffinity labeling therefore appeared to be a complementary method of studying the interaction of both types of peptide agonists with the NK-1 receptor. Photolabeling of the rat NK-1 receptor with a SP and a NKA photoactivatable analog has been reported recently, and subsequent mapping studies have established that the site of photoinsertion was located in the same segment of the second extracellular loop (E2) of the receptor, probably on an identical residue Met181 (13).
In this study, we have used the same "one-pot" strategy of photolabeling, enzymatic digestion, and purification before matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis described previously (14), with two photoactivatable analogs of SP designed to screen the two binding sites associated with the NK-1 receptor. Both photoactivatable SP analogs, [BAPA-Lys6-(p-Bz)Phe8-Pro10-Met(O2)11]SP-(7-11) and [BAPA-(p-Bz)Phe8]SP, contain a biotin sulfo maleate at the N-terminal end, which is separated from the first amino acid by an aminopentanoic acid flexible spacer (BAPA). After photolabeling and enzymatic digestion(s), this biotin sulfo maleate is used to purify the fragment of interest via streptavidin-coated magnetic beads (14, 15). The substance P receptor fragment covalent complex is then released from the magnetic beads directly with the matrix solution used for MALDI-TOF mass spectrometry analysis (14).

Using this strategy, we have previously shown that the SP analog photoactivatable in position 8, [BAPA-Lys6-(p-Bz)Phe8-Pro10-Met(O2)11]SP-(7-11) or [BAPA-(p-Bz)Phe8]SP, specifically interact with Met174 in the second extracellular loop of the human NK-1 receptor (14). Using a similar photoactivatable SP analog, [[125I]BH-(p-Bz)Phe8-Pro10-Met(O2)11]SP-(7-11) or [BAPA-(p-Bz)Phe8]SP in 50 mM Tris-Cl buffer, pH 7.4, containing 1 mM EDTA, 10 mM MgCl2, 0.1 mM PMSF, 5 μg/ml soybean trypsin inhibitor, and 400 mg/ml bovine serum albumin. The membrane preparation was then irradiated on ice for 40 min using an ultraviolet light at 365 nm (HPR 125-watt lamp) at a distance of 6 to 10 cm. After irradiation, [Pro9]SP (10 μM) was added for 10 min to the membrane preparation prior to centrifugation for 2 min at 13,000 rpm (MSE Micro Centaur). The sample was then washed with Tris-Cl buffer and centrifuged again. Finally, photolabeled membranes were incubated for 2 h at room temperature in a denaturation buffer consisting of 17 mM dithiothreitol and 3% SDS in 50 μl of Tris-Cl 50 mM, pH 8.0.

Tryptic Digestion of Photolabeled Receptors—After denaturation, photolabeled membranes were digested for 5–24 h at 22 °C in 1 ml of 50 mM NH4HCO3, pH 8.0, with 100 μg of trypsin (ketone-treated 1-toylsiamide-2-phenylethylchloromethyl, Sigma). Digestion was stopped by adding 10 μl of PMSF (100 mM) and 10 μg of soybean trypsin inhibitor (5 μg/ml, 1).

Streptavidin-coated Magnetic bead Purification—The tryptic digested sample was incubated with 100 μg of streptavidin-coated magnetic beads (Dynabeads M280, Dynal) for 2 to 12 h under gentle agitation. The beads were then washed as described previously (14). The sample was then either submitted to further digestion with endo-GluC or analyzed by MALDI-TOF mass spectrometry.

Endo-GluC Digestion of Tryptic Fragments onto Streptavidin-coated Magnetic beads—After purification and washing (14), the beads were incubated at 37 °C for 15 h with 20 μg of endo-GluC (Roche Molecular Biochemicals) in 20 μl of 100 mM Tris-Cl, pH 7.8. Digestion was stopped by the addition of 10 μl of PMSF (100 mM) and 10 μg of soybean trypsin inhibitor (10 μg/ml, 1). 100 μg of streptavidin-coated magnetic beads were again added for 2 h. Purification and washing steps were performed as described (14). The sample was then either submitted to further digestion with carboxypeptidase Y or analyzed by MALDI-TOF mass spectrometry.

