Characterization of the Cocaine Binding Site on the Sigma-1 Receptor†

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ABSTRACT: The cocaine photoaffinity label 3-iodo-4-azidococaine ([^{125}I]IACoc) binds to the sigma-1 receptor with an affinity that is 2−3 orders of magnitude higher than the parent compound cocaine [Kahoun, J. R., and Ruoho, A. E. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1393−1397]. In the present study, the binding properties of several cocaine derivatives to the guinea pig liver sigma-1 receptor were determined. The results from assessing the affinity of various derivatives of cocaine which were substituted on the phenyl ring indicated that an important determinant of binding to the guinea pig sigma-1 receptor binding site may be the development of a dipole in the ring in which the π electron density of the phenyl ring is reduced. This implies that an electron-rich source is present in the sigma-1 receptor binding site, such as the π system of an aromatic ring or other electron-rich side chains, which interact with the phenyl ring of cocaine. The precise [^{125}I]IACoc derivatization site in the guinea pig sigma-1 receptor was identified using chemical cleavage and purification of the resulting labeled peptides. Cyanogen bromide cleavage of the [^{125}I]IACoc photolabeled sigma-1 receptor followed by radiosequencing identified Asp188, which is located in the putative steroid binding domain-like II (SBDL II) near the carboxyl terminus, as the site of [^{125}I]IACoc insertion. Systematic truncation of the C-terminus indicated the requirement for the last 15 amino acid residues of the receptor for [^{125}I]IACoc photolabeling.

Sigma receptors are unique receptors, originally identified and classified as opioid and phencyclidine (PCP)[1] receptors (*1*, *2*). Since the term "opioid" refers to a receptor's ability to bind naloxone or naltrexone and the sigma receptor binds with extremely low affinity to both of these ligands (*3*), the sigma receptor was reclassified as a nonopiate receptor. Additionally, the sigma receptor was determined not to be the site of high-affinity PCP binding, which was identified as the *N*-methyl-D-aspartate (NMDA) receptor (*4*, *5*). With the identification of sigma receptor selective ligands, it is now well-established that the sigma receptors represent a unique binding site in the CNS and peripheral organs.

Sigma receptors, of which there are two subtypes reported, sigma-1 and sigma-2, are ubiquitously distributed in tissues. While many pharmacologic responses have been linked to the sigma receptors, the function of the sigma receptor is not completely understood. Several reports demonstrate that the sigma-1 receptor regulates voltage-gated potassium channels (*6*−*9*), intracellular Ca^{2+} release (*10*), and intracellular trafficking of galactosylceramide-enriched membrane microdomains (*11*). Sigma-1 binding sites have been identified not only in many areas of the brain but also in the peripheral tissues of the endocrine and immune systems, such as the adrenal gland, testis, ovary, spleen, and blood leukocytes. This broad distribution has led to speculation that sigma receptors can mediate various cellular functions including increasing extracellular acetylcholine levels (*12*), neuroprotective effects (*13*−*15*), and inhibition of proliferative response to mitogens (*16*). The modulation of ion channels (mainly voltage-gated K^+ channels) in the plasma membrane could be a reasonable mechanism to regulate membrane excitability (*8*, *9*, *17*−*20*) and modulation of the effects of cocaine (*21*−*25*).

The generation of a sigma-1 receptor knockout mouse has led to further findings on the behavioral effects of the sigma-1 receptor. While there is no overt phenotypic change in sigma-1 receptor knockout mouse as compared to wild-type littermates, several pharmacological and behavioral changes were observed. The most pronounced was the loss of the hypermobility response when the knockout mice were treated with the sigma-1 receptor selective ligand SKF 10,047 (*26*). Another change reported was the decrease in tonic, nonacute pain induced by formalin (*27*). Ligand binding to the sigma-2 receptor was unaffected in the knockout mouse (*26*).

Far less is known about the sigma-2 receptor. It has been suggested that the sigma-2 receptor is capable of modulating the apoptotic response of cells (*28*) and the release of Ca^{2+} through an IP_3-independent mechanism (*29*). The presence of large amounts of sigma-2 receptor in tumor cells has led

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[1] Abbreviations: IACoc, 3-iodo-4-azidococaine; SBD I, steroid binding domain I; SBD II, steroid binding domain II; SBDL I, steroid binding domain-like I; SBDL II, steroid binding domain-like II; CNBr, cyanogen bromide; DABCO, 1,4-diazabicyclo[2.2.2]octane; PCP, phencyclidine; NMDA, *N*-methyl-D-aspartate; SKF 10,047, *N*-allylnormetazocine; PPP+, (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine; PVDF, polyvinylidene fluoride; HRP, horseradish peroxidase.

