0026-895X/96/050808-06$3.00/0

MOLECULAR PHARMACOLOGY, 49:808–813 (1996).

Photoaffinity Labeling of the Human Substance P (Neurokinin-1) Receptor with $[^3H_2]$Azido-CP-96,345, a Photoreactive Derivative of a Nonpeptide Antagonist

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Received November 2, 1995; Accepted February 7, 1996

SUMMARY

An azido derivative of $[^3H_2]$(2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-((2-methoxyphenyl)methyl)-1-azabicyclo[2.2.2]octan-3-amine (CP-96,345), a potent nonpeptide antagonist of the substance P (SP) (neurokinin-1) receptor, was synthesized and shown to have an affinity for the human SP receptor similar to that of the parent compound, CP-96,345. When Chinese hamster ovary cells expressing the human SP receptor were photolabeled with this compound and analyzed with the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography, several radioactive bands were observed, including a major band centered at molecular mass 80 kDa, the expected value for the SP receptor expressed in Chinese hamster ovary cells. Only the labeling of the 80-kDa protein was specific: nonradiolabeled CP-96,345 but not its optical enantiomer, CP-96,344, was a potent inhibitor of photoincorporation. SP prevented photolabeling only at concentrations higher than expected from its binding affinity but similar to those shown in a competition binding assay to displace a radioiodinated analogue of CP-96,345. Antiserum generated against a synthetic peptide corresponding to the carboxyl terminus of the human SP receptor immunoprecipitated only the 80-kDa photoaffinity labeled protein, confirming that it is the human SP receptor. Interestingly, a second antiserum that was generated against the third extracellular loop of this G protein-coupled receptor no longer immunoprecipitated the receptor when covalently labeled with $[^3H_2]$azido-CP-96,345. This result indicates either that attachment of the antagonist modified the antigenic region directly, suggesting involvement of this domain in the binding of CP-96,345, or that the loss of recognition by the antiserum is secondary to a change in conformation induced by the covalent attachment of the antagonist at a different site.

SP is an 11-amino acid peptide neurotransmitter that has been implicated in many physiological responses, including the transmission of pain and neurogenic inflammation. Thus, there has been considerable interest in the development of nonpeptide antagonists to the SP (neurokinin-1) receptor (1).

CP-96,345 is a potent nonpeptide antagonist that is selective for the human SP receptor. It was the first to be described and was discovered through empirical file screening and subsequent structure-activity relationship-directed modification. This process was necessary because at the present level of understanding, it is not possible to design, *de novo*, nonpeptide ligands with high affinity for peptide receptors. CP-96,345 is a member of a group of structurally diverse antagonists that may act at a similar site (2). CP-96,345 has been well characterized, with a K_d for the human SP receptor of 1 nM (3), and its binding sites in the guinea pig brain are colocalized to areas of SP binding (4). Additional studies have determined that the action of CP-96,345 is stereoselective, with the 2*R*,3*R* optical enantiomer, CP-96,344, being pharmacologically inactive and interacting with low affinity to the SP receptor (3).

Although CP-96,345 seems to act in a competitive manner in binding and functional assays, recent studies suggest that agonist and antagonist binding domains on the G protein-coupled SP receptor are not equivalent (5). Of particular significance is a report of a genetically designed SP/neurokinin-2 receptor chimera containing the sixth transmembrane helix, the third extracellular loop, and the outer portion of the seventh transmembrane helix of the neurokinin-2 receptor (6). Although this chimera retains SP specificity, it is

ABBREVIATIONS: SP, substance P; CP-96,345, (2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-((2-methoxyphenyl)methyl)-1-azabicyclo[2.2.2]octan-3-amine; CHO, Chinese hamster ovary; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; $[^{125}I]$BH-SP, radioiodinated Bolton-Hunter substance P; $[^{125}I]$BH-Bpa^8-SP, radioiodinated Bolton-Hunter *p*-benzoyl-L-phenylalanine^8 substance P; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RIPA, radioimmunoprecipitation assay.

This work was supported by United States Public Health Service Grant NS31346 from the National Institutes of Health.

inhibited by the neurokinin-2 antagonist SR-48,968 and not by CP-96,345. Additional evidence for site heterogeneity is that the binding of [^{3}H]CP-96,345 to the SP receptor in brain membrane preparations of different species shows marked differences in affinity and that [^{3}H]SP binds with an equivalent affinity across species (7). The determination of the locations and possible overlap between the binding sites of the peptide agonist SP and the antagonist CP-96,345 is important for understanding the underlying mechanism of antagonism. In this report, we describe the synthesis of [^{3}H$_2$]azido-CP-96,345 and demonstrate its usefulness as a specific photoaffinity probe of the antagonist binding domain of the SP receptor.