Carboxypeptidase Y Digestion of Tryptic/Endo-GluC Fragments onto Streptavidin-coated Magnetic beads—After purification and washing (14), the beads were incubated at 37 °C for 2 to 24 h with 0.05 μg of carboxypeptidase Y in 20 μl of 100 mM Tris-Cl, pH 7.0. After incubation the beads were washed as described (14).

MALDI-TOF Mass Spectrometry Analysis—Peptide fragments were eluted from the magnetic beads with 3 μl of MALDI matrix (α-cyano-4-hydroxy-cinnamic acid in 4:1 (v/v) CH3CN/H2O (0.1% trifluoroacetic acid). After a 10-min incubation, 1 μl of bead-free supernatant was deposited on the sample holder for MALDI-TOF MS analysis. MALDI-TOF mass spectra (averaged over 256 laser shots) were obtained in positive mode on a Voyager Elite (PerSeptive Biosystems) mass spectrometer in the reflector mode. For weak ion signals, a better signal/noise ratio was obtained by averaging 10 mass spectra. External calibration was applied using standard peptides deposited on the MALDI-TOF target very close to the studied sample. In the following, the measured and indicated m/z values are monoisotopic. Peptide receptor domains corresponding to the mass peaks obtained from MALDI-TOF MS analysis were identified using the Protein Analysis WorkSheet freeware edition (ProteoMics; http://www.proteomics.com) and applied to the NK-1 receptor (human and rat) using the different proteases.
Pharmacological profile of photoactivatable analogs of SP in intact CHO cells expressing the human or the rat NK-1 receptor

Binding assays were performed with [1H]Pro[9]SP (for NK-1M binding site) and [1H]propionyl-Met(O2)[11]SP-(7–11) (for NK-1m binding site), and potencies to activate phospholipase C and adenylate cyclase were determined as described under “Experimental Procedures.” Data presented are the mean ± S.E. of at least three independent experiments performed in duplicate. PLC, phospholipase C; ND, not determined.

Table I

| NK-1 species | Peptide analog | PLC | Adenylate cyclase |
|--------------|----------------|-----|------------------|
| Human        | [BAPA-Lys6,(p-Bz)Phe8]SP | 1.3 ± 0.3 | 10 |
|              | [BAPA-Lys6,Pro6,Met(O2)11]SP-(7–11) | 0.2 0.1 | 10 |
| Rat          | [BAPA-Lys6,(p-Bz)Phe8]SP | 1.1 ± 0.1 | 10 |
|              | [BAPA-Lys6,Pro6,Met(O2)11]SP-(7–11) | 0.2 0.1 | 10 |

Data taken from Ref. 7.

RESULTS

Pharmacological Profile of the Photoactivatable SP Analog—The photoactivatable analog [BAPA0,((p-Bz)Phe8]SP binds NK-1M and NK-1m binding sites with nanomolar affinities similar to those of SP for rat or human NK-1 receptor expressed in CHO cells (Table I). This analog also activates with potencies similar to those of SP the phospholipase C and adenylate cyclase second messenger pathways (Table I). In CHO cells expressing the human NK-1 receptor, the receptor-unselected C-terminal analog, [BAPA-Lys6,((p-Bz)Phe8,Pro9,Met(O2)11]SP-(7–11), had a 4-fold better affinity for the less abundant NK-1m binding site than the NK-1m selective ligand, propionyl[Met(O2)11]SP-(7–11) (Table I). The affinity of [BAPA-Lys6,((p-Bz)Phe8,Pro9,Met(O2)11]SP-(7–11) for the more abundant NK-1M binding site was also increased 70-fold compared with propionyl[Met(O2)11]SP-(7–11). As expected, the increase in the affinity of [BAPA-Lys6,((p-Bz)Phe8,Pro9,Met(O2)11]SP-(7–11) for NK-1M and NK-1m binding sites was accompanied by higher potencies of this analog to stimulate phospholipase C and adenylate cyclase compared with those of propionyl[Met(O2)11]SP-(7–11).

