Characterization of Non-peptide Antagonist and Peptide Agonist Binding Sites of the NK1 Receptor with Fluorescent Ligands*

(Received for publication, November 13, 1996, and in revised form, February 21, 1997)

Gerardo Turcati‡‡, Sannah Zoffmann§§, John A. Lowe III, Susan E. Drozda, Gérard Chassaing**, Thue W. Schwartz‡, and André Chollet‡‡

From the Geneva Biomedical Research Institute, Glaxo Wellcome, CH-1228 Geneva, Switzerland, the Laboratory for Molecular Pharmacology, Department of Protein Chemistry, Molecular Biology Institute, University of Copenhagen, DK-2100, Denmark, the Pfizer Central Research, Groton, Connecticut 06340, and the **Université Pierre et Marie Curie, CNRSURA 493, F-75252 Paris, France

Ligand recognition of the NK1 receptor (substance P receptor) by peptide agonist and non-peptide antagonist has been investigated and compared by the use of fluorescent ligands and spectrofluorometric methods. Analogues of substance P (SP) labeled with the environment-sensitive fluorescent group 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) at either position 3, 8, or 11 or with fluorosecin at the Nα position were synthesized and characterized. Peptides modified at the ω-amino group or at positions 3 or 11 conserved a relatively good affinity for NK1 and agonistic properties. Modification at position 8 resulted in an 18,000-fold decrease in affinity. A fluorescent dansyl analogue of the non-peptide antagonist CP96,345 was prepared and characterized. The quantum yield of fluorescence for dansyl-CP96,345 was much higher than for any of the dansyl-labeled peptides indicating that the micro-environment of the binding site is more hydrophobic for the non-peptide antagonist than for the peptide agonists. Comparison of collisional quenching of fluorescence by the water-soluble hydroxy-Tempo compound showed that dansyl-CP96,345 is buried and virtually inaccessible to aqueous quenchers, whereas dansyl- or fluorosecineyl-labeled peptides were exposed to the solvent. Anisotropy of all fluorescent ligands increased upon binding to NK1 indicating a restricted motional freedom. However, this increase in anisotropy was more pronounced for the dansyl attached to the non-peptide antagonist CP96,345 than for the fluorescent probes attached to different positions of SP. In conclusion, our data indicate that the environment surrounding non-peptide antagonist and peptide agonists are vastly different when bound to the NK1 receptor. These results support recent observations by mutagenesis and cross-linking work suggesting that peptide agonists have their major interaction points in the N-terminal extension and the loops forming the extracellular face of the NK1 receptor. Our data also suggest that neither the C terminus nor the N terminus of SP appears to penetrate deeply below the extracellular surface in the transmembrane domain of the receptor.

Many peptide hormones and neuropeptides act via known receptors belonging to the superfamily of G protein-coupled receptors characterized by a seven membrane-spanning topology. There is considerable interest in understanding ligand-receptor recognition and the mechanisms of action of both non-peptide ligands and natural peptides for peptide receptors. The tachykinin substance P (SP) is a peptide transmitter that plays an important role in pain perception and neurogenic inflammation (1, 2). The cellular actions of SP are mediated by the tachykinin (neurokinin) NK1 receptor, a G protein-coupled receptor. Therefore, the NK1 receptor has been the target for the development of multiple non-peptide antagonists. The prototype NK1 non-peptide antagonist is the quinuclidine compound CP96,345, which acts as a high affinity and highly selective non-peptide inhibitor of SP in both binding and functional assays (3, 4).

Traditionally, the identification of binding domains for peptide agonists and non-peptide antagonists has been investigated by site-directed mutagenesis of receptors and by the construction of chimeric receptors (5). In the case of NK1, studies by different groups have localized a binding pocket for the antagonist CP96,345 in the outer portion of the transmembrane domain of the receptor with presumed contacts points clustering on transmembrane domains TM-III, -IV, -V, VI, and VII and facing the interior of the seven-helix bundle (Fig. 1) (6–13). In contrast, the binding site for substance P appears to involve multiple domains on the extracellular side of NK1 including the N-terminal segment, the first extracellular loop, and the top of TM-III and -VII (Fig. 1) (10–12). One still open and debated question is whether substance P makes additional contacts within the transmembrane domains. So far mutational analysis of this region has failed to give any clear answer to this (12, 13). The main problem with mutational mapping experiments is that they do not necessarily reveal direct contact points between amino acids on the receptor and ligand functional group. Loss of binding affinity can be due to either a true contact residue or to indirect allosteric effect on the receptor folding. Covalent labeling of NK1 with photolabile SP analogues has identified agonist peptide-binding domains of NK1 in proximity of the binding site.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.

¶ To whom correspondence should be addressed: Geneva Biomedical Research Institute, 14 Chemin des Aulx, CH-1228 Plan-les-Ouates, Switzerland. Tel.: 41 22 706 9666; Fax: 41 22 794 6965; E-mail: arc3029@ggr.co.uk.
the N-terminal region and the second extracellular loop using ligands labeled at either position 3 or 8 of the peptide (14, 15, 35).

It is therefore very important to complement the mutational approach with biochemical and spectroscopic techniques. Recently, we developed novel fluorescence methodology to investigate ligand-receptor recognition and to map subdomains of binding sites with fluorescent ligands. In the NK2 receptor system, we demonstrated that agonist and antagonist peptides of similar molecular size have distinct binding sites (16), and we built a molecular model for ligand-receptor interactions using experimental determination of distances by fluorescence energy transfer between a fluorophore on the ligand and another fluorophore placed at specific sites in the receptor through biosynthetic incorporation by suppression of nonsense codons (17).