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to speculation that the sigma-2 may serve as a marker for certain tumors. Recent reports show that sigma-2 receptors in rat liver membranes are localized to lipid rafts and that activation of the sigma-2 results in increases in ceramide and sphingosylphosphorylcholine, which may play a role in the apoptotic signal transduction from sigma-2 receptors (*28*). The sigma-2 receptor has not been cloned.

The sigma-1 receptor, originally cloned from guinea pig liver (*30*) and then from several other sources (*31*−*34*), consists of 223 amino acids and shares about 90% identity and 95% similarity across species. The amino acid sequence of the guinea pig (GP) sigma-1 receptor has significant homology (30% identity and 67% similarity) with a sterol C8−C7 isomerase from yeast (ERG2), which is involved in postsqualene sterol biosynthesis (*30*). Hydrophobicity analysis indicates that the sigma receptor and fungal sterol isomerases are topologically similar, each containing three hydrophobic regions. One proposed model suggests that the first hydrophobic segment is likely to be a transmembrane domain (TMD) (*30*), with the two additional hydrophobic segments (highly conserved regions), which we propose to be steroid binding domain-like (SBDL I and SBDL II). The sigma receptor itself does not have sterol isomerase activity, nor can it rescue sterol isomerase-deficient yeast (*35*). Data derived from N- and C-terminal GFP-sigma-1 receptor expression in *Xenopus* oocytes (*7*) support the conclusion that the sigma-1 receptor contains two transmembrane domains, resulting in an extracellular loop of approximately 50 amino acids and an intracellular C-terminal domain of approximately 120 amino acids. Several splice variants of the sigma-1 receptor have been identified (*36*), but no function has been defined for these variants.

Several classes of ligands, including neurosteroids, neuroleptics, dextrobenzomorphans, and psychostimulants, such as cocaine, have been shown to bind to sigma receptors. The relationship between the sigma receptors and cocaine action has been reported and previously reviewed, and ligands for the sigma-1 receptor will block the sensitization and attenuate the locomotor response to cocaine (*24*, *37*, *38*). In addition, some of the excitatory effects of methamphetamine have been suggested to be due to interactions with the sigma-1 receptor (*39*−*41*).

While there have been numerous reports on the possible function of sigma receptors in recent years, there is relatively little information regarding the structure of the sigma receptor or its ligand binding site. In the present study, we assessed the contribution of the benzoyl moiety of cocaine with regard to sigma-1 receptor binding and applied the technique of photoaffinity labeling to identify the cocaine binding site on the sigma-1 receptor. Photoaffinity labeling approaches have been used intensively to identify the binding sites of the β-adrenergic receptor, monoamine transporters, G-proteins, G-protein-linked effectors, and the sigma receptor (*42*−*48*). Photoaffinity labels have also been applied to the study of sigma-1 receptors (*9*, *48*−*50*). The cocaine analogue [^{125}I]-3-iodo-4-azidococaine ([^{125}I]IACoc) is a high-affinity sigma-1 receptor photoaffinity label that specifically identified the 26 kDa sigma-1 receptor in brain homogenates, PC12 cells, and membranes derived from several organs (*9*, *48*). In this paper, we present data that support the idea that a dipole in the phenyl ring of cocaine results in high-affinity binding to the sigma-1 receptor, and further, we identified aspartate 188 as the site of covalent derivatization by [^{125}I]-IACoc.

EXPERIMENTAL PROCEDURES

Chemical Syntheses. Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are reported uncorrected. NMR spectra were recorded on a Varian 300 spectrometer with the free base form of the compounds except where noted. Spectra were obtained in deuterated chloroform with TMS as an internal standard. (−)-Cocaine hydrochloride was purchased from Sigma-Aldrich.