Yield of Photoaffinity Labeling—In human or rat NK-1 receptors—Membrane preparations from CHO cells expressing the human or the rat NK-1 receptor were irradiated in the presence of 2 nM [BAPA-((ε-CT)CTCO)Lys6,((p-Bz)Phe8,Pro9,Met(O2)11]SP-(7–11) or [BAPA-((ε-CT)CTCO)Lys6,((p-Bz)-Phe8,Pro9,Met(O2)11]SP (3700 GBq/mmol). After photolysis and extensive washing of the membrane preparation with Tris-Cl buffer plus 10–2 M [Pro9]SP, the yield of photolabeling was determined to be in the range of 70 to 85% from one experiment to the other.
(data not shown). This 70–85% yield of photolabeling refers to the receptor fraction, which initially had ligand bound that was subsequently modified covalently. No covalently bound radioactivity could be detected in nontransfected CHO cells (not shown).

Tryptic Digestion of Photolabeled Rat and Human NK-1 Receptor—After photoinertion of [BAPA-Lys6,p-Bz]Phe8,Pro9,Met(O2)11]SP-(7-11) or [BAPA-Lys6,p-Bz]Phe8]SP bound to CHO cells expressing either the rat or the human NK-1 receptor, the covalent complex was digested for 5–24 h with 1-tosylamide-2-phenyl-ethylchloromethyl ketone-treated trypsin. The ligand-receptor fragment covalent complex was then purified via streptavidin-coated magnetic beads and analyzed by MALDI-TOF MS. Tryptic digestion of the covalent complex after photoinertion of [BAPA-Lys6,p-Bz]Phe8,Pro9,Met(O2)11]SP on the rat or the human NK-1 receptor gave, by MS analysis, two peptides with MH+ at m/z 2980.4 and 2817.4 (Fig. 1a). The presence and relative intensity of these two peptides were dependent on the time of trypsin digestion, the higher mass peptide disappearing with a time of 15 h. The peptide corresponding to the highest mass was the more abundant at short incubation times with trypsin, whereas after long periods of incubation the peptide with the lowest mass was the only one observed. After subtracting the mass of the photoactivatable peptide, domains Tyr168–Arg177 and Ser169–Arg177 of the NK-1 receptor were again identified as the site of photoinsertion.

Subcleavage on Beads of the Tryptic Fragments by Endo-GluC—After trypsin digestion and purification of the covalent peptide-receptor fragment complex on streptavidin-coated magnetic beads, endo-GluC digestion was performed for 15 h at 22°C. For [BAPA-Lys6,p-Bz]Phe8]SP covalently linked either to the rat or human NK-1 receptor, the ions of the trypsin fragments at m/z 2980.41 and 2817.34 were shifted to a single peak at m/z 2399.1 (Fig. 1, b and c), corresponding to the domain Thr173–Met-Pro-Ser-Arg177 (590.23 atomic mass units measured and 590.28 atomic mass units expected from the sequence) of the receptor. Similarly, the trypsin peptides obtained for [BAPA-Lys6,p-Bz]Phe8,Pro9,Met(O2)11]SP-(7-11) covalently linked to the rat or human NK-1 receptor (MH+ at m/z 2446.1 and 2283.1, respectively) when digested with endo-

Fig. 1. Partial MALDI-TOF mass spectra of the peptide fragments eluted from magnetic beads after digestion of membranes covalently linked to [BAPA-Lys6,p-Bz]Phe8]SP with a, trypsin (9 h), human NK-1 receptor; b, endo-GluC digestion (15 h) of tryptic (24 h) fragments, human NK-1 receptor; c, endo-GluC digestion (8 h) of tryptic (24 h) fragments, rat NK-1 receptor; d, carboxypeptidase Y digestion (7 h) of combined trypsin (24 h)endo-GluC (9 h) digestion fragment, human NK-1 receptor; and e, carboxypeptidase Y (7 h) digestion of the combined trypsin (24 h)endo-GluC (9 h) digestion fragment, rat NK-1 receptor. *nonspecific peaks found without photolabeling.