Here we have used site-specific fluorescent-labeled analogues of substance P at positions 1, 3, and 11 and a fluorescent CFp6,345 analogue to probe the polarity and solvent accessibility of NK1 binding pockets and to measure the motional freedom of receptor-bound ligands. In this study we present spectrofluorometric evidence for the existence of different ligand binding pockets on the NK1 receptor for substance P and the antagonist CFp6,345, respectively.

**EXPERIMENTAL PROCEDURES**

**Synthesis of Fluorescent Ligands**

N²-Dansyl-Lys²-SP (Dns-Lys²-SP)—To 2.34 mg of SP (1.73 µmol) in 360 µl of 50 mM sodium borate, pH 9.0, at 4 °C was added 42 µl of 50 mM dansyl chloride in acetone (2.07 µmol). The mixture was stirred for 2 h at 4 °C. The mono-dansylated SP derivative was recovered as the minor peak of the mixture. Electrospray MS: calculated, 1519.86; found, 1519.69.

S-((2-Dansylamino)-ethyl)-Hcy¹¹-SP—Dns-Hcy¹¹-SP was prepared as described by Chassaing et al. (19).

N²-Fluorescin thioarbazyl-SP (N²-Flu-SP)—To 4.35 mg of SP (2.84 µmol) in 500 µl of 25 mM sodium borate, pH 9.0, at 4 °C was added 62 µl of 50 mM fluorescein isothiocyanate in N,N-dimethylformamide. The mixture was stirred for 3 h at 4 °C, and then 100 µl of N,N-dimethylformamide and 500 µl of 0.2 mM acetic acid were added. Monofluorescin SP was recovered by high performance liquid chromatography (same conditions as above) as the major peak of the mixture. Yield: 2.3 mg (47%). Electrospray-MS, calculated, 1737.1; found, 1736.9.

Edman degradation: cycle 1 yielded Arg-PTH for SP but not for [N²-Flu-SP]; cycles 2 and 3 yielded Pro-PTH and Lys-PTH respectively, for both SP and N²-Flu-SP.

(←)-Diphenylmethyl-3-(6-methoxy-5-aminophenyl)methylamino)-1-azabicyclo[3.2.2]octane—To a 150-ml round-bottomed flask equipped with condenser and N₂ inlet were added 3.42 g (11.71 mmol) of (←)-diphenylmethyl-1-azabicyclo[3.2.2]octan-3-amine, prepared as described in Ref. 20, 2.51 g (15.23 mmol) of 5-nitroanisaldehyde, prepared according to Ref. 21, and 60 ml of methanol. The solution was stirred 40 min at room temperature, and the resulting precipitate was collected by filtration and dried. It was then taken up in 46 ml of dry tetrahydrofuran, 28 ml (56 mmol) of a 2.0 M solution of borane methyl sulfide in tetrahydrofuran added, and the reaction refluxed for 4 days. The reaction was cooled, evaporated, and taken up in 50 ml of ethanol, treated with 5 g of sodium carbonate and 4 g of cesium fluoride. The resulting mixture was refluxed 3 days, cooled, and taken up in methylene chloride and water. The organic layer was separated, dried over sodium sulfate, and evaporated. The residue was chromatographed on silica gel using methanol/methylene chloride as eluant and then triturated with diethyl ether to afford 2.088 g (42% overall) of a white solid, m.p. 185–189 °C, after evaporation from methanol chloride. ¹H NMR (δ, CDCl₃): 1.30 (m, 1H), 1.57 (m, 1H), 1.66 (m, 1H), 1.97 (m, 1H), 2.10 (m, 1H), 2.68 (m, 1H), 2.84 (m, 2H), 2.97 (m, 1H), 3.30 (m, 1H), 3.40 (dd, J = 8, 12, 1H), 4.49 (d, J = 12, 1H), 5.80 (d, J = 2, 1H), 6.5–7.5 (m, 12H). ¹³C NMR (δ, CDCl₃): 20.1, 24.7, 25.5, 42.0, 45.7, 49.3, 49.6, 54.2, 55.9, 61.7, 111.5, 114.0, 116.6, 125.9, 126.2, 127.5, 127.7, 128.4, 128.9, 129.1, 139.5, 143.6, 145.5, 150.7. MS (%): 428 (parent + 1, 1), 291 (20), 274 (18), 260 (100), 136 (28). High Resolution MS: calculated for C₂₈H₃₃N₅O, 427.2624; found, 427.2613. [α]D = −16.6° (c = 1, CH₂Cl₂).

C₂₈H₃₃N₅O · 1/₄ CH₂Cl₂

Calculated: C 75.60 H 7.52 N 9.36

Found: C 75.20 H 7.53 N 9.26

(←)-Diphenylmethyl-3-(6-methoxy-5-aminophenyl)methylamino)-1-azabicyclo[3.2.2]octane (Dns-CFP6,345)—To a 150-ml round-bottomed flask equipped with condenser and N₂ inlet were added 100 mg (0.234 mmol) (←)-diphenylmethyl-3-
Ligand Recognition of the NK1 Receptor

6.41 (d, 1H), 1.42 (m, 1H). 8.15 (m, 1H), 8.42 (m, 1H), 8.51 (m, 1H). 13C NMR (CDCl3): 1.25 (m, 1H), 2.6–2.8 (m, 2H), 3.61 (dd, 1H), 4.40 (d, J = 12, 1H), 6.26 (broad s, 1H), 6.41 (J = 6, 1H), 6.77 (m, 1H), 7.0–7.3 (m, 12H), 7.44 (m, 1H), 7.60 (m, 1H), 7.84 (m, 1H). 110.2, 115.2, 118.7, 122.7, 123.1, 124.6, 126.0, 126.2, 127.5, 127.6, 128.3, 128.5, 128.9, 129.0, 129.7, 129.8, 130.2, 130.6, 134.5, 143.2, 145.3, 152.1, 155.5. MS (%): 644 (parent + 1, 1), 493 (40), 291 (100), 125 (70), 105 (75), 96 (60), 84 (70). High resolution MS: calculated for C29H42N2O6S2: 660.13154; found, 660.13281.

