Further Definition of the Substance P (SP)/Neurokinin-1 Receptor Complex

MET-174 IS THE SITE OF PHOTINSERTION OF p-BENZOYLPHENYLALANINE4 SP*

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The covalent attachment site of a substance P (SP) analogue containing the photoreactive amino acid p-benzoyl-L-phenylalanine (Bpa) in position 8 of the C-terminal portion of the peptide was identified previously as Met-181 on the neurokinin-1 (NK-1) receptor. In this study, a second photoreactive SP analogue, Bpa8-SP, in which the Bpa residue is located in the N-terminal portion of the peptide, was used to define further the peptide-receptor interface. This NK-1 receptor, expressed in Chinese hamster ovary cells, was specifically and efficiently photolabeled with a radiiodinated derivative of Bpa8-SP. Fragmentation analysis of the photolabeled receptor restricted the site of photoincorporation of Bpa8-SP to an amino acid within the sequence Thr-173 to Arg-177 located on the N-terminal side of the E2 loop. To identify the specific amino acid in this sequence that serves as the covalent attachment site for Bpa8-SP, a small photolabeled receptor fragment was generated by chemical cleavage with cyanogen bromide. Matrix-assisted laser desorption/ionization time of flight mass spectrometric analysis of the purified fragment identified a single protonated molecular ion with a molecular mass of 1801.3 ± 1.8, indicating that upon irradiation, the bound photoligand covalently attaches to the terminal methyl group of a methionine residue. This result, taken together with the results of the peptide mapping studies, establishes that the site of Bpa8-SP covalent attachment to the NK-1 receptor is Met-174.

Substance P (SP)1 is a peptide neurotransmitter that has a high affinity (10−10 M) interaction with the NK-1 receptor (also Substance P receptor) (1). Identification of side-chain interactions between SP and the NK-1 receptor is important to understand the molecular basis for high affinity peptide binding and receptor activation. In the absence of high resolution structural data, molecular biological and biochemical approaches, particularly when combined, have provided useful information that allows the localization of specific interactions between SP and the NK-1 receptor. We have developed a methodology using the direct approach of photoaffinity labeling for the determination of contact sites between a peptide ligand and its receptor (1). In previous work, an analogue of SP in which a photoreactive amino acid p-benzoyl-L-phenylalanine (Bpa) was incorporated into position 8 of the peptide Bpa8-SP was used to covalently label the NK-1 receptor (1, 3–5). Importantly, the introduction of the Bpa residue into this position does not alter the ability of the SP analogue to bind the NK-1 receptor with high affinity or to induce a functional response. Furthermore, Bpa8-SP was shown to be a specific and an efficient photoprobe of the NK-1 receptor expressed in Chinese hamster ovary (CHO) cells. The site of Bpa8-SP covalent attachment was identified by peptide mapping strategies and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry to be Met-181 of the E2 loop of the receptor (2, 3).

In the present study, to define further the peptide binding domain of the NK-1 receptor as well as to orient the SP peptide within the binding pocket, a second photprobe, Bpa4-SP, in which proline 4 is replaced by the photoreactive Bpa residue (Bpa4-SP), has been used to covalently label the NK-1 receptor. The introduction of the Bpa residue into this position does not alter the ability of this analogue to bind to the NK-1 receptor with high affinity or to function as an NK-1 receptor agonist. The attachment site of radioiodinated 125I-Bpa4-SP was identified by peptide mapping and MALDI-TOF mass spectrometry to be Met-174.

EXPERIMENTAL PROCEDURES

Materials—p-Benzoyl-L-phenylalanine4-substance P (Bpa4-SP) was synthesized and radioiodinated with a specific activity of 2200 Ci/mmol using the Bolton-Hunter reagent as described previously (1, 2).

Cell Transfection and Culture—CHO cells were stably transfected with the rat SP receptor cDNA as described previously (6). The transfected CHO cells expressing 1 × 105 SP binding sites/cell were maintained in α-minimum Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum and 0.8 mg/ml G418 (Life Technologies, Inc.). For preparative experiments, cells were obtained in quantity by culture in suspension using spinner flasks to a cell density of 2–3 × 106 cells/ml.

Receptor Binding Assay—For the saturation binding experiments, transfected CHO cells were incubated at 4°C for 2 h with increasing concentrations of 125I-Bpa4-SP (0.1–5 nM) in HEPES buffer (20 mM HEPES, 120 mM NaCl, 5 mM KCl, 2.2 mM MgCl2, 1 mM CaCl2, pH 7.4), supplemented with 6 mM glucose and 0.6 mg/ml bovine serum albumin. Binding experiments using photoactivity ligands were performed in the dark. Nonspecific binding was determined by the addition of excess unlabeled SP (1 μM). Binding was terminated by the addition of

* This work is sponsored by National Institutes of Health Grants NS 3134 (to N. D. B.) and NCRR P41-RR10888 (to C. E. C.). 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.

