Identification of Methionine as the Site of Covalent Attachment of a p-Benzoyl-Phenylalanine-containing Analogue of Substance P on the Substance P (NK-1) Receptor*

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Previously we have been able to restrict the site of covalent attachment of a photolabile and radiolabeled derivative of substance P (SP), p-benzoylphenylalanine8-substance P (Bpa8-SP), to residues 178–183 located on the second extracellular loop (E2) of the SP (NK-1) receptor (Boyd, N. D., Kage, R., Dumas, J. J., Krause, J. E., and Leeman, S. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 433–437). To ascertain the specific amino acid in this sequence that serves as the site of covalent attachment for 125I-Bolton-Hunter reagent (BH)-Bpa8-SP, we have employed here a novel solid-phase approach to cyano gen bromide cleavage of the photolabeled receptor followed by mass spectrometric analysis of a purified labeled fragment. SP receptors on transfected Chinese hamster ovary cells were photolabeled with 125I-BH-Bpa8-SP. A membrane preparation of the photolabeled receptors was adsorbed onto C-18-derivatized silica gel and cleaved with cyano gen bromide. A single radiolabeled fragment containing 63% of the photoincorporated radioactivity was generated and purified by high performance liquid chromatography. Mass spectrometric analysis identified a single molecular ion with a molecular mass of 1751.4 ± 2, establishing that upon irradiation the bound photoligand forms a covalent link with the methyl group of a methionine residue at the peptide binding site. In view of our previous findings, this methionine is Met-181 on the primary sequence of the SP receptor.

Substance P (SP), a peptide that is widely but selectively distributed throughout the central and peripheral nervous system, participates in multiple physiological roles and in particular has been implicated in the mediation of painful and inflammatory stimuli (2). The amino acid sequence of the SP receptor, also termed the NK-1 receptor, has been identified by measuring UV absorbance at 262 nm (for 127I-BH-Bpa8-SP) or 280 nm (for 125I-BH-Bpa8-SP) and used to deduce the corresponding cDNA for several species, including human (3–5). The receptor is a typical G protein-coupled receptor in that it has an extracellular amino terminus, seven membrane-spanning domains, intracellular and extracellular connecting loops, and an intracellular carboxyl terminus (6, 7). The location of the peptide binding region within this proposed structure has attracted much interest. To address this question, we have developed a photoaffinity derivative of substance P, p-benzoyl-l-phenylalanine8-substance P (125I-BH-Bpa8-SP) (8), which efficiently and specifically photolabels the substance P receptor in membrane preparations (8) or on intact cells (9). Enzymatic fragmentation using trypsin, V8-protease, and endoproteinase Arg-C coupled with identification by SDS-polyacrylamide gel electrophoresis and immunodetection allowed us to restrict the site of covalent attachment of the photoaffinity ligand to the sequence Val-Val-Cys-Met-Ile-Glu (residues 178–183) of the receptor (1), a result in agreement with the study by Li et al. (10). In the present study, we provide evidence establishing that the covalent attachment site of iodinated Bpa8-SP is the methionine at position 181.

EXPERIMENTAL PROCEDURES

Materials—p-Benzoyl-l-phenylalanine8-substance P (Bpa8-SP) was synthesized as described (6). 125I-Bolton-Hunter reagent was obtained from DuPont NEN. 127I-Bolton-Hunter reagent was synthesized by B. Tomczuk of Eastman Kodak Co. and generously made available to us.

Preparation of Iodinated Bpa8-SP—Bpa8-SP was coupled to the Bolton-Hunter reagent (11), either iodinated with the radioactive isotope 125I or the nonradioactive isotope 127I, as described previously (9). In short, Bpa8-SP was dissolved in ice-cold sodium borate buffer, pH 8.4, and incubated in the dark with iodinated Bolton-Hunter reagent for 2 h. The reaction products were separated by high performance liquid chromatography (HPLC) using a C-18-derivatized silica gel column (250 × 4.75 mm, Spherisorb ODS-2, Alltech) and an acetonitrile/water/trifluoroacetic acid solvent system. The acetonitrile concentration in the eluant was raised by 0.7%/min using a gradient controller/pump system (LC10-AD, Shimadzu, Kyoto, Japan) at a flow rate of 1.5 ml/min. Fractions were collected every minute and the reaction products identified by measuring UV absorbance at 262 nm (for 127I-BH-Bpa8-SP) or by γ-emission spectrometry (for 125I-BH-Bpa8-SP). Tandem mass spectrometry was used to determine that the 125I-BH reagent had coupled to the e-amino group of Lys-3 of Bpa8-SP. The Bpa8-SP derivatives labeled with either 125I-BH or 127I-BH reagent were shown to co-elute on the HPLC system described above.