Cell Culture

Fluorescence experiments were performed with either CHO cells stably transfected with the human NK1 receptor and expressing about 150,000 NK1 receptors per cell and obtained using the Semliki forest virus system as described (22). All cells were cultured as monolayers in a humidified 5% CO2 atmosphere at 37 °C, in Dulbecco’s modified Eagle’s medium/Ham’s F12 medium supplemented with 10% fetal calf serum, 2% (v/v) Pen-Strep, and 0.5% (v/v) Nystatin. Cells were harvested at 80% confluence with PBS containing 1 mM EDTA and washed with ice-cold PBS. Membrane fractions were prepared as described previously (23).

For radioligand binding assays, the expression plasmids containing the receptor cDNAs were transiently transfected into COS-7 cells by the calcium phosphate precipitation methods as described previously (24).

Competition Binding Experiments

Monoiodinated 125I-Bolton-Hunter-labeled substance P (125I-BH-SP) was prepared and purified as described (24). The transfected COS-7 cells were transferred to 12-well culture plates. 0.1–0.6 × 10^6 cells/well, 1 day after transfection and 24 h before performing the binding experiments. The number of cells per well was adjusted according to the mitotic index. The number of cells per well was adjusted according to the mitotic index. The number of cells per well was adjusted according to the mitotic index. The number of cells per well was adjusted according to the mitotic index. The number of cells per well was adjusted according to the mitotic index.

Collisional Quenching Experiments

Fluorescence collisional quenching experiments were performed at 20 °C by adding increasing amounts of quencher stock solution in water to suspended NK1/CHO cell membranes. Typically, a complete quenching experiment was performed in 6–8 min. Under these conditions, the contribution of fluorescence signal from dissociated ligand was negligible. The quencher stock solutions were 0.135 M KI containing 1 mM NaZnO2 to prevent Ir formation or 0.1 M Tempo or 0.1 M hydroxy-Tempo. Changes in fluorescence due to the addition of quencher were corrected by subtracting the fluorescence measured in parallel samples in which quencher was added to membranes in PBS or membranes saturated with an excess (1000-fold) of non-fluorescent CP99,994 (36). Moreover, in quenching experiments with the nitroxide radical compounds Tempo and hydroxy-Tempo, the fluorescence intensities were corrected for the absorption increments caused by added quenchers at the excitation and emission wavelengths (inner filter effect) as described (25). The quenching of the fluorescence emission at the wavelength of maximum emission was calculated with the Stern-Volmer equation (26): F/F0 = 1 + Ksv [Q], where F/F0 is the ratio of the fluorescent ligand to NK1 in CHO cell membranes was made as described above. The limiting anisotropy A0 in the absence of rotation was calculated with the Perrin equation (Equation 2) for the case of a spherical molecule (26):

\[ A = \left[ \frac{\langle I_1 \rangle - Q}{\langle I_2 \rangle + Q} \right] - 1 \]  

where \( I_1/I_2 \) is the ratio of the vertical and horizontal emission intensities when the excitation polarizer is in the vertical position (V) and the emission-polarizer either in the horizontal (H) or vertical position. The emission intensities, in respective V and H positions, were corrected by subtracting the corresponding background signals from the cells or membrane suspensions and converted to anisotropy (A), see Equation 1:

\[ A = \left[ \frac{\langle I_1 \rangle - Q}{\langle I_2 \rangle + Q} \right] - 1 \]  

where \( I_1/I_2 \) is the ratio of the vertical and horizontal emission intensities when the excitation polarizer is in the vertical position, and G is the same ratio when the excitation light is horizontally polarized. Binding of fluorescent ligands to NK1 in CHO cell membranes was made as described above. The limiting anisotropy A0 in the absence of rotation was calculated with the Perrin equation (Equation 2) for the case of a spherical molecule (26):

\[ A = A_0^{-\frac{1}{6}} \left( 1 + \frac{1}{6} T \right) = A_0^{-\frac{1}{6}} \left( 1 + \frac{3}{2} T V_n n \right) \]  

where \( T = \frac{V_n}{V_w} \) is the fractional volume of a hydrated sphere, and \( V_n \) is the viscosity. Perrin plots were constructed by measuring anisotropy of free ligands, 0.1 to 10 μM in PBS solution, or bound to membranes from NK1/CHO cells, as a function of either temperature or viscosity. Anisotropy on free, unbound ligand was also measured in the absence of fluorescence binding. Membranes were then washed twice with ice-cold PBS, pH 7.2, resuspended in ice-cold PBS, and homogenized by sonication in an ice-cold bath for 3 min immediately prior fluorescence measurements. During fluorescence recordings, membranes were kept in suspension by continuous stirring. Fluorescence was corrected for light scattering and background fluorescence from control samples. For dansyl compounds excited at 343 nm, the scattering caused by irradiation of membranes particles was not important in the 520–540-nm emission region. For the fluoresein ligand N'-Flu-SP, the effect of scattering was low in comparison to the high quantum yield of this fluorophore.