Materials and Methods

Synthesis of [^{3}H$_2$]Azido-CP-96,345

A tritiated azido derivative of CP-96,345, the potent nonpeptide antagonist of the human SP receptor, was synthesized according to the procedure outlined in Fig. 1 and described in detail below.

(−)-(4′,4′-Dibromo)diphenylmethyl-3-((2-methoxy-5-aminophenyl)methylamino)-1-azabicyclo[3.2.2]octane. To a 100-ml round-bottomed flask equipped with condenser and N$_2$ inlet, we added 583 mg (1.295 mmol) of (4′,4′-dibromo)diphenylmethyl-1-azabicyclo[3.2.2]octan-3-amine, prepared as described previously (8); 351 mg (1.943 mmol) of 5-nitroanisaldehyde, prepared as described previously (9); and 13 ml of methanol. The solution was stirred for 40 min at room temperature, and the resulting precipitate was collected through filtration and dried. It was then taken up in 13 ml of dry tetrahydrofuran and 3.8 ml (7.8 mmol) of a solution of borane methyl sulfide in tetrahydrofuran was added, and the reaction was refluxed 4 days. The reaction was cooled, evaporated, and taken up in 20 ml of ethanol and treated with 1 g of sodium carbonate and 0.6 g of cesium fluoride. The resulting mixture was refluxed for 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 with methanol/methylene chloride as eluant and then triturated with isopropyl ether to produce a white solid (m.p., 184–187°; 261 mg) (34%). This intermediate was confirmed by the following analytical measurements [^{1}H NMR (δ, CDCl$_3$), δ 1.24 (m, 1 H), 1.48 (m, 1 H), 1.61 (m, 1 H), 1.85 (m, 1 H), 2.04 (m, 1 H), 2.59 (m, 1 H), 2.73 (m, 2 H), 2.84 (m, 1 H), 3.14 (m, 1 H), 3.38 (ABq, J = 13 Hz, Δν = 107, 2 H), 3.49 (s, 3 H), 4.41 (d, J = 12 Hz, 1 H), 5.98 (bs, 1 H), and 6.4–7.4 (series of multiplets, 10 H); ^{13}C NMR (δ, CDCl$_3$), δ 19.8, 24.5, 25.3, 41.9, 46.5, 48.0, 49.5, 53.9, 55.9, 61.7, 111.5, 114.4, 117.1, 120.0, 120.3, 129.3, 129.5, 131.6, 132.1, 132.2, 139.4, 141.7, 143.8, and 150.7; MS (%), 586 (parent + 1, 1), 260 (100), 136 (60), and 125 (70); $[\alpha]_D$ = −13.5° (c = 1, CH$_2$Cl$_2$); combustion analysis for $C_{28}H_{31}N_3OBr_2 \cdot 1/2H_2O$, C 56.58, H 5.43, N 7.07; found, C 56.53, H 5.60, N 6.94].