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† The abbreviations used are: SP, substance P; 125I-Bpa4-SP, (125I-labeled Bolton-Hunter conjugate) Lys3-substance P; Bpa4-SP, (125I-labeled Bolton-Hunter conjugate) Lys3-p-benzoyl-L-phenylalanine4-substance P; Bpa, p-benzoyl-L-phenylalanine; CHO, Chinese hamster ovary; E2, second extracellular loop; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; NK-1, neurokinin-1; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; HPLC, high pressure liquid chromatography; Tricine, N,N,N-[2-hydroxy-1,1-bis(hydroxymethyl)glycine].
5 ml of ice-cold HEPES buffer and rapid filtering through a glass fiber filter (Whatman GF/C) that had been soaked for at least 2 h in polyethyleneimine (0.1%). The filters were then assayed for radioactivity by γ spectrometry. Binding affinity \( K_d \) was calculated from the Scatchard transformation of the binding data.

**Internal Calcium Measurement**—Cells were plated on 9 × 22-mm glass coverslips in a tissue culture dish and maintained in α-minimum Eagle’s medium containing 10% fetal bovine serum and 0.8 mg/ml G418. When the cells reached 70–90% confluence, the coverslips were washed with a HEPES-buffered saline solution (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM MgSO\(_4\)·7H\(_2\)O, 1.8 mM CaCl\(_2\), 10 mM glucose, pH 7.4). The cells were then washed with the fluorescent calcium indicator, 4 μM Fura-2/AM (Molecular Probes), with 0.01% pluronic acid (v/v) in 2 ml of HEPES-buffered saline and incubated for 25 min at room temperature. The coverslip was then washed with HEPES-buffered saline and placed into a fluorescence spectrophotometer in a cuvette containing HEPES-buffered saline. Upon the addition of peptide ligands, fluorescent emission was measured at 510 nm with excitation wavelengths of 340 and 380 nm (Fura-2 bound and unbound to calcium, respectively). The amount of trypsin used per mg of membrane protein is specified in the figure legends. The digestion was stopped by the addition of SDS-PAGE sample buffer (10% glycerol, 1% SDS, 0.05% mercaptoethanol) to a final concentration of 10% in the sample. 127I-Bpa4-SP was treated with 50 mg/ml CNBr in 0.1 M Tris-HCl, pH 6.8. The solubilized peptide fragments were then eluted passively in either extraction buffer (0.1% acetonitrile, 0.05% trifluoroacetic acid) or extraction buffer (0.1% trifluoroacetic acid) at room temperature. The coverslip was then washed with HEPES-buffered saline and placed into a fluorescence spectrophotometer in a cuvette containing HEPES-buffered saline. Upon the addition of peptide ligands, fluorescent emission was measured at 510 nm with excitation wavelengths of 340 and 380 nm (Fura-2 bound and unbound to calcium, respectively). The amount of trypsin used per mg of membrane protein is specified in the figure legends. The digestion was stopped by the addition of SDS-PAGE sample buffer (10% glycerol, 1% SDS, 0.05% mercaptoethanol) to a final concentration of 10% in the sample.

**Photoaffinity Labeling of Transfected CHO Cells**—Cells were resuspended at 10⁶ cells/ml in HEPES buffer and incubated with 125I-labeled Bpa4-SP (0.1 nM) for 2 h at 4 °C in the dark. The cells were then irradiated at a distance of 6 cm from a 100-watt long-wave (365 nm) UV lamp (Blak-ray, San Gabriel, CA) for 15 min on ice. For large scale preparative experiments, a small receptor fragment (2 kDa) generated by CNBr treatment of the intact receptor was further purified by preparative HPLC using an Alltech C18 column (4.6 × 250 mm) and a Vydac C4 column (4.6 × 250 mm). The columns were eluted with increasing Solvent B (20% acetonitrile/80% water/0.01% trifluoroacetic acid) and monitored with a UV detector set at 210 nm.