Fluorescence Anisotropy Measurements—Steady-state anisotropy measurements were recorded using a Jasco FP-777 spectrofluorometer equipped with a model ADP-301 fluorescence polarization accessory. The polarizer and analyzer were placed in the thermostatic sample chamber. The emission intensity was measured by setting the excitation and emission wavelengths (inner filter effect) as described (25). The quenching of the fluorescence emission at the wavelength of maximum emission was calculated with the Stern-Volmer equation (26): F/F0 = 1 + Ksv [Q], where F/F0 is the ratio of the fluorescent ligands Tempo and hydroxy-Tempo, the fluorescence intensities were corrected for the absorption increments caused by added quenchers at the excitation and emission wavelengths (inner filter effect) as described (25). The quenching of the fluorescence emission at the wavelength of maximum emission was calculated with the fracture anisotropy equation (26): F/F0 = 1 + Ksv [Q], where F/F0 is the ratio of the fluorescent ligands Tempo and hydroxy-Tempo, the fluorescence intensities were corrected for the absorption increments caused by added quenchers at the excitation and emission wavelengths (inner filter effect) as described (25).
samples were measured at corresponding temperatures after 4–5 min only, indicating that the measured anisotropy accounted for NK1 bound rather than dissociated ligand. Alternatively, the viscosity was varied by addition of increasing amounts of glycerol (0–80% w/v) at constant temperature (20 °C). The viscosity at each temperature was determined from standard tables (40). Anisotropy measurements were made using different membrane concentrations (39).

RESULTS

Design and Synthesis of Fluorescent Ligands—A series of undecapeptides derived from substance P and labeled with either dansyl (Dns) or fluoresceinyl (Flu) fluorophores at different positions in the sequence were prepared (Fig. 2). The control of selectivity for the N-terminal amino group or the ε-amino group in the lysyl side chain during chemical modification of peptides can be controlled by the pH of the reaction and the nature of the electrophilic reagent. Thus, substance P was derivatized with an equimolar amount of dansyl chloride. Coupling of the remaining seven amino acids was then resumed. The fluorescent peptide Dns-Dap8-SP, in which Phe8 of SP is replaced by β-dansylaminopropionic acid, was prepared by solid phase peptide synthesis. After the third coupling cycle with Dap, the β-amino group of Dap was selectively deprotected and derivatized with dansyl chloride. Coupling of the remaining seven amino acids was then resumed. The S-dansyl homocysteine derivative Dns-Hcy11-SP, in which Met11 of SP is replaced by S-(2-dansylamino)ethyl homocysteine was prepared as reported before (19).

The quinuclidine derivative Dns-CP96345, a dansyl-labeled analogue of the well known NK1 antagonist CP96,345 (3), was prepared as shown in Fig. 3.

![Scheme for the synthesis of dansyl-CP96345](image)

Fluorescence Properties of Ligands—The excitation and emission maxima of dansyl- and fluorescein-labeled ligands are shown in Table I. The quantum yield of dansyl fluorescence emm2 of dansyl label at pH 9.0 to afford the mono-labeled compound Dns-Lys3-SP. Reaction of SP with fluorescein at the N-terminal position (Nα-Flu-SP) or at position 11 as in Dns-Hcy11-SP resulted in a 70–100-fold decrease in affinity compared with SP. More dramatically, Dns-Dap8-SP showed a 18,000-fold decrease in affinity compared with SP that prevented its further use as a fluorescent probe of the binding pocket. Compounds Nα-Flu-SP, Dns-Lys3-SP, and Dns-Hcy11-SP were still agonists at NK1 (Table II), and 2) evoke Ca2+-dependent chloride currents in Xenopus oocytes expressing NK1 (data not shown).

![Fluorescence properties of a representative dansyl-labeled NK1 ligand in solvents of different polarity](image)

Pharmacological Characterization of Fluorescent Peptide Ligands—The fluorescent ligands shown in Figs. 2 and 3 were assayed for NK1 binding affinity by competitive binding analysis with [125I]BH-SP using COS-7 cells (Fig. 5). The results are summarized in Tables II and III. Labeling of SP with dansyl at position 3 (Dns-Lys3-SP) maintained a high affinity for NK1, in the nanomolar range and comparable to the parent SP. Modification of SP with fluorescein at the N-terminal position (Nα-Flu-SP) or at position 11 as in Dns-Hcy11-SP resulted in a 70–100-fold decrease in affinity compared with SP. More dramatically, Dns-Dap8-SP showed a 18,000-fold decrease in affinity compared with SP that prevented its further use as a fluorescent probe of the binding pocket. Compounds Nα-Flu-SP, Dns-Lys3-SP, and Dns-Hcy11-SP were still agonists at NK1 as assayed by their ability to 1) mobilize intracellular Ca2+ in CHO cells stably transfected with NK1 (Table II), and 2) evoke Ca2+-dependent chloride currents in Xenopus oocytes expressing NK1 (data not shown).