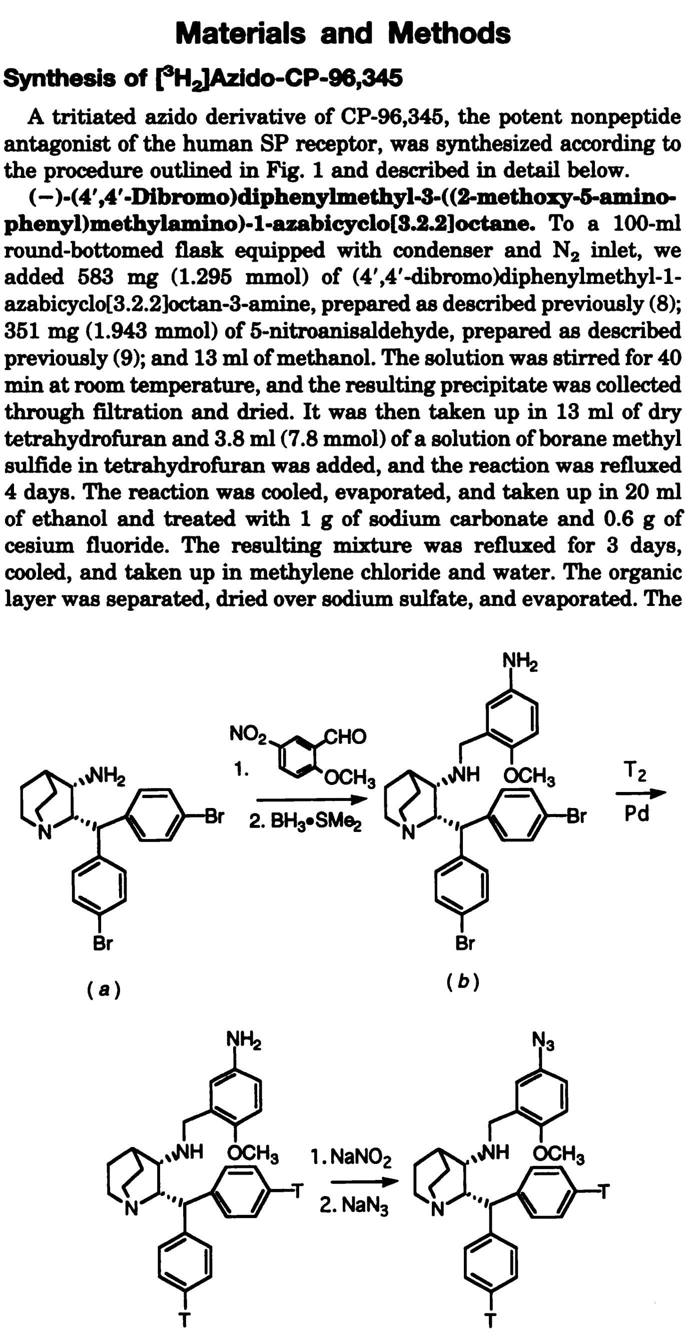

Fig. 1. Synthesis of [^{3}H$_2$]azido-CP-96,345.

The tritium/bromine exchange (10) and conversion of the tritiated intermediate, c, to the final azido compound, d, were carried out at DuPont-NEN Products (Boston, MA) by Drs. Crist Filer and Judith Egan according to appropriate procedures for handling radioactive substances. This resulted in [^{3}H$_2$]azido-CP-96,345 (specific activity, 31.9 Ci/mmol).

The synthesis of the nonradiolabeled azido-CP-96,345 was carried out as described below, beginning with a nonbrominated derivative of(−)-(4′,4′-dibromo)diphenylmethyl-3-((2-methoxy-5-aminophenyl)methylamino)-1-azabicyclo[3.2.2]octane.

(−)-Diphenylmethyl-3-((2-methoxy-5-aminophenyl)methylamino)-1-azabicyclo[3.2.2]octane. (−)-Diphenylmethyl-3-((2-methoxy-5-aminophenyl)methylamino)-1-azabicyclo[3.2.2]octane was prepared as described in the previous example with the use of diphenylmethyl-1-azabicyclo[3.2.2]octan-3-amine, which was prepared as described (8), in 53% yield (m.p. 185–189° after evaporation from methylene chloride). This intermediate was confirmed by the following analytical measurements [^{1}H NMR (δ, CDCl$_3$), δ 1.30 (m, 1 H), 1.57 (m, 1 H), 1.66 (m, 1 H), 1.97 (m, 1 H), 2.10 (m, 1 H), 2.68 (m, 1 H), 2.84 (m, 2 H), 2.97 (m, 1 H), 3.30 (m, 1 H), 3.40 (ABq, J = 15 Hz, δν = 64, 2 H), 3.52 (s, ^{3}H), 3.68 (dd, J = 8, 12 Hz, 1 H), 4.49 (d, J = 12 Hz, 1 H), 5.80 (d, J = 2 Hz, 1 H), and 6.5–7.5 (m, 12 H); ^{13}C NMR (CDCl$_3$), δ 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, 127.8, 128.4, 128.9, 129.1, 139.5, 143.6, 145.5, and 150.7; MS (%), 428 (parent + 1, 1), 291 (20), 274 (18), 260 (100), 136 (65), and 106 (28); HRMS, calculated for $C_{28}H_{33}N_3O$, 427.2624; found, 427.26131; $[\alpha]_D$ = −16.6° (c = 1, CH$_2$Cl$_2$); combustion analysis for $C_{28}H_{33}N_3O \cdot 1/4CH_2Cl_2$, C 75.60, H 7.52, N 9.36; found, C 75.20, H 7.53, N 9.26].

(−)-Diphenylmethyl-3-((2-methoxy-5-azidophenyl)methylamino)-1-azabicyclo[3.2.2]octane (azido-CP-96,345). To a 25-ml round-bottomed flask equipped with N$_2$ inlet we added 500 mg (1.17 mmol) (−)-diphenylmethyl-3-((2-methoxy-5-aminophenyl)methylamino)-1-azabicyclo[3.2.2]octane, 4 ml of water, and 0.8 ml of concentrated sulfuric acid. The reaction turned dark brown, cooled to 0°, and then treated dropwise with a solution of 107.5 mg (1.56 mmol) of sodium nitrite in 2 ml of water over 5 min. The reaction was warmed to room temperature and stirred for 1 hr; then, 23.2 mg (0.39 mmol) urea was added, and the stirring continued for 15 min. Next, 127 mg (1.96 mmol) of sodium azide was added, producing bubbling, and the stirring was continued overnight. The precipitate that had formed was filtered, taken up in ethyl acetate, and washed with 3 N aqueous sodium hydroxide solution; the aqueous layer was reextracted with ethyl acetate, and the combined organic layers were dried over sodium sulfate and evaporated. The residue was chromatographed on silica gel with methanol/methylene chloride as eluant, and the resulting tan solid crystallized from methylene chloride/isopropanol produced 89 mg (17%) (m.p. 164–166°). The structure of this final product was confirmed by the following analytical measurements [^{1}H NMR (δ, CDCl$_3$), δ 1.28 (m, 1 H), 1.54 (m, 1 H), 1.64 (m, 1 H), 1.92 (m, 1 H), 2.08 (m, 1 H), 2.62 (m, 1 H), 2.78 (m, 2 H), 2.89 (m, 1 H), 3.20 (m, 1 H), 3.36 (ABq, J = 15 Hz, Dn = 100, 2 H), 3.55 (s, 3 H), 3.70 (m, 1 H), 4.47 (d, J = 12 Hz, 1 H), and 6.41 and 6.6–7.4 (m, 13 H); MS (%), 453 (parent, 2), 452 (parent − 1, 10), 286 (50), 221 (30), 167 (35), 136