Cell Transfection and Growth—Chinese hamster ovary cells that had been stably transfected with cDNA containing the entire coding sequence of the rat SP receptor together with an aminoglycoside resistance gene (12) were grown as monolayers in α-MEM, Life Technologies, Inc.) supplemented with 10% fetal calf serum and 1 mg/ml Geneticin (Life Technologies, Inc.) and grown to 85–90% confluency in an atmosphere of 95% air and 5% CO2 at 37 °C. For preparative experiments, cells were grown in suspension to a concentration of 106 cells/ml using spinner-culture flasks without the addition of Geneticin.

Photoaffinity Labeling of Transfected Chinese Hamster Ovary Cells—Chinese hamster ovary cells were photolabeled with 125I-BH-Bpa8-SP isotopically diluted with 127I-BH-Bpa8-SP to a specific activity of 2

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†The abbreviations used are: SP, substance P; Bpa8-SP, p-benzoyl-l-phenylalanine8-substance P; 125I-BH-Bpa8-SP, reaction product of Bpa8-SP and the 125I-Bolton-Hunter reagent; MALDI, matrix-assisted laser desorption/ionization; HPLC, high performance liquid chromatography.
Tris-EDTA buffer (5 mM Tris, 1 mM EDTA, pH 7.0) using the smallest previously (1) from photolabeled cells by homogenizing the cells in ice-cold Krebs-Ringer buffer and irradiated on ice for 15 min at a distance of 6 cm using a 100-W UV lamp (wavelength maximum 365 nm, Blak-Ray).

Membrane Preparation—Membranes were prepared as described previously (1) from photolabeled cells by homogenizing the cells in Tris-EDTA buffer (5 mM Tris, 1 mM EDTA, pH 7.0) using the smallest probe of an homogenizer (Tissumizer, Tekmar) twice for 15 s on ice. The homogenate was spun at 1000 × g for 5 min. After discarding this first pellet, membranes were pelleted from the supernatant by centrifugation at 48,000 × g for 20 min. The membranes were then washed twice by resuspension and centrifugation in Tris-EDTA buffer.

Cyanogen Bromide Treatment—Photolabeled membranes were adsorbed onto C-18-derivatized silica gel (1 g of gel/100 mg of membrane protein, EM-Science) which had been washed with 100% acetonitrile followed by 0.1% trifluoroacetic acid in water. The silica gel was washed twice with 10 g volumes of 0.1% trifluoroacetic acid and then washed three times with 10 g volumes of acetonitrile/trifluoroacetic acid/water (70:0.1:29.9 v/v/v). The silica gel was equilibrated by washing twice with 10 g volumes of 0.1% trifluoroacetic acid followed by washing twice with 10 g volumes of 0.1% trifluoroacetic acid. Fraction was diluted with 0.1% trifluoroacetic acid in water to a final acetonitrile concentration of less than 10% and subjected to chromatography on a C-18 derivatized silica gel column (4.6 x 250 mm, Alltech) equilibrated with 0.1% trifluoroacetic acid. Peptides were then washed twice by resuspending the fractions and centrifuging in Tris-EDTA buffer.

To evaluate the effects of cyanogen bromide on the photoligand, a mixture of radioactive and nonradioactive Bolton-Hunter Bpa<sup>8</sup>-SP (molecular ratio of 1:100) was treated with cyanogen bromide under the same conditions described for the photolabeled membranes and purified by HPLC on a C-18 column as described below.

**High Performance Liquid Chromatography**—The eluate containing the cyanogen bromide-generated fragment from the membrane preparation was diluted with 0.1% trifluoroacetic acid in water to a final acetonitrile concentration of less than 10% and applied onto a HPLC column filled with C-4-derivatized silica gel (4.6 x 250 mm, Alltech) equilibrated with 0.1% trifluoroacetic acid in water and connected to a gradient pump/controller system (Shimadzu L6-A) and a variable UV wavelength detector set at 220 nm. Using a constant flow rate of 1.5 ml/min at room temperature, peptides were eluted from the column by an acetonitrile gradient formed by mixing solvent A (0.1% trifluoroacetic acid) with solvent B (70% acetonitrile in 0.1% trifluoroacetic acid). Fractions were collected every minute and the radioactivity measured by γ-emission spectrometry. The fraction with the highest radioactivity was diluted with 0.1% trifluoroacetic acid in water to a final acetonitrile concentration of less than 10% and subjected to chromatography on a diphenyl-derivatized silica gel column (4.6 x 250 mm, Waters) equilibrated with 0.1% trifluoroacetic acid. Peptides were then again eluted from the column by raising the acetonitrile concentration in the solvent to a final of 70%. Peaks were collected manually by observing the UV absorbance. The fraction containing the highest radioactivity as determined by γ-emission spectrometry was diluted with 0.1% trifluoroacetic acid in water to a final acetonitrile concentration of less than 10% and subjected to chromatography on a diphenyl-derivatized silica gel column (4.6 x 250 mm, Waters) equilibrated with 0.1% trifluoroacetic acid. Peptides were then again eluted from the column and purified by HPLC on a C-18 column as described below.