Pharmacological Characterization of the Fluorescent Nonpeptide Dns-CP96345—Dns-CP96345 had a high, nanomolar affinity for NK1 with only an 8-fold decrease compared with the

![Fluorescence properties of a representative dansyl-labeled NK1 ligand in solvents of different polarity](image)
parent compound CP96,345 (Fig. 5). To compare the mode of binding of Dns-CP96,345 to that of CP96,345, binding constants were determined in a series of NK1 point mutants (Table III). These mutations included sites that were known to be important for CP96,345 binding. For most of the mutants tested, the change in affinity compared with wild-type receptor \( f_{\text{mut}} \) was similar for CP96,345 and Dns-CP96,345. However, for mutant F264A near the top of TM-VI, the binding affinity for Dns-CP96,345 increased 2-fold. In contrast, the affinity of the parent CP96,345 decreased 17-fold in this mutant (Table III). A similar but less pronounced effect was observed for mutants F267A and F268A. There was only one mutant, F264Y, for which Dns-CP96,345 lost significantly more affinity than did CP96,345. Taken together, these data indicate that the dansyl group of Dns-CP96,345 could have the same binding determinants on the NK1 receptor. The mutants F267A and F268A. There was only one mutant, F264Y, for which Dns-CP96,345 lost significantly more affinity than did CP96,345. Taken together, these data indicate that the dansyl group of Dns-CP96,345 could have the same binding determinants on the NK1 receptor. The data also suggest that the dansyl group of Dns-CP96,345 could be located in the vicinity of residue Phe\(^{264}\) and, possibly, residues Phe\(^{267}\) and Phe\(^{268}\).

**Interactions of Fluorescent Ligands with the NK1 Receptor—** Fluorescence emission of ligands bound to NK1 was measured in a suspension of membranes from stably transfected CHO cells with an estimated total concentration of binding sites of 3–5 nm. Fig. 6 shows for each ligand the fluorescence spectra of total observed fluorescence, the nonspecific fluorescence due to binding of ligands into the membrane and determined in the presence of a 1000-fold excess of unlabeled ligand, and the fluorescence of an equivalent amount of compound (5 nm) in solution without membranes. The nonspecific binding of fluorescent ligands in membranes was about 5% for \( N^a \)-Flu-SP, 25% for Dns-Hcy\(^{11}\)-SP, and 45% for Dns-CP96,345. The comparison of the specific fluorescence intensity emission for the dansyl-labeled ligands bound to NK1 and in solution, after correction for the fluorescence of membranes labeled in the presence of an excess of nonfluorescent ligand, is a direct indication of the relative polarity of the environment around the bound fluorescent group. For Dns-CP96,345 and Dns-Hcy\(^{11}\)-SP, there was a 22- and 10-fold increase, respectively, in fluorescence intensity accompanied by a blue-shift of the emission maximum from 540 to 515 ± 5 nm and 525 ± 5 nm, respectively, upon binding to NK1. This indicates an increase in hydrophobicity around the fluorescent group. In contrast, for Dns-Lys\(^3\)-SP the lack of detection of fluorescence signal indicated a different, more hydrophilic environment. Flu-N\(^{-}\)-SP which is brighter and less sensitive to the polarity than dansyl was used to probe the N terminus of SP.

**Probing the Accessibility of Bound Ligands by Collisional Quenching—** The nitroxide radical compounds hydroxy-Tempo (water-soluble) and Tempo (lipid-soluble) were used to probe the accessibility of bound dansyl-labeled ligands. In aqueous solution, intermolecular quenching of singlet excited state radicals occurs via an electron exchange (transfer) mechanism (27). The fluorescence of ligands shown in Figs. 2 and 3 free in solution was efficiently quenched by addition of increasing amounts of Tempo or hydroxy-Tempo to the solution. The very weak fluorescence emission of NK1-bound Dns-Lys\(^3\)-SP precluded quenching experiments with this ligand. Iodide anion and neutral hydroxy-Tempo were used as fluorescein quenchers in experiments with Flu-N\(^{-}\)-SP. Linear Stern-Volmer plots were obtained indicating collisional quenching. The Stern-Volmer constants (Table IV), which are the slopes of \( F_0 \) as a function of the quencher concentration, provide a relative measure of the degree of accessibility of the fluorescent group to the quencher. The Tempo compounds efficiently quenched the dansyl fluorescence of Dns-CP96,345 and Dns-Hcy\(^{11}\)-SP in solution with \( K_{SV} \) values of 27–28 m\(^{-1}\) comparable to the previously reported value of 20 m\(^{-1}\) for quenching of dansyl-choline in 1-butanol (28). The \( K_{SV} \) value for hydroxy-Tempo quenching of fluorescein in the Flu-N\(^{-}\)-SP peptide in PBS was 21 m\(^{-1}\), a value comparable to the previously reported value of 20 m\(^{-1}\) for fluorescein-labeled compounds (29).

The specific fluorescence of Dns-CP96,345 bound to NK1 was not quenched by either water-soluble hydroxy-Tempo or lipid-soluble Tempo (Fig. 7). However, nonspecifically bound Dns-CP96,345 was quenched by collision with Tempo in the mem-

**Table II**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>IC(_{50})</th>
<th>EC(_{50}) curve</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nm</td>
<td>% of maximum SP</td>
</tr>
<tr>
<td>SP</td>
<td>0.29 ± 0.06</td>
<td>0.60 ± 0.16</td>
</tr>
<tr>
<td>Dns-Lys(^3)-SP</td>
<td>1.1 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>Dns-Dap(^8)-SP</td>
<td>5150 ± 1440</td>
<td>18,000</td>
</tr>
<tr>
<td>Dns-Hcy(^{11})-SP</td>
<td>29.2 ± 6.4</td>
<td>100</td>
</tr>
<tr>
<td>N-(\alpha)-Flu-SP</td>
<td>21.1 ± 3.7</td>
<td>13.55 ± 3.61</td>
</tr>
</tbody>
</table>

\( ^{a} \) ND, not determined.