(40), and 91 (100); $[\alpha]_D = -5.22°$ (c = 1, CH_2Cl_2); combustion analysis for $C_{28}H_{31}N_5 \cdot 1/3H_2O$, C 73.18, H 6.94, N 15.24; found, C 73.24, H 7.01, N 14.95].

HPLC analysis was used to authenticate the radiolabeled azido derivative of CP-96,345. This nonradiolabeled azido derivative was also used in equilibrium displacement binding assays to assess the effect of the azido group on binding affinity.

Photolabeling of Transfected Cells and Membrane Preparation

Stably transfected human SP receptor in CHO cells expressing 490,000 receptors/cell were provided by Dr. J. E. Krause (Washington University, St. Louis, MO). These cells were grown in α-modified essential medium containing 10% fetal bovine serum and 800 mg/ml geneticin (GIBCO-BRL) (11) and harvested with Enzyme Free Cell Dissociation Buffer (Specialty Media, Lavellette, NJ). Cell pellets of transfected cells were obtained through centrifugation at 100 × *g* for 10 min and were suspended in HEPES buffer (20 mM HEPES, 1 mM $CaCl_2$, 2.2 mM $MgCl_2$, 5 mM KCl, 120 mM NaCl, pH 7.4) supplemented with 6 mg/ml glucose and 0.6 mg/ml BSA. Tritiated azido-CP-96,345 was added to this suspension to give a final concentration of 1–2 nM. After a 1-hr incubation in the dark at 4° with gentle agitation, the mixture was transferred to a 100-mm Petri dish, diluted 1:1 with HEPES buffer, and exposed to short-wave UV irradiation on ice in a UV Stratalinker 1800 (Stratagene, La Jolla, CA) at a distance of 6 cm for 15 min. Under these conditions, quantitative analysis of photoincorporated radioactivity into the 80-kDa band indicated that ~5% of the bound $[^3H_2]$azido-CP-96,345 became covalently attached on UV irradiation.

Photolabeled cells were obtained through centrifugation, resuspended in Tris/EDTA buffer (50 mM Tris·HCl, 1 mM EDTA, pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride and sonicated for 10 sec twice with a Sonicator Cell Disrupter (Heat Systems-Ultrasonics). Nuclear and cellular debris was removed through centrifugation at 500 × *g* for 10 min, and membranes were then obtained through centrifugation at 30,000 × *g* for 30 min. The final pellet was resuspended in Tris/EDTA buffer, frozen in liquid nitrogen, and stored at −70°. Intact cells rather than membrane preparations were used for labeling because in preliminary photolabeling experiments with membranes, a large number of nonspecifically radiolabeled bands were observed.

Human SP receptor-transfected CHO cells were also photolabeled with $[^{125}I]$BH-Bpa^8-SP (12) as previously described (11).

SDS-PAGE and Fluorography or Autoradiography

$[^3H_2]$Azido-CP-96,345 or $[^{125}I]$BH-Bpa^8-SP photoaffinity labeled membranes were solubilized in sample buffer (0.125 M Tris, pH 6.8, 2% SDS, 10% glycerol, and 0.01% bromphenol blue) and subjected to SDS-PAGE as described by Laemmli (13). After electrophoresis, gels containing tritiated samples were prepared for fluorography with use of the ENTENSIFY Universal Autoradiography Enhancer procedure (DuPont-NEN). The gels of both tritiated and iodinated samples were dried and exposed to X-ray film (XAR 5, Kodak). Molecular weight markers from Bio-Rad and prestained Rainbow molecular weight markers from Amersham were used. Photoincorporation was quantified through the use of densitometric scanning and analysis with ImageQuant (Molecular Dynamics).