**Mass Spectrometry**—The purified cyanogen bromide-generated fragment of the photolabeled receptor as well as the cyanogen bromide-treated Bpa<sup>8</sup>-SP was subjected to mass spectrometric analysis (by C.E.C.) at the MIT Mass Spectrometry Resource using matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry on a linear Vestec VT 2000 instrument equipped with a LSI nitrogen laser (337 nm, 3 ns pulse duration) and sinapinic acid as the matrix (13).
RESULTS

Starting with 2 nmol of SP receptor present in membranes from a large scale preparation of Chinese hamster ovary cells expressing $2 \times 10^8$ SP receptors/cell, approximately 545 pmol of photolabeled receptor remained adsorbed onto the octadecylsilica gel. Following treatment with cyanogen bromide and elution with organic solvent, 63% of the radioactivity was eluted from the solid phase. The eluted material was resolved by HPLC on a C-4-silica gel column (Fig. 1A). A major radioactive peak containing greater than 90% of the total radioactivity eluted from the column along with several minor components. These minor components, which were not identified, may represent chemically modified fragments such as those produced by oxidation rather than representing fragments generated from other incorporation sites of iodinated BH-Bpa8-SP produced by oxidation rather than representing fragments generated by subsequent HPLC steps using a diphenyl-derivatized silica gel column (Fig. 1B). The total amount of radioactivity recovered after each chromatographic step was at least 80% of the radioactivity applied. The yield of the fragment recovered after each purification step is summarized in Table I.

Mass spectrometry of this product (Fig. 2) showed a single protonated molecular ion ($M + H)^+$ with an average mass/charge ($m/z$) of 1751.4 ± 2 confirming the purity of the fragment. This molecular mass defines the chemical structure of the isolated fragment (Fig. 3). This product, with the calculated $m/z$ of 1752.8, could only have occurred if bound $^{127}$I-BH-Bpa8-SP upon photoligation had covalently attached to the methyl group of a methionine to methionine 181 of the SP receptor and the amino acid sequence Val-Val-Cys-Met-Ile-Glu (residues 178–183) present in the second extracellular loop of the SP receptor (1). Since the only methionine residue in this sequence is the methionine at position 181, the methionyl group that serves as the covalent attachment site is on this residue. It is noteworthy that this residue is adjacent to cysteine 180, which participates in a disulfide bond with cysteine 105, a bond essential for high affinity binding (1).

A solid phase approach to the cleavage of the photolabeled SP receptor with cyanogen bromide was used because in preliminary experiments using conventional methodology a low yield of the cleavage product was obtained, perhaps due to receptor aggregation that occurs in the acidic solvent. We chose C-18-derivatized silica gel as the solid phase because it is known that large hydrophobic proteins such as the SP receptor adsorb almost irreversibly onto this matrix and cannot be eluted with organic solvents. However, hydrophilic proteins, as well as lipids, which are present in the membrane preparations, can be eluted with organic solvents and thus this procedure provides a significant purification step. In addition, non-covalently bound photoaffinity ligand is also eluted before cleavage with cyanogen bromide, insuring that free probe does not contaminate the resulting product.

The demonstration that Bpa8-SP is photoincorporated into the methyl group of the methionine in position 181 indicates that the activated carbonyl group of the benzophenone is close to this group when the photoligand is bound to the receptor. Indeed, for the initial hydrogen abstraction step to occur, theoretical considerations supported by experimental data dictate that the distance between the ketone oxygen of the benzophenone and the abstractable hydrogen of the CH$_3$ group on the methionine side chain cannot be greater than 3.1 Å (14). In addition to these spatial considerations, efficient covalent attachment also depends on the reactivity of the hydroxyl donor moiety. C–H bonds that are predicted to be particularly reactive include tertiary centers such as the Cγ–H bond of leucine, the Cβ–H bond of valine, and C–H bonds adjacent to heteroatoms. Residues that have been reported to be sites of covalent attachment for a chemically diverse group of benzophenone containing ligands include methionine (15–17), glycine (16, 17), glutamine (18), asparagine (18), and serine (19).

That there is an order to the reactivity of different side chains of amino acid residues, and that the benzophenone group will exhibit some flexibility even when bound, means that it cannot be concluded that the methionine that serves as
the site of covalent attachment is necessarily the residue closest to the phenyl group on the side chain at position 8 of SP. Nevertheless our data provide direct evidence that this methionine residue is located within the peptide binding pocket of the receptor.

Studies on the structure-function relationship of the SP receptor have been performed by other groups by construction of chimeric receptors as well as by point mutations. Mutations at multiple sites have resulted in losses of high affinity binding. However, data derived from mutated receptors do not permit the conclusion that the identified residues are directly involved in ligand binding. It is thus particularly significant that a chimeric SP receptor in which residues 176–183 (containing methionine 181) were replaced by the corresponding sequence in the NK-3 receptor, showed a marked loss in binding affinity (20). The convergence of the results from these two different approaches strengthens the identification of this region of the receptor as part of the binding site for SP.

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