![Figure 5](http://www.jbc.org/)

**Figure 5.** Competition binding experiments in human NK1 receptor. Binding experiments were performed on COS-7 cells as described under "Experimental Procedures." Upper panel, competition binding of \( ^{125}\)I-BH-SP by SP (●), \( N^a \)-Flu-SP (○), Dns-Lys\(^3\)-SP (×), Dns-Hcy\(^{11}\)-SP (■), or Dns-Dap\(^8\)-SP (▲). Lower panel, competition binding of \( ^{125}\)I-BH-SP by Dns-CP96,345 (●) or CP96,345 (○).
TABLE III

Comparison of competition binding of SP, CP96,345, and Dns-CP96,345 for $^{125}$I-BH-SP on NK1 in COS-7 cells. The data are the mean ± S.E. of triplicates. The number of independent experiments is shown in parentheses.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>SP IC$_{50}$</th>
<th>Dns-CP96,345 IC$_{50}$</th>
<th>CP96,345 IC$_{50}$</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.29 ± 0.02 (52)</td>
<td>3.3 ± 0.2 (11)</td>
<td>0.42 ± 0.03 (19)</td>
<td>1</td>
</tr>
<tr>
<td>H108A</td>
<td>2.9 ± 0.4 (11)</td>
<td>2.3 ± 0.2 (3)</td>
<td>1.23 ± 0.23 (5)</td>
<td>3</td>
</tr>
<tr>
<td>N109A</td>
<td>0.30 ± 0.05 (8)</td>
<td>13.5 ± 1.2 (5)</td>
<td>1.70 ± 0.53 (5)</td>
<td>4</td>
</tr>
<tr>
<td>P112A</td>
<td>0.23 ± 0.04 (7)</td>
<td>34 ± 6 (4)</td>
<td>1.47 ± 0.26 (4)</td>
<td>4</td>
</tr>
<tr>
<td>P112D</td>
<td>0.12 ± 0.01 (10)</td>
<td>150 ± 180 (4)</td>
<td>88 ± 8 (7)</td>
<td>200</td>
</tr>
<tr>
<td>Q185A</td>
<td>0.36 ± 0.06 (6)</td>
<td>92 ± 17 (4)</td>
<td>12.0 ± 1.6 (3)</td>
<td>30</td>
</tr>
<tr>
<td>H197A</td>
<td>0.32 ± 0.10 (5)</td>
<td>230 ± 50 (4)</td>
<td>42 ± 8 (4)</td>
<td>100</td>
</tr>
<tr>
<td>I198A</td>
<td>0.21 ± 0.03 (7)</td>
<td>55 ± 0.8 (3)</td>
<td>0.65 ± 0.11 (5)</td>
<td>1.5</td>
</tr>
<tr>
<td>V200A</td>
<td>0.14 ± 0.03 (12)</td>
<td>1.32 ± 0.39 (4)</td>
<td>0.30 ± 0.07 (5)</td>
<td>0.7</td>
</tr>
<tr>
<td>T201A</td>
<td>0.26 ± 0.03 (10)</td>
<td>1.94 ± 0.92 (3)</td>
<td>0.56 ± 0.11 (9)</td>
<td>1.3</td>
</tr>
<tr>
<td>F264A</td>
<td>5.0 ± 1.5 (3)</td>
<td>1.80 ± 0.44 (4)</td>
<td>7.3 ± 2.1 (4)</td>
<td>17</td>
</tr>
<tr>
<td>F264Y</td>
<td>0.36 ± 0.04 (23)</td>
<td>1.07 ± 0.18 (4)</td>
<td>2.1 ± 0.3 (10)</td>
<td>5</td>
</tr>
<tr>
<td>H265A</td>
<td>0.20 ± 0.03 (16)</td>
<td>4.6 ± 0.9 (3)</td>
<td>2.6 ± 0.7 (6)</td>
<td>6</td>
</tr>
<tr>
<td>H265F</td>
<td>0.34 ± 0.03 (20)</td>
<td>27 ± 7 (4)</td>
<td>1.47 ± 0.31 (7)</td>
<td>4</td>
</tr>
<tr>
<td>F267A</td>
<td>0.43 ± 0.09 (2)</td>
<td>3.9 ± 0.6 (3)</td>
<td>3.8 ± 1.8 (3)</td>
<td>9</td>
</tr>
<tr>
<td>F267Y</td>
<td>0.30 ± 0.04 (11)</td>
<td>0.88 ± 0.10 (3)</td>
<td>0.29 ± 0.05 (4)</td>
<td>0.7</td>
</tr>
<tr>
<td>F268A</td>
<td>1.20 ± 0.22 (3)</td>
<td>18.5 ± 3.2 (3)</td>
<td>18.6 ± 1.2 (3)</td>
<td>40</td>
</tr>
<tr>
<td>Y272A</td>
<td>0.14 ± 0.03 (6)</td>
<td>4.6 ± 0.3 (3)</td>
<td>3.2 ± 0.8 (3)</td>
<td>8</td>
</tr>
<tr>
<td>I290A</td>
<td>0.37 ± 0.03 (4)</td>
<td>1.17 ± 2.4 (3)</td>
<td>1.43 ± 0.32 (3)</td>
<td>3</td>
</tr>
</tbody>
</table>

$^a$ f$_{mut}$ = IC$_{50}$ (mutation)/IC$_{50}$ (wild type receptor).

$^b$ f$_{mut}$ CP96,345/f$_{mut}$ Dns-CP96,345.