Immunoprecipitation by Site-Directed Antipeptide Antisera

Antiserum (CT) was generated against a synthetic peptide corresponding to the last 15 residues of the carboxyl terminus of the human SP receptor, residues 391–407, according to the *m*-maleimidobenzoyl-*N*-hydroxysuccinimide coupling method (14) and was characterized by ELISA. The resulting antiserum was ammonium sulfate precipitated (50% ammonium sulfate, 18 hr, 4°) and then centrifuged at 3000 × *g* for 30 min. The pellet was resuspended in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 5.4 mM NaH_2HPO_4, 1.8 KH_2PO_4, pH 7.2), transferred to a Centricon-10 microconcentrator (Amicon, Beverly, MA), centrifuged at 5000 × *g* for 1 hr, and washed twice with 1 ml phosphate-buffered saline followed by centrifugation. The antiserum was absorbed onto Protein A beads for 1 hr at room temperature and washed twice with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5). $[^3H_2]$Azido-CP-96,345 or $[^{125}I]$BH-Bpa^8-SP photoaffinity labeled human SP receptors were solubilized in RIPA buffer and added to the antibodies preabsorbed onto Protein A beads and allowed to mix for 18 hr at 4° in the absence and presence of the antigenic peptide. The Protein A beads were pelleted by centrifugation and washed three times with RIPA buffer. The immunoprecipitates and supernatants were analyzed for tritium content with the use of scintillant (EcoLite, ICN, Costa Mesa, CA) and a scintillation counter or the ^{125}I content was analyzed with the use of γ spectrometry. The immunoprecipitates were also analyzed with SDS-PAGE and fluorography after solubilization in sample buffer.

A second antiserum (E3), described previously (15–17) and generated against a synthetic peptide corresponding to the third extracellular loop of the human SP receptor, residues 273–287, was kindly provided by Dr. J. E. Krause (Washington University, St. Louis, MO) and was also used in the immunoprecipitation assay described above.

Equilibrium Displacement Binding Assays

Human SP receptor-transfected CHO cell membranes were prepared as described above and incubated with radiolabeled ligand, $[^{125}I]$BH-SP or $[^{125}I]$-L-703,606, a radioiodinated analogue of CP-96,345 (18), purchased from DuPont-NEN. Binding was measured alone or in the presence of increasing concentrations of nonradiolabeled SP, CP-96,345, CP-96,344, or azido-CP-96,345 for 30 min in Tris/$MgCl_2$/EGTA buffer (50 mM Tris, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.4) containing peptidase inhibitors (5 μg/ml leupeptin, 3 μg/ml chymostatin, 30 μg/ml bacitracin, and 200 μg/ml BSA). These experiments were conducted on membrane preparations rather than on intact CHO cells, because in preliminary experiments, and as reported by others (18), intact cells contain a low affinity/high capacity uptake system for the radioiodinated antagonist that is not related to the SP receptor. After incubation, the membranes were filtered through Whatman GF/C filter paper and washed three times in 4° Tris/$MgCl_2$ buffer (50 mM Tris, 10 mM $MgCl_2$, pH 7.4) with a Brandel Harvester apparatus (Gaithersburg, MD), and the radioactivity was quantified with γ spectrometry.

Results

Synthesis and characterization of azido-CP-96,345 and $[^3H_2]$azido-CP-96,345. Nonradiolabeled azido-CP-96,345 was synthesized as described in Materials and Methods, and its structure was confirmed with the use of NMR, mass spectrometry, optical rotation, and combustion analysis. Azido-CP-96,345 was a potent inhibitor of $[^{125}I]$BH-SP binding, with an IC_{50} of 0.2 nM, which is similar to the value of the parent compound, CP-96,345 (Fig. 2). In contrast, the 2*R*,3*R* optical enantiomer, CP-96,344, inhibits binding only at high concentrations (>100 nM). The agonist SP inhibited $[^{125}I]$BH-SP binding, with an IC_{50} of 0.15 nM. These results confirm previous findings (3) that CP-96,345 is a potent and stereoselective inhibitor of SP binding to the SP receptor and that the addition of the azido moiety did not adversely affect binding affinity. A tritiated derivative, $[^3H_2]$azido-CP-96,345 (specific activity, 31.9 Ci/mmol), was also synthesized, as outlined in Fig. 1. The structure of this photoreactive, radiolabeled derivative was established through high performance