**Enzymatic and Chemical Cleavage of Photoaffinity Labeled CHO 1-K Receptors**—Membrane preparations containing the photolabeled receptor were partially digested for 18 h with 1:1-1:10,000 proteases such as trypsin, pronase, or chymotrypsin. The digested complexes were then separated on a Tricine gel using the Tricine gel system of Schagger and von Jagow (8). Following electrophoresis, the gel was dried and exposed to x-ray film (Kodak XAR-5) with an intensifying screen (3). The molecular masses of the radiolabeled fragments were determined using molecular mass markers from Amersham Pharmacia Biotech (2.35–46 kDa).

**Receptor Fragment Peptide Analysis by Mass Spectrometry**—The molecular mass of the purified 2-kDa receptor fragment was determined at the Mass Spectrometry Resource at Boston University School of Medicine using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in less than picoquantum mode. The Finnigan Vision 2000 instrument (Applied Biosystems, Franklin, MA) is equipped with an LSI nitrogen laser (337 nm, 3-ns pulse duration). The matrix, 2,5-dihydroxybenzoic acid, was dissolved in 1:1 water/acetone (10 mg/ml).

**Characterization of Iodinated Bpa4-SP**—The addition of a 125I-labeled Bolton-Hunter conjugate of Bpa4-SP to transfected CHO cells expressing the NK-1 receptor induced a Ca\(^{2+}\) response comparable with that induced by the same concentration of the parent peptide SP (Fig. 1). The response evoked by both peptides was completely inhibited in the presence of 1 μM RP-67,580, a specific nonpeptide antagonist of the rat NK-1 receptor.

The binding affinity of radiiodinated 125I-Bpa4-SP to intact CHO cells expressing the NK-1 receptor was determined by saturation binding experiments. The observed equilibrium binding was characterized by \( K_d = 1.4 \pm 0.2 \) nM, a value about 5-fold higher than that previously reported for the corresponding radiiodinated conjugate of the parent peptide (3). In contrast, the number of NK-1 receptors/cell \( (N_{max}) \) was the same for both derivatives.

**Photoaffinity Labeling of the Rat NK-1 Receptor and Fragmentation Analysis**—NK-1 receptors expressed in intact CHO cells were photolabeled with 125I-Bpa4-SP, and the resulting ligand-receptor complex was analyzed by SDS-PAGE and autoradiography. Photolabeled receptors were observed as a broad band centered at 80 kDa (Fig. 2, lane 1). The diffusion of the band is attributable to heterogeneous glycosylation of NK-1 receptors expressed in CHO cells (5). Qualitative analysis of the radioactivity incorporated into the NK-1 receptor indicated that photolabeling was highly efficient; ~40% of the

![Fig. 1. SP and 125I-Bpa4-SP induced calcium mobilization in CHO cells expressing the rat NK-1 receptor.](http://www.jbc.org/Downloaded from http://www.jbc.org/)

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**RESULTS**

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protease. After cleavage of the 5-kDa fragment with *S. aureus* V8 protease, a small fragment of 2.4 kDa in agreement with the calculated mass (2.3 kDa) of the photoligand covalently attached to a receptor fragment extending from residues 173 to 177 (TMPSR) was obtained (Fig. 4, lane 2). The *M*, 5000 limit tryptic fragment was also treated with CNBr, a reagent specific for cleavage after Met, generating a fragment of *M* ~2000 (Fig. 4, lane 3), a value that is close to that of the photoligand itself.

Identification of the Residue on the NK-1 Receptor That Is Covalently Labeled by Bpa4-SP—To obtain a sufficient amount of the 2-kDa fragment for accurate determination of molecular mass MALDI-TOF mass spectrometric analysis, transfected CHO cells (10⁹) were photolabeled with 125I-Bpa4-SP isotopically diluted with 127I-Bpa4-SP to a specific activity of 2 Ci/mm and then subjected to solid-phase CNBr cleavage (2). Approximately 100 pmol of the photolabeled receptor was absorbed onto C18-derivatized silica beads. Following treatment with CNBr, the generated fragments were resolved using Tricine-SDS-PAGE. The 2-kDa radiolabeled fragment was eluted and further purified by HPLC using both C18 and C4 columns (Fig. 5, A and B). The final yield of the purified 2-kDa fragment was 8 pmol, representing 8% of the starting photolabeled receptor, an amount more than sufficient to permit MALDI-TOF mass spectrometric analysis.