(photoactivatable SP analog, [BAPA-Lys6,p-Bz]Phe8]SP, is (MH+) = 1808.89, the two peptides identified by MS analysis corresponded to peptide fragments from the receptor with a mass of 1171.5 and 1008.5 atomic mass units. Taking into account the uncertainties on the mass measurements (<0.1 atomic mass unit) and considering that the ligand-receptor complex was digested both by trypsin and also, as described previously, by trypsin-derived chymotryptic enzymatic activities (14), these two peptides (Protein Analysis WorkSheet analysis) could only correspond to the sequences Tyr168–Thr179–Thr–Glu–Thr–Met–Pro–Ser–Arg177 (1171.57 atomic mass units expected) and Ser169–Thr178–Thr–Glu–Thr–Met–Pro–Ser–Arg177 (1008.54 atomic mass units expected) in the second extracellular loop of the rat or human receptor. The trypsin-derived chymotrypsin activity was not inhibited even by treatment with 1-1-tosylamide-2-phenyl-ethylchloromethyl ketone (0.02 mg/ml) in the course of trypsin digestion (15 h). The trypsin digestion of [BAPA-Lys6, p-Bz]Phe8,Pro9,Met(O2)11]SP itself was also detected by MALDI-TOF analysis. Indeed, after incubation of [BAPA-Lys6, p-Bz]Phe8,Pro9,Met(O2)11]SP with trypsin under the same experimental conditions as those used for the receptor, three peptide fragments, BAPA-Arg-Pro-Lys-Pro-Gln-Gln-GluCOOH (MH+ at m/z 1257.6), BAPA-Arg-Pro-Lys-Pro-Gln-GluCOOH (MH+ at m/z 982.4), and BAPA-Arg-Pro-Lys-GluCOOH (MH+ at m/z 757.3), could be identified (data not shown). Tryptic digestion of the covalent complex after photoinertion of [BAPA-Lys6, p-Bz]Phe8,Pro9,Met(O2)11]SP-(7-11) on the rat or human NK-1 receptor gave by MS analysis two peptides with quasi-molecular ions at m/z 2446.1 and 2283.0 (Fig. 2a), respectively. The peptide corresponding to the highest mass was the more abundant at short incubation times with trypsin, whereas after long periods of incubation the peptide with the lowest mass was the only one observed. After subtracting the mass of the photoactivatable peptide, domains Tyr168–Arg177 and Ser169–Arg177 of the NK-1 receptor were again identified as the site of photoinsertion.
GluC led to a single peak at \( m/z \) 1864.9, which corresponded again to domain Thr\textsubscript{173}–Arg\textsubscript{177} (590.29 atomic mass units measured and expected) of the receptor. It should be mentioned that an oxidized form for the species at \( m/z \) 1864.9 was also observed (Fig. 2\textit{d}, inset) indicating that the receptor fragment contains a residue that can be oxidized, likely the methionine in the sequence Thr\textsubscript{173}–Arg\textsubscript{177}.

**Carboxypeptidase Y Digestion on Beads of the Tryptic/Endo-GluC Fragments**—Combined tryptic/endo-GluC fragments from the human NK-1 receptor linked to [BAPA\textsubscript{0},(p-Bz)Phe\textsubscript{8}]SP were further submitted to carboxypeptidase Y digestion on beads before MS analysis. Carboxypeptidase Y digestion from 6 to 24 h led to peptides corresponding to the removal of both Arg\textsubscript{177} and Ser\textsubscript{176} from the C terminus of the tryptic fragment Ser\textsubscript{169}–Arg\textsubscript{177} or of the combined tryptic/endo-GluC digest Thr\textsubscript{173}–Arg\textsubscript{177} (Fig. 1, d and e). We have previously established that after CNBr cleavage and MALDI-TOF analysis of the fragment, the methyl of the Met\textsubscript{174} side chain was the site of covalent insertion of \( p \)-benzoyl probe from both [BAPA\textsubscript{6},(p-Bz)Phe\textsubscript{8}]SP and [BAPA\textsubscript{6},(p-Bz)Phe\textsubscript{8},Pro\textsubscript{9}]SP (15). Altogether, experiments reported in this study support this result and Met\textsubscript{174} is also likely to be the site of covalent attachment of [BAPA-Lys\textsubscript{6},(p-Bz)Phe\textsubscript{8},Pro\textsubscript{9},Met(O\textsubscript{2})\textsubscript{11}]SP-(7–11).