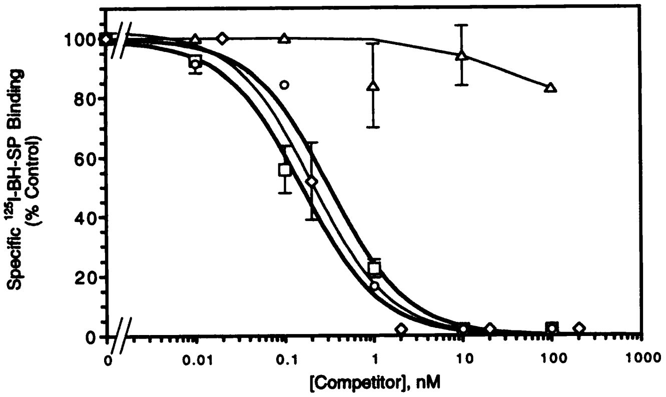

Fig. 2. Binding of [^{125}I]BH-SP to human SP receptor in the presence of competitors. Membrane preparations of CHO cells expressing the human SP receptor were incubated for 30 min at room temperature with [^{125}I]BH-SP and the indicated concentrations of the parent antagonist, CP-96,345 (○); the azido derivative of CP-96,345 (●); the optical enantiomer of CP-96,345, CP-96,344 (△); and the peptide agonist SP (□). The specific binding of [^{125}I]BH-SP was determined through ultrafiltration assay as described in Materials and Methods. These data are shown as percentage of the specific control binding determined in the absence of added competitor. The experiments were performed in duplicate and are representative of a typical experiment that was repeated at least twice.

liquid chromatography analysis with the nonradiolabeled azido derivative as the standard.

Photoaffinity labeling of CHO cells expressing human SP receptor with [$^3H_2$]azido-CP-96,345 or [^{125}I]BH-Bpa^8-SP. CHO cells transfected with the human SP receptor were equilibrated with [$^3H_2$]azido-CP-96,345 and irradiated with short-wave UV light. The photolabeled products were analyzed with SDS-PAGE and fluorography, and a band centered at 80 kDa, as well as several other bands, were observed (Fig. 3A, *lane 1*). Densitometric analysis of the fluorograph established that the addition of nonradiolabeled CP-96,345 at 1 μM (*lane 3*) completely inhibited the photolabeling of the 80-kDa protein, whereas the addition of the inactive optical enantiomer, CP-96,344, at the same concentration (*lane 2*) decreased photolabeling of the 80-kDa protein but by only ~38%. SP (1 μM) inhibited photolabeling of the 80-kDa protein by >90–95% (*lane 4*) but by only 53% at 100 nM. As shown in Fig. 2, SP can inhibit the binding of [^{125}I]BH-SP in the subnanomolar range; thus, SP is considerably less potent in protecting against photolabeling by [$^3H_2$]azido-CP-96,345.

For comparative purposes, the SP receptor was also photolabeled with the photoreactive SP analogue [^{125}I]BH-Bpa^8-SP, which has been shown to photolabel the SP receptor with high specificity (11, 12). CHO cells expressing the human SP receptor were photolabeled with this photoreactive agonist and analyzed with SDS-PAGE and autoradiography. The human SP receptor photolabeled with [^{125}I]BH-Bpa^8-SP was shown to be specific and was detected as a single radioactive band centered at molecular mass of 80 kDa (Fig. 3B).

Immunoprecipitation of photoaffinity labeled human SP receptors. To provide further evidence that the protein specifically photolabeled with [$^3H_2$]azido-CP-96,345 was the human SP receptor, we performed immunoprecipitation assays with two human SP receptor-specific antisera. The antiserum CT was generated against a synthetic peptide corresponding to the last 15 amino acid residues of the carboxyl terminus of the human SP receptor. The ability of this antiserum to recognize the human SP receptor was established with the use of immunoprecipitation of the SP receptor specifically photolabeled with [^{125}I]BH-Bpa^8-SP. When CHO cell membranes photolabeled with [$^3H_2$]azido-CP-96,345 were solubilized under nondenaturing conditions and incubated with the CT antiserum, the only radiolabeled protein recognized by this antibody was the specifically photolabeled 80-kDa protein (Fig. 3C). These results further establish the identity of the 80-kDa protein as the SP receptor.

Immunoprecipitation assays were also performed with E3 antiserum generated against a synthetic peptide with a sequence that corresponds to the third extracellular loop of both the human and rat SP receptors. The E3 antiserum recognizes the unoccupied human and rat SP receptors (19, 17), as well as the rat SP receptor, when photolabeled with the agonist [^{125}I]BH-Bpa^8-SP (16). This antibody recognized the human SP receptor when photolabeled by [^{125}I]BH-Bpa^8-SP but not by [$^3H_2$]azido-CP-96,345, suggesting that photo-