MALDI-TOF mass spectrometric analysis of the CNBr-generated fragment yielded a (M+H)⁺ with an *m/z* value of 1801.3 ± 1.8 (Fig. 6). This mass spectrum not only confirms the purity of the isolated fragment but also defines the chemical structure of the fragment. The observed molecular mass (1801.3 ± 1.8) could only be generated from a photolabeled receptor by the covalent attachment of the probe 125I-Bpa4-SP (molecular weight = 1776.9) to the methyl group of a methionine residue, followed by CNBr cleavage of the bond between the γ-carbon and sulfur on the methionine side chain to form a thiocyanate (–CH₂SCN) derivative. In addition, the C-terminal methionine amide of the covalently attached probe is converted by CNBr to a homoserine lactone. The observed (M+H)⁺ of *m/z* 1801.3 ± 1.8 of the final cleavage product is in good agreement with the calculated (M+H)⁺ of *m/z* 1802.9.

Because there is only one methionine residue (Met-174) in the photolabeled receptor fragment (residues 173–177), we can conclude that the site of photoincorporation of Bpa4-SP is Met-174 (Fig. 7).

**DISCUSSION**

Our previous results have shown that Bpa4-SP, which contains the photoreactive Bpa residue in the eighth position of the SP peptide within the conserved C-terminal region that defines the tachykinin peptide family, covalently attaches to a single residue, Met-181, on the NK-1 receptor (2). The present study characterizes the site of a covalent attachment of a second photoreactive analogue of SP with the photoreactive residue

### Fig. 2. Limited tryptic digestion and CNBr cleavage of the 125I-Bpa4-SP labeled SP receptor.

Rat NK-1 receptors expressed in CHO cells were photoaffinity-labeled with 125I-Bpa4-SP (lane 1), and a membrane preparation derived from these cells was subjected to limited cleavage by trypsin (0.5 mg/ml) (lane 2) or by CNBr (1 mg/ml) (lane 3) under conditions described under "Experimental Procedures." The cleavage fragments were analyzed by Tricine SDS-PAGE under nonreducing conditions followed by autoradiography. Lane 4 is the photoaffinity probe, 1.8 kDa. Isolated fragments from limited tryptic digestion were treated with 100 mM DTT, reanalyzed by Tricine SDS-PAGE under nonreducing conditions followed by autoradiography. Lane 4 is the photoaffinity probe, 125I-Bpa4-SP.

### FIG. 3.

Reduction of the disulfide bond within the photolabeled tryptic fragments. Isolated fragments from limited tryptic digestion were treated with 100 mM DTT, reanalyzed by Tricine SDS-PAGE, and visualized by autoradiography. Lanes 1 and 2, 22-kDa fragment; lanes 3 and 4, 14-kDa fragment; lanes 5 and 6, 5-kDa fragment.

MW (kDa)
located in the nonconserved N terminus of the peptide, specifically position 4 (Bpa 4-SP).

The substitution of the 4th position of SP (Pro-4) by Bpa is well tolerated by the receptor, as evidenced by its high affinity binding to the NK-1 receptor and its ability to stimulate an increase in intracellular calcium in the in vitro assay. The results presented in this report show that Bpa 4-SP covalently attaches to a single residue Met-174, implying that position 4 of SP is in close spatial proximity to Met-174 when bound to the NK-1 receptor.

Interestingly, in a separate study, Girault et al. (9) identified Met-174 as the attachment site on the human NK-1 receptor for the SP analogue Bpa 8-Pro 9-SP, the same residue that is labeled in the present study by Bpa 4-SP. Although the basis for the discrepancy in these studies is unknown, the introduction of a proline residue at the 9th position of SP could potentially alter the confirmation of the peptide in such a manner that the Bpa residue of Bpa 8-Pro 9-SP is positioned in close proximity to Met-174 rather than Met-181. Additional explanations could include (i) species differences (human versus rat) or (ii) experimental differences (e.g. labeling of membrane preparations versus labeling of intact cells).

The results obtained here with radioiodinated Bpa 4-SP, when combined with our previous findings (2, 3) with radioiodinated Bpa 8-SP, establish the importance of the initial sequence of the E2 loop extending beyond transmembrane 4 of the NK-1 receptor (in peptide binding). Based on these findings, it is likely that the bound SP peptide is oriented parallel to this region of the receptor with its 4th position adjacent to Met-174 and its 8th position adjacent to Met-181. Because of its location in the E2 loop just after it emerges from the lipid bilayer, Met-174 is positioned close to the membrane. Furthermore, although Met-181 is located in the middle of the E2 loop,
it is adjacent to Cys-180, which participates in a disulfide bond with Cys-105 (3), a residue that is close to the membrane interface. Thus, Met-181 is also positioned near the membrane interface of the peptide. Interestingly, we have recently obtained evidence (10) that these two methionines on the E2 loop are also spatially close to each other.

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J. Biol. Chem. 2001, 276:10589-10593.
doi: 10.1074/jbc.M007397200 originally published online December 14, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M007397200

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