*Preparation of Methyl (1R-2-exo-3-exo)-8-Methyl-8-azabicyclo[3.2.1]octane-2-carboxylate (Ecgonine Methyl Ester; See Scheme 1) (**1**).* (−)-Cocaine hydrochloride (10.0 g, 0.029 mol) was dissolved in 200 mL of methanol, combined with 20 mL of concentrated H_2SO_4, and refluxed overnight. The reaction mixture was allowed to cool to room temperature. Excess solvent was evaporated under reduced pressure. The residual yellow oil was taken up in a small amount of water and neutralized with saturated Na_2CO_3 solution at 0 °C. The product was extracted four times with 50 mL portions of Et_2O. The combined Et_2O extracts were dried with Na_2SO_4, filtered, and evaporated to dryness. The resulting yellow oil was used in the following reaction without further purification. The crude product was purified by column chromatography (silica gel, toluene−Et_2NH, 20:1): yield >95%. Yellow oil. ^{1}H NMR: δ 4.80 (m, 1 H), 4.2 (m, 1 H), 4.08 (s, 1 H, OH), 3.80 (s, 3 H, OMe), 3.59 (m, 1 H), 2.90 (s, 3 H, NMe), 2.46−2.20 (m, 7 H). ^{13}C NMR: 180, 68.2, 65.13, 61.8, 51.72, 50.24, 41.31, 35.68, 25.79, 25.50.

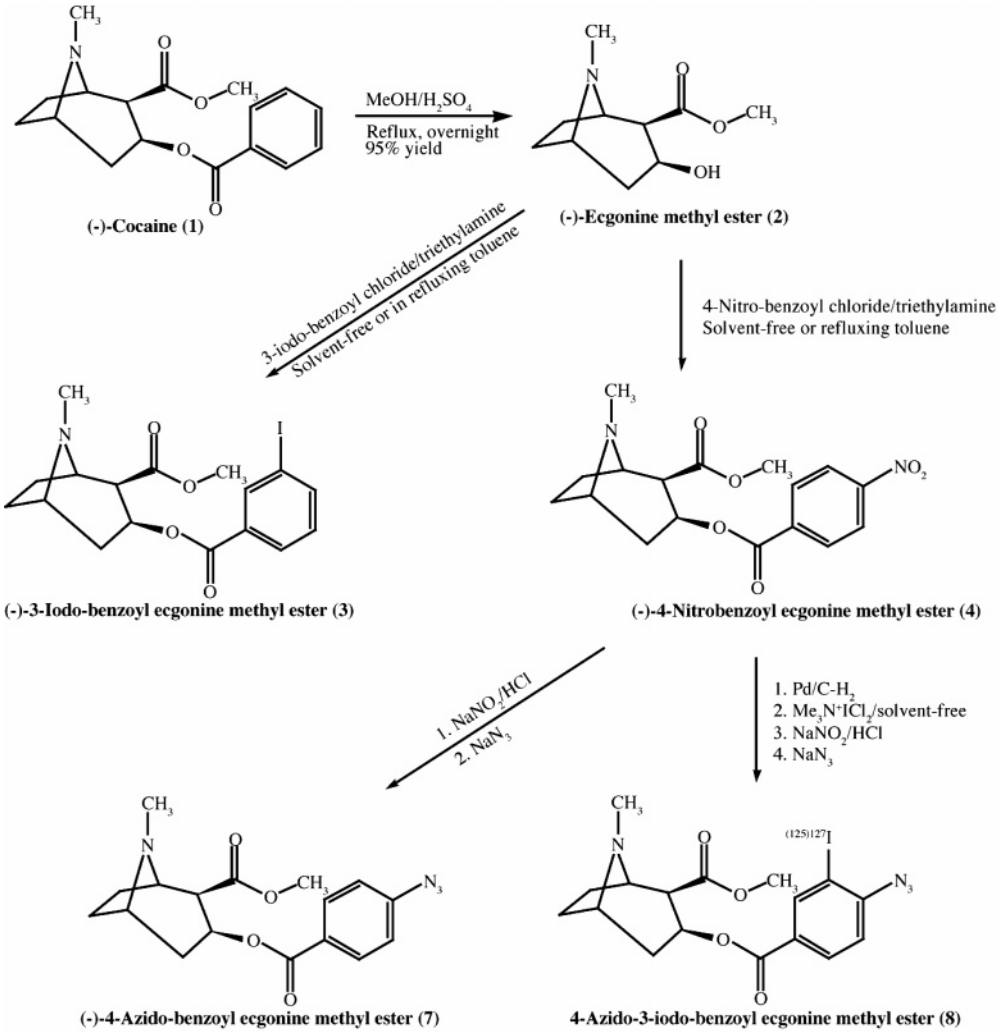
General Method for (−)-4-Nitrobenzoylecgonine Methyl Ester (4-Nitrococaine) and (−)-3-Iodobenzoylecgonine Methyl Ester (3-Iodococaine). Method A. In a mortar a mixture of (−)-ecgonine methyl ester (free base) (1 mmol, 0.2 g), the appropriate arylbenzoyl chloride (1.1 mmol), and 1,4-diazabicyclo[2.2.2]octane (DABCO, 1 mmol, 0.12 g) was ground with a pestle at room temperature; the reaction mixture was dissolved in 3 mL of MeOH and was purified by column chromatography (silica gel, toluene−Et_2NH, 20:1) to afford pure product in >90% yield.