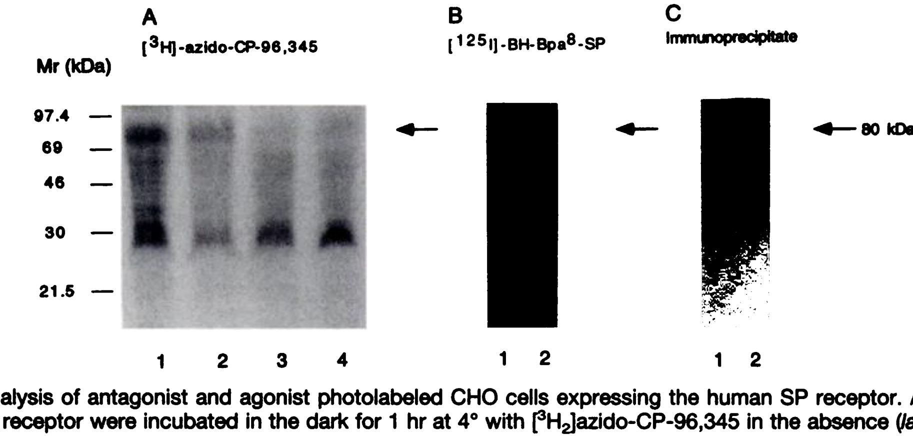

Fig. 3. SDS-PAGE analysis of antagonist and agonist photolabeled CHO cells expressing the human SP receptor. A, CHO cells (~1.4 × 10^6) expressing human SP receptor were incubated in the dark for 1 hr at 4° with [$^3H_2$]azido-CP-96,345 in the absence (*lane 1*) and presence (1 μM) of CP-96,344 (*lane 2*), CP-96,345 (*lane 3*), or SP (*lane 4*). The cells were then irradiated for 15 min, and membranes were prepared as described in Materials and Methods and subjected to SDS-PAGE and fluorography. B, CHO cells (~1 × 10^6) expressing human SP receptor were incubated in the dark for 1 hr at 4° with [^{125}I]BH-Bpa^8-SP alone (*lane 1*) or in the presence of 1 μM SP (*lane 2*). The cells were then irradiated for 30 min, and membranes were prepared as described in Materials and Methods and subjected to SDS-PAGE and autoradiography. C, Solubilized [$^3H_2$]azido-CP-96,345 photolabeled membranes were incubated with CT antiserum preabsorbed to Protein A beads. Immunoprecipitation in the presence (*lane 1*) or absence (*lane 2*) of the antigenic peptide (1 μM) was analyzed with SDS-PAGE and fluorography. The radioactivity detected at >100 kDa (~33% of the total) represents aggregated receptor.

labeling with the antagonist but not the agonist results in a modification of the antigenic site.

Inhibition of [^{125}I]-L-703,606 binding. The requirement of high concentrations of SP to protect against photolabeling of the human SP receptor by [$^3H_2$]azido-CP-96,345 was examined in equilibrium displacement binding assays with [^{125}I]-L-703,606, a radioiodinated analogue of CP-96,345 that has a high specific activity (Fig. 4). CP-96,345 inhibits the binding of [^{125}I]L-703,606 with an IC$_{50}$ of 0.2 nM. This value is similar to the IC$_{50}$ determined in Fig. 2 for the inhibition of [^{125}I]BH-SP binding and is in agreement with values obtained in similar assays (3). In contrast, inhibition of [^{125}I]-L-703,606 binding by SP was complex, occurring over a concentration range of 3 orders of magnitude. Although this requirement of high SP concentrations to completely displace [^{125}I]-L-703,606 cannot be explained simply, it parallels the high concentrations of SP required to protect against photolabeling by [$^3H_2$]azido-CP-96,345.

Discussion

This report demonstrates the first photolabeling of a peptide receptor with a nonpeptide antagonist. The protein photolabeled by [$^3H_2$]azido-CP-96,345 was identified as the human SP receptor on the basis of (i) mobility on SDS-PAGE, (ii) stereoselectivity of antagonist inhibition of photolabeling, (iii) inhibition by SP, albeit at high concentrations, and (iv) recognition by antiserum to the carboxyl terminus of the human SP receptor. In addition, immunological evidence was obtained suggesting that the site of covalent attachment of this photoreactive antagonist is a residue within or conformationally linked to the third extracellular loop of the SP receptor.

Previously, we reported that the SP receptor expressed in CHO cells, when photolabeled with [^{125}I]BH-Bpa^8-SP, migrates as a single, specific 80-kDa band (12) that, upon enzymatic deglycosylation, is converted to a discrete band of 46 kDa, the calculated molecular mass of the SP receptor (11).