A B C

Fig. 6. Spectrofluorometric analysis of binding of fluorescent ligands to NK1. Specific (traces 1) and nonspecific (traces 2) fluorescence spectra for ligands bound to human NK1 receptors transiently expressed in CHO cell membranes and for ligands in PBS solution in the absence of membranes (traces 3). A, dansyl nonpeptide antagonist Dns-CP96,345. B, Dns-Hcy$^{11}$-SP. C, N$^\gamma$-Flu-SP. Spectra are corrected for autofluorescence and scattering of membranes. See "Experimental Procedures" for details.

TABLE IV

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Quencher</th>
<th>$K_{SV}$ free</th>
<th>$K_{SV}$ nonspecific-bound</th>
<th>$K_{SV}$ specific-bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dns-CP96,345</td>
<td>Tempo</td>
<td>28 ± 2</td>
<td>25 ± 2</td>
<td>0.6 ± 2</td>
</tr>
<tr>
<td>Dns-CP96,345</td>
<td>Hydroxy-tempo</td>
<td>28 ± 3</td>
<td>0 ± 2</td>
<td>−1.0 ± 2</td>
</tr>
<tr>
<td>Dns-Hcy$^{11}$-SP</td>
<td>Hydroxy-tempo</td>
<td>27 ± 2</td>
<td>ND$^*$</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>N$^\omega$-Flu-SP</td>
<td>Hydroxy-tempo</td>
<td>21 ± 1</td>
<td>ND</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>N$^\omega$-Flu-SP</td>
<td>Sodium iodide</td>
<td>11 ± 1</td>
<td>ND</td>
<td>10 ± 1</td>
</tr>
</tbody>
</table>

$^*$ ND, not determined.

brane (Table IV). From this we conclude that the antagonist Dns-CP96,345 bound to NK1 is buried in a hydrophobic pocket that is inaccessible to the solvent and not in direct contact with the membrane lipids, most likely in the middle of the bundle formed by the seven membrane-spanning domains of the NK1 receptor. Fig. 8 shows the Stern-Volmer plots obtained for the agonists Dns-Hcy$^{11}$-SP and Flu-N$^\gamma$-SP. The Stern-Volmer constants for each Flu-N$^\gamma$-SP and Dns-Hcy$^{11}$-SP were comparable to those of ligands free in solution, indicating that both the fluorescein group at the N-terminal position and the dansyl group at the C-terminal position, respectively, were fully accessible and exposed to the solvent.

Probing the Molecular Mobility of Fluorescent Ligands Bound to NK1—Fluorescence anisotropy for Dns-CP96,345, Dns-Hcy$^{11}$-SP, and Flu-N$^\gamma$-SP bound to NK1 or free in solution was measured in the 2–37 °C temperature range and is shown in the Perrin plots on Fig. 9. Comparable plots were obtained at constant temperature (20 °C) by varying the viscosity (data not
shown). Limiting anisotropy, defined as the anisotropy in the absence of all rotational freedom, was calculated from the intercepts of Perrin plots on the y-axis. Limiting values of anisotropy \((A_0)\), ranging from 0.35 for Dns-CP96,345 and for Flu-Na-SP to 0.24 for Dns-Hcy11-SP, were calculated for the ligands bound to NK1 (Table V). \(A_0\) values for each ligand free in solution or bound to NK1 were similar. The binding of ligands to NK1 receptors considerably increased the dansyl or fluoresceinyl anisotropies compared with those of the ligands in solution (Table V and Fig. 9). For Dns-CP96,345, the slope of the Perrin plot for the total bound was lower than the slope for the nonspecifically bound, indicating indirectly that the motional freedom of the dansyl group in the ligand bound specifically to NK1 was more restricted than that of the ligand bound nonspecifically to membranes.

**DISCUSSION**

In this paper, we have utilized a fluorescent group in ligands to compare the binding sites of SP and the non-peptide antagonist CP96,345 in the NK1 receptor. We and others (16, 30, 37, 38) had shown that fluorescent ligands may serve as probes to investigate ligand-receptor interactions and to obtain insight into the microenvironment of binding sites. Introducing fluorescent groups in ligands while maintaining high binding affinity is a prerequisite to this approach. This is generally easier to achieve for medium to large size peptides than for small molecular weight non-peptide ligands. SP was labeled at four different sites with the fluorophore dansyl to probe all regions of the peptide, both the N and C terminus and two internal positions at residue numbers 3 and 8. Modification at position 8 with the bulky dansyl group decreased drastically NK1 affinity and was not used further. It is worth noting, however, that other SP derivatives in which Phe at position 8 was replaced by larger aromatic side chains are still binding to the NK1 receptor with high affinity (14, 15). Dansyl-labeled SP analogues at positions 1, 3, and 11 conserved reasonable affinity for SP. Dansyl was introduced into CP96,345 at a position known to tolerate different functional groups; see, for example, the use of an azide function at this position as a potent photolabeling version of CP96,345 (31). Functionally, the fluorescently labeled SP analogues maintained agonist activity, and Dns-CP96,345 was still capable of antagonizing SP at the NK1 receptor.