Method B. To a mixture of (−)-ecgonine methyl ester (free base) (1 mmol, 0.2 g) stirred with 1 equiv of K_2CO_3 in toluene was added the appropriate amount of arylcarbonyl chloride (1.1 mmol) over 5 min. The mixture was refluxed for 2 h and allowed to cool to room temperature. After filtration, the solvent was removed at reduced pressure to give a yellow oil. The crude products were purified by column chromatography (silica gel, toluene−Et_2NH, 20:1) to afford pure product in >90% yield.

*(−)-3-Iodobenzoylecgonine Methyl Ester (3-Iodococaine, **3**).* Yellow semisolid. ^{1}H NMR: δ 8.30 (d, J = 1.8 Hz, 1 H), 7.8 (m, 2 H), 7.28 (d, J = 8.4 Hz, 1H), 5.2 (m, 1 H), 3.60 (s, 3 H, OMe), 3.5 (m, 1 H), 3.22 (m, 1 H), 2.29 (s, 3 H, NMe), 2.46−1.2 (m, 7 H). ^{13}C NMR: 180, 170, 149, 138, 135, 120, 118, 117, 65.2, 65.13, 60.78, 50.92, 50.22, 41.31, 35.68, 25.79, 25.50.

*(−)-4-Nitrobenzoylecgonine Methyl Ester (4-Nitrococaine, **4**).* Yellow oil. ^{1}H NMR: δ 8.3 (d, J = 6.8 Hz, 2 H), 8.2 (d, J = 6.8 Hz, 2 H), 5.3 (m, 1 H), 3.78 (s, 3 H, OMe), 3.5 (m, 1 H), 3.32 (m, 1 H), 2.29 (s, 3 H, NMe), 2.46−1.2 (m, 7 H).

Scheme 1



^{13}C NMR: 180, 170, 135, 132, 128, 126, 68.4, 65.13, 60.78, 50.92, 50.22, 41.31, 35.68, 25.79, 25.50.

*(−)-4-Aminobenzoylecgonine Methyl Ester (4-Aminococaine, **5**).* Yellow solid, mp 180−182 °C. ^{1}H NMR: δ 7.76 (d, J = 8.4 Hz, 2 H), 6.62 (d, J = 8.4 Hz, 2 H), 5.25 (m, 1 H), 4.80 (br, 2 H, NH_2), 3.71 (s, 3 H, OMe), 3.60 (m, 1 H), 3.32 (m, 1 H), 3.07 (m, 1 H), 2.24 (s, 3 H, NMe), 2.46−1.2 (m, 7 H). ^{13}C NMR: 175, 170, 150, 135, 132, 128, 126, 68.4, 66.23, 61.78, 50.92, 50.22, 41.31, 35.68, 25.79, 25.50.

*(−)-3-Iodo-4-aminobenzoylecgonine Methyl Ester (3-Iodo-4-aminococaine, **6**).* Yellow solid, mp 195−198 °C. ^{1}H NMR: δ 8.32 (d, J = 1.8 Hz, 1 H), 7.80 (dd, J = 1.8, 8.4 Hz, 1 H), 6.69 (d, J = 8.4 Hz, 1 H), 5.25 (m, 1 H), 4.50 (br, 2 H, NH_2), 3.74 (s, 3 H, OMe), 3.60 (m, 1 H), 3.32 (m, 1 H), 3.07 (m, 1 H), 2.24 (s, 3 H, NMe), 2.46−1.2 (m, 7 H). ^{13}C NMR: 175, 170, 150, 135, 132, 128, 126.3, 126.0, 68.4, 66.23, 61.78, 50.92, 50.22, 41.31, 35.68, 25.79, 25.50.