**Molecular Modeling of the Human NK-1 Receptor**—This study suggests a spatial proximity in the binding site between Met\textsubscript{174} and residue Phe\textsubscript{8} of substance P, whereas studies from Boyd and colleagues (16) indicated that Met\textsubscript{181} was the major site of photoinsertion. Both methionines belong to the E2 loop connecting transmembrane helices H4 and H5; this E2 loop may be either part of or proximal to the binding site in the NK-1 receptor. To get insight into the molecular basis of SP recognition, a model of the human NK-1 receptor was built to predict the conformation of the E2 loop and the positions of Met\textsubscript{174} and Met\textsubscript{181}. The three-dimensional structure of the human NK-1 receptor was modeled on the basis of its structural similarity with bovine rhodopsin, the only G-protein-coupled receptor for which the structure is known at atomic resolution (30). The sequence alignment of transmembrane helices was based on the structure and analysis of conserved residues in the G-protein-coupled receptor (32). The N-terminal extremity and the cytoplasmic tail beyond helix H8 were not considered in the sequence alignment, because no similarities could be detected between the NK-1 receptor and rhodopsin in these regions. The extracellular and the intracellular loops were included in the alignment. The degree of identity over 296 aligned positions is 23%. The sequences of the E2 loops were aligned by making one deletion in the human NK-1 receptor at the end of helix H4. Analysis of the sequences (Fig. 3) indicated that the E2 loops in both proteins show common properties, including conservation of the disulfide bridge between Cys\textsubscript{180} and Cys\textsubscript{105} in transmembrane helix H3 and similar lengths and distribution of polar and nonpolar residues around Cys\textsubscript{180} (Fig. 3). Furthermore, several algorithms predicted an extended secondary structure in the region around Cys\textsubscript{180}, as observed in...
the crystal structure of rhodopsin (Fig. 3). These elements suggested that rhodopsin could be used as a template to model not only the transmembrane regions but also the E2 loop. The best homology model of the NK-1 receptor is shown in Fig. 4. The r.m.s. deviation between 296 aligned Cα positions of human NK-1 receptor and bovine rhodopsin is 1.7 Å. The model is consistent with mutagenesis analysis based on engineered zinc binding sites (33). The E2 loop adopts a central position on the extracellular face of the receptor, with residues 170–183 forming a β-hairpin. The first β-strand dives down into the transmembrane domain, whereas the second β-strand is more external. The positions of the two photolabeled Met residues are shown in Fig. 4a. Met181 and Met171 lie in the inner and the outer strand of the β-hairpin, respectively. In bovine rhodopsin, the inner strand is part of the retinal binding pocket. Interestingly, the two photolabeled residues in the NK-1 receptor, Met174 and Met181, correspond to two residues in rhodopsin (Glu181 and Gly183, respectively), which are part of the retinal binding pocket. In the homology model, the side chain of Met181 of the NK-1 receptor is buried in the core of transmembrane helix bundle and close to the position occupied by the polypene chain of retinal in rhodopsin. The side chain of Met174 is also buried, but its more peripheral position on the extracellular face could make it more accessible to a photoreactive probe. The two Met residues are quite close with a distance of about 6 Å between Cα atoms. However, the relative inaccessibility of Met residues in the model led us to search for alternative conformations of the E2 loop not based on homology modeling.

Conformational Properties of the Isolated E2 Loop in Solution—To investigate the conformational preferences of the amino acid sequence of the E2 loop, we examined the solution conformation of a synthetic peptide corresponding to residues 168–196 of the human NK-1 receptor (herein termed E2 peptide). The chosen sequence encompasses the entire E2 loop plus amino acid sequence of the E2 loop, we examined the solution conformation of the synthetic E2 peptide are indicated below the NK-1 receptor sequence.