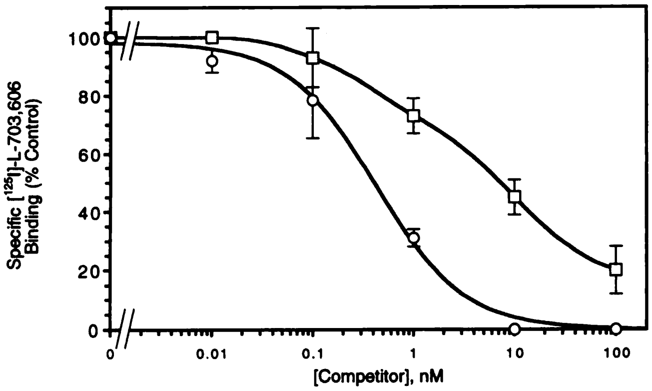

Fig. 4. Competition binding of [^{125}I]L-703,606, a radioiodinated analogue of CP-96,345. Membrane preparations of CHO cells expressing the human SP receptor were incubated for 30 min at room temperature with [^{125}I]L-703,606, a radioiodinated analogue of CP-96,345, and the indicated concentrations of the parent antagonist, CP-96,345 (○), and the peptide agonist SP (□). The specific binding of [^{125}I]L-703,606 was determined with an ultrafiltration assay as described in Materials and Methods. These data are shown as percentage of the specific control binding determined in the absence of added competitor. The experiments were performed in duplicate and are representative of a typical experiment that was repeated at least three times.

In the present study, when CHO cells expressing the human SP receptor were photolabeled with [$^3H_2$]azido-CP-96,345, the only specifically photolabeled protein detected also migrated at 80 kDa, which is consistent with its identification as the SP receptor.

Although CP-96,345 has been shown to be a very potent inhibitor of radiolabeled SP binding and biological activity, its *2R,3R* optical enantiomer, CP-96,344, lacks appreciable affinity for the human SP receptor (20). This stereoselectivity of antagonist binding was also demonstrated in [$^3H_2$]azido-CP-96,345 photolabeling experiments by showing that these two enantiomers differed widely in their ability to protect against photolabeling. The requirement of high concentrations of SP to protect against photolabeling by [$^3H_2$]azido-CP-96,345 seems to be related to our observation that similarly high concentrations of SP are necessary to compete with a radioiodinated analogue of CP-96,345. The low potency of SP to compete with radiolabeled nonpeptide antagonists [also noted by others (4, 21)] is not consistent with a simple competitive interaction but rather suggests a more complex relationship, possibly involving interactions with different sites and/or different conformational states. Because of this complexity in the pharmacological characterization of the radiolabeled 80-kDa band, we sought additional evidence through the use of immunodetection, a highly specific method for protein identification. The CT antiserum, generated against the last 15 residues of the carboxyl terminus of the human SP receptor, immunoprecipitated only the 80-kDa photolabeled protein, establishing this protein as the human SP receptor. This antibody should also be useful in future experiments to isolate the photolabeled receptor from nonspecifically labeled proteins, a necessary first step in peptide mapping studies of the antagonist binding site.

In contrast to the CT antisera, another SP receptor-specific antisera, E3, which recognizes the third extracellular loop of the SP receptor, no longer immunoprecipitates the human SP receptor when it is covalently labeled by [$^3H_2$]azido-CP-96,345. This result is noteworthy because it suggests that the antigenic sequence (residues 273–287 of the SP receptor) has been modified on photoaffinity labeling. Studies of chimeric and mutant receptors have implicated residues at the boundary between the E3 loop and the sixth and seventh transmembrane helixes in the binding of CP-96,345 (23–26). The simplest interpretation of our results is that the site of covalent attachment of the antagonist is to a residue within the antigenic sequence, proposed to be composed of the third extracellular loop and possibly part of the seventh transmembrane helix (22). An alternative possibility, which cannot be ruled out, is that covalent attachment to a residue outside the antigen sequence results in a change in conformation such that the E3 domain is no longer accessible to the antibody.

In contrast, the E3 antiserum does recognize the SP receptor when covalently labeled with the photoreactive agonist [^{125}I]BH-Bpa^8-SP. We previously used peptide mapping of the agonist photolabeled receptor to identify the site of covalent attachment and found that it is to a residue located within the second extracellular loop of the SP receptor (16, 27). Although similar mapping studies will be necessary to define more closely the binding site for nonpeptide antagonists, our results provide biochemical evidence that the binding pockets for the peptide agonist SP and the nonpeptide antagonist CP-96,345 are located at distinct regions of the receptor. A heterogeneity in binding sites is supported by results of molecular genetic studies that demonstrate that the agonist but not the antagonist binding site is conserved in evolution. The SP receptor from multiple species binds the peptide agonist with equal affinity but exhibits large species variations in antagonist binding affinities (26). Point mutation analyses have also suggested heterogeneity in the binding sites for SP and the nonpeptide antagonists (5). The possibility that peptide agonists and nonpeptide antagonists interact at distinct sites suggests that even though CP-96,345 seems to inhibit the action of SP in a competitive manner, a more complex allosteric mechanism, perhaps involving different functional states, may be involved.

Acknowledgments

We thank Drs. Crist Filer and Judith Egan (DuPont-NEN, Boston, MA) for the tritiation and final conversion to the azido derivative of [^{3}H]azido-CP-96,345.

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