The putative binding site for CP96,345 has been mapped through an extensive mutational analysis of the transmembrane regions and extracellular loops of the NK1 receptor (for a review, see Ref. 32). The results from several groups suggest that CP96,345 fits into a cavity delineated by TM-III, -IV, -V, -VI, and -VII and make specific contacts by polar interactions with receptor residues as shown in Fig. 1. The mutational binding analysis of the present study shows that Dns-
CP96,345, despite the presence of the bulky dansyl group in the molecule, has a binding profile similar to that of its parent compound CP96,345, as determined by the ability to displace 125I-BH-SP binding to NK1. The only significant differences were found at positions 264, 267, and 268 which all have Phe in wild-type NK1. These positions are located on the face of TM-VI which is presumed to be turned inward and toward TM-VII (Fig. 10). Single mutations to alanine at each of these three sites affected CP96,345 more than Dns-CP96,345. Conceivably, removal of bulky phenyl side chain disturbs the shape complementarity between the non-peptide ligand and the binding cavity. A possible explanation for why this effect was less pronounced for Dns-CP96,345 could be that the dansyl group could occupy the empty space created by the Phe to Ala mutation. In contrast, adding bulk in the cavity by introduction of an hydroxyl group in the F264Y mutation decreases Dns-CP96,345 affinity more than that of CP96,345, presumably by creating an unfavorable interaction between the tyrosyl chain and the dansyl group. Overall we conclude that Dns-CP96,345 binds to NK1 in the same mode as CP96,345 thus validating its use as a reporter for the binding site.

Fluorescent reporter groups on ligands may provide important information on the binding pocket in the receptor. First, the polarity of the binding pocket can be estimated by using environment-sensitive fluorophores. Dansyl is very sensitive to the polarity of the medium with high fluorescence in low polarity environment. Second, we can access information on the mobility of the ligand when bound to the receptor by measuring the fluorescence anisotropy. Third, we can obtain information on the accessibility of the bound ligand by using the technique of collisional quenching.

The first spectrofluorometric evidence for the existence of different binding pockets for SP and CP96,345 was given by the differences in quantum yield of fluorescence for ligands bound to NK1 (Fig. 6). Dns-CP96345 must occupy a very hydrophobic pocket in the NK1 receptor. In sharp contrast, all labeled sites on SP were found to be located in a more hydrophilic environment. The N-terminal moiety of SP (positions 1 and 3) was in a more hydrophilic environment than the C-terminal moiety. The rotational mobility of the fluorescence-labeled ligands bound to NK1 was assessed by fluorescence anisotropy measurements. Anisotropy of fluorescent ligands increased upon binding to NK1 on CHO cells. The mostly temperature-insensitive anisotropy values of the different ligands bound to NK1 demonstrate

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Anisotropy at 20 °C</th>
<th>Limiting anisotropy ((A_0))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dns-CP96,345</td>
<td>0.049 ± 0.001</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>Dns-Hcy11-SP</td>
<td>0.035 ± 0.005</td>
<td>ND*</td>
</tr>
<tr>
<td>N-α-Flu-SP</td>
<td>0.039 ± 0.005</td>
<td>0.12 ± 0.01</td>
</tr>
</tbody>
</table>

* ND, not determined.
that ligand motions are restricted at the receptor binding site(s) in the nanosecond time range. This immobilization was more important for Dns-CP96,345 than for the SP analogues, thus suggesting a more densely packed environment surrounding the receptor binding site for Dns-CP96,345.

Collisional quenching of fluorescence can be used to probe solvent accessibility of receptor-bound ligand (16). Our quenching data clearly show important differences for receptor recognition between Dns-CP96,345 and the SP analogues. Dns-CP96,345 binds in a buried pocket that is shielded from the solvent and is not in contact with the membrane lipids surrounding the receptor. Therefore we conclude that Dns-CP96,345 docks inside the pore formed by the transmembrane helices, below the extracellular water-membrane interface which is in agreement with the mutational data. In contrast, both N- and C-terminal positions of SP were fully accessible to the solvent, an observation consistent with the binding of SP in the extracellular part of the NK1 receptor. This supports the identification of contact sites between photoactivable SP analogues and the extracellular domains of NK1 (14, 15, 35). However, one important implication of our findings is that it is not necessary for SP to reach deep into the bundle of transmembrane segments to activate NK1. Interactions with the extracellular loops are sufficient to stabilize the active conformation of the receptor. In support of this hypothesis, it has been recently shown that the bradykinin B2 receptor can be activated by antibodies raised against extracellular loop peptides (34). Also, mutations in the extracellular loops of the thrombin receptor (34) can produce active receptor conformations.

In summary, our results demonstrate the existence of distinct, although possibly overlapping, binding sites for SP and the non-peptide antagonist CP96,345 in the NK1 receptor. CP96,345 binds into a hydrophobic pocket buried into the receptor and shielded from the solvent. In contrast, SP binds in the hydrophilic environment of the extracellular parts of NK1, and apparently all parts of the undecapeptide SP remain accessible to the solvent. Our data imply that receptor activation by SP does not require the peptide to make contacts deep in the transmembrane regions although we cannot totally exclude that the central portion of SP could be partly buried in the transmembrane domains. In the future, fluorescence energy transfer experiments may help define more accurately the position of the bound ligands (17). The work presented here demonstrates the complementarity of fluorescence spectroscopy and site-directed mutagenesis in investigating ligand-receptor recognition in the absence of high resolution structural data.

Acknowledgments—We thank Karin Nemeth for help with the cell culture, Charles Bradshaw for peptide synthesis, and Dr. Jonathan Knowles for enthusiastic support during these studies.

REFERENCES

Downloaded from http://www.jbc.org/ by guest on September 24, 2017
Characterization of Non-peptide Antagonist and Peptide Agonist Binding Sites of the NK1 Receptor with Fluorescent Ligands
Gerardo Turcatti, Sannah Zoffmann, John A. Lowe III, Susan E. Drozda, Gérard Chassaing, Thue W. Schwartz and André Chollet

doi: 10.1074/jbc.272.34.21167

Access the most updated version of this article at http://www.jbc.org/content/272/34/21167

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 37 references, 15 of which can be accessed free at http://www.jbc.org/content/272/34/21167.full.html#ref-list-1