The NMR study indicates that two regions of the 168–196 fragment of the NK-1 receptor have some helical propensity. The observed helical conformation of the peptide C-terminal extremity is in agreement with the sequence localization in the receptor, as it is expected to be part of transmembrane helix H5. To investigate whether the conformation of the E2 loop observed in solution could be accommodated in the context of the full receptor, we modeled the structure of the E2 loop based on the NMR study. The experimental restraints determined for the isolated E2 loop peptide in solution were incorporated into the homology model of the NK-1 receptor. The structure was submitted to simulated annealing to satisfy the new set of restraints in the E2 loop, with the position of transmembrane helices being held. Our analysis of 30 calculated structures indicates that the E2 loop can adopt two topologies within the receptor differing by the orientation of their central helix (Fig. 4). In the structure seen in Fig. 4b, this helix lies in the center of the C-terminal extremity, whereas the second extremity is in agreement with the sequence localization in the receptor. In the major form (residues 176–182), the E2 loop conformation is more stable in the presence of SDS. Because the NMR spectra recorded in 25% TFE exhibited the highest quality in terms of chemical shift dispersion and proton line width, we used these solvent conditions to determine the three-dimensional structure of the peptide.
Chemical affinity labeling is a powerful tool used to establish the spatial proximity between photolabile residues within a ligand and its receptor. Time-consuming steps in this strategy include the identification of the residues or fragments in the protein that are covalently linked to the photoactivatable ligand. A combination of different strategies is required to identify the site of covalent attachment, such as enzymatic and/or chemical digestion(s), immunoprecipitation with antibodies against specific domains of the receptor, radiochemical sequencing, and high-performance liquid chromatography (HPLC) separation or SDS-PAGE analysis. However, G-protein-coupled receptors are particularly challenging due to their hydrophobic nature and high tendency to aggregate, which precludes working on pure or isolated receptors. Therefore, the benefit of our strategy is to bypass most of the isolation procedures, allowing the direct identification of a fragment or a residue as the site of photoinsertion without the need for radioactivity.

In this study, the use of [BAPA-(p-Bz)Phe8]SP and [BAPA-Lys6,(p-Bz)Phe8,Pro9,Met(O2)11]SP(7–11) led to the unambiguous identification of the tripeptide Thr173-Met-Pro175 in the second extracellular loop of both rat and human NK-1 receptors as the site of interaction of the photoactivatable amino acid p-benzoyl-l-phenylalanine, incorporated in position 8 of both SP analogs. All of these experiments were performed more than 10 times and led unambiguously to this fragment of the NK-1 receptor, whatever the NK-1 receptor species or the photoactivatable SP analog used. The data do not pinpoint any new anchoring point for substance P in the NK-1 receptor. However, they indicate that two different photoreactive peptide analogs that differ in their pharmacological profiles and are activatable at the same position (8) in their sequence do interact with the same domain of the NK-1 receptor, whether rat or human species. We had previously established, using cyanogen bromide cleavage and MALDI-TOF analysis, that the covalent attachment is on the methyl of the Met174 side chain (15). A steric factor induced by this modification on the adjacent methionine probably interrupts herein the cleavage at Pro175 by carboxypeptidase Y, because digestion of a model peptide, ACTH-(17–39), shows that removal of a proline by carboxypeptidase Y may indeed occur, as demonstrated by MALDI-TOF analysis (data not shown).

These two photoreactive analogs were designed to screen the two binding sites described for the NK-1 receptor. The results obtained show that these two analogs interact with the same sequence of the NK-1 receptor within the E2 loop. However, the two binding sites of the NK-1 receptor might still be different conformations or protein isoforms.

In all of these experiments we always found Met174 as the site of photoinertion, whereas Kage et al. (16) found Met181 as the site of covalent attachment of [125I-BH](p-Bz)Phe8]SP on the rat NK-1 receptor. But in a more recent study, the same group has shown that both Met181 and Met174 are labeled in the same position of the receptor whereas in the alternative conformation in Fig. 4c it is more peripheral. The positions of residues Met174 and Met181 are shown in Fig. 4. In the NMR-based conformations seen in Fig. 4b, the two Met residues are more accessible than in the homology model. The photolabeling data support the model in Fig. 4b rather than in Fig. 4c because both Met residues are accessible and in close proximity.

**DISCUSSION**

Affinity labeling is a powerful procedure used to establish the spatial proximity between photolabile residues within a ligand and its receptor. The time-consuming step in this strategy is the identification of the residue, or fragment of residues, in the protein that is covalently linked to the photoactivatable ligand. A combination of different strategies is indeed required to identify the site of covalent attachment, *i.e.* enzymatic and/or chemical digestion(s), immunoprecipitation with antibodies against specific domains of the receptor combined with either HPLC separation or SDS-PAGE analysis, and finally in some cases radiochemical sequencing. With G-protein-coupled receptors this procedure is further hampered by the hydrophobicity of these proteins and their high tendency to aggregate, which precludes working on pure or isolated receptors. Therefore, the benefit of our strategy is to bypass most of the isolation procedures, allowing the direct identification of a fragment or a residue as the site of photoinsertion without the need of radioactivity.

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These two photoreactive analogs were designed to screen the two binding sites described for the NK-1 receptor. The results obtained show that these two analogs interact with the same sequence of the NK-1 receptor within the E2 loop. However, the two binding sites of the NK-1 receptor might still be different conformations or protein isoforms.

In all of these experiments we always found Met174 as the site of photoinertion, whereas Kage et al. (16) found Met181 as the site of covalent attachment of [125I-BH](p-Bz)Phe8]SP on the rat NK-1 receptor. But in a more recent study, the same group has shown that both Met181 and Met174 are labeled in the same position of the receptor whereas in the alternative conformation in Fig. 4c it is more peripheral. The positions of residues Met174 and Met181 are shown in Fig. 4. In the NMR-based conformations seen in Fig. 4b, the two Met residues are more accessible than in the homology model. The photolabeling data support the model in Fig. 4b rather than in Fig. 4c because both Met residues are accessible and in close proximity.
Gly166 at the junction of helix H4 and the E2 loop induces a change in tachykinin ligand selectivity and alters the conformation of the receptor. Furthermore, the binding of substance P is abolished by reducing agents, indicating that the Cys105–Cys160 disulfide bridge is of major importance in maintaining the conformation of the receptor and of the E2 loop in particular (34).

The conformation of the E2 loop has been modeled on the hypothesis of its structural similarity with the E2 loop of bovine rhodopsin. In the calculated homology model, the two Met residues are in close proximity, ~6 Å. However the side chains of Met174 and Met181 are not likely to be directly accessible to photoreactive probes. This accessibility may depend on the activation-deactivation state of the receptor, and/or the desensitization process. Importantly, the template used for homology modeling is the structure of rhodopsin covalently bound to 11-cis retinal, which therefore corresponds to an inactive state. It seems likely that the homology model of the NK-1 receptor also corresponds to an inactive state. The conformational changes that occur upon agonist binding probably involve movements of transmembrane helices (45), but so far very little is known about the putative conformational changes of transmembrane helices in their outer part or of extracellular loops.

In an attempt to model alternative conformations of the E2 loop, we have analyzed the structural preferences of the amino acid sequence of the E2 loop. NMR studies of a synthetic fragment 168–196 of human NK-1 receptor showed that the region 176–182 has a helix propensity in the presence of TFE or SDS. These solvents were used to stabilize secondary structures and mimic the water-membrane interface. MacDonald et al. (46) have applied a similar approach in a synthetic fragment 162–198 of rat NK-1 receptor in phospholipid vesicles. Despite different peptide sequences and solvent conditions, the two structures are very similar. The conformational preferences of the E2 loop in solution were used to generate other models in which the Met residues are more accessible. The structure in Fig. 4b is very similar to the model described by Pellegrini et al. (47) and is in good agreement with photolabeling data.

Two strategies have been used herein to model the E2 loop of the NK-1 receptor, one based on sequence homology to rhodopsin and the other based on the secondary structure of the E2 loop in solution. Homology modeling of the E2 loop yields interesting information, as it indicates that the structure of the extracellular domain of the NK-1 receptor is not completely identical to the one of rhodopsin in the dark state. So far, very few biophysical studies have been carried out to analyze the conformational changes in the extracellular part of G-protein-coupled receptors that occur during activation. Therefore the proposed conformations of the E2 loop remain working models. What can be concluded here from the modeling data is that they are in good agreement with results obtained from the photolabeling study. In the homology model as well as in the NMR-based model, Met174 and Met181 are spatially very close, which might explain why these two amino acids can be photolabeled. In the NMR-based model, the two Met residues are well positioned to be cross-linked by photoreactive analogs of substance P. In our view, it will be necessary to identify more anchoring points from photolabeling to go further into the characterization of the interaction between SP and the NK-1 receptor. However, the question of the dynamics within the ligand–receptor complex or during the recognition process will probably limit this approach. Therefore, complementary strategies to mutagenesis and photolabeling studies will be required.

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Involvement of the Second Extracellular Loop (E2) of the Neurokinin-1 Receptor in the Binding of Substance P: PHOTOAFFINITY LABELING AND MODELING STUDIES
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