*(−)-4-Azidobenzoylecgonine Methyl Ester (4-Azidococaine, **7**).* Yellow solid, mp 168−170 °C (dec). ^{1}H NMR: δ 8.3 (d, J = 7.6 Hz, 2 H), 8.2 (d, J = 7.6 Hz, 2 H), 5.24 (m, 1 H), 3.74 (s, 3 H, OMe), 3.65 (m, 1 H), 3.35 (m, 1 H), 3.09 (m, 1 H), 2.26 (s, 3 H, NMe), 2.50−1.69 (m, 7 H). ^{13}C NMR: 175, 170, 135, 130, 125, 123, 68.4, 66.23, 61.78, 50.92, 50.22, 41.31, 35.68, 25.79, 25.50.

*(−)-3-Iodo-4-azidobenzoylecgonine Methyl Ester (3-Iodo-4-azidococaine, **8**).* Yellow solid, mp 202−204 °C. ^{1}H NMR: δ 8.40 (d, J = 1.8 Hz, 1 H), 8.20 (dd, J = 1.8, 8.4 Hz, 1 H), 7.2 (d, J = 8.4 Hz, 1 H), 5.25 (m, 1 H), 3.88 (s, 3 H, OMe), 3.60 (m, 1 H), 3.32 (m, 1 H), 3.07 (m, 1 H), 2.24 (s, 3 H, NMe), 2.46−1.2 (m, 7 H). ^{13}C NMR: 175.20, 170.10, 158.90, 139.10, 135.25, 130.20, 128.10, 127.40, 68.40, 66.23, 61.78, 50.92, 50.22, 41.31, 35.68, 25.79, 25.50.

Transient Expression of Full-Length Guinea Pig Sigma-1 in COS-7 Cells. The pCDNA vectors encoding recombinant GP sigma-1 cDNA were introduced into COS-7 cells by electroporation at 226 V and 950 μF in PBS. The cells were grown under standard conditions in a 15 cm plates in Dulbecco's modified medium supplemented with 10% cosmic calf serum (Hyclone), penicillin, and streptomycin (Irvine Scientific) at 37 °C in 5% CO_2. At approximately 75% confluency, the cells were detached with 6.5 mL of trypsin and transferred to a 15 mL tube, collected by centrifugation at about 500*g* for 15 s. The cell pellet was washed with 10 mL of sterile PBS, collected by centrifugation, and resuspended in 0.7 mL of calcium- and magnesium-free PBS. Approximately 15 μg of plasmid DNA was added to the cells, and the entire solution was transferred to electroporation cuvettes (0.4 cm gap; Bio-Rad) and electroporated (0.4 kV, 960 μF). The cells were transferred to a fresh 15 cm plate with 25 mL of media and grown for 3 days following transfection.

Preparation of COS-7 Cell Homogenates. Transfected cells were detached from the plate with trypsin, collected by centrifugation at 500*g*, and resuspended in 1 mL of cold S/H buffer (0.32 M sucrose, 10 mM HEPES−KOH, pH 7.4) containing 5 mM Mg-EGTA, 2 μg/mL leupeptin, and 0.1 mM approtinin for each plate. Cells were then disrupted manually with a custom-made cell cracker (*51*) using 50 strokes. The cell homogenates were frozen at −80 °C for later use. Protein concentrations were determined to be approximately 3 mg/mL using the Bio-Rad protein assay.

Photolabeling of COS-7 Cell Homogenates. Carrier-free [^{125}I]IACoc was prepared as described previously by Kahoun and Ruoho (*48*). The photolysis experiment was carried out in a final volume of 0.1 mL in S/H buffer. COS-7 homogenate (approximately 60 μg of protein) was incubated in the presence of 1 μL of protecting drug or vehicle control for 30 min in ice. [^{125}I]IACoc (final concentration 1.0 nM) was added, and the incubation was continued for 7.5 min on ice. Photolysis was performed in Pyrex Sorvall centrifuge tubes at 4 °C for 5 s, 10 cm from a 1 kW high-pressure AH6-mercury lamp. Proteins were separated by SDS−PAGE, and the radioactivity was detected using a Phosphorimager 445 SI (Molecular Dynamics Inc.).

Determination of IC_{50}'s. Evaluation of the relative inhibitor potency of phenyl-substituted cocaine derivatives was performed by a modification of the method of Kahoun and Ruoho (*48*). Briefly, guinea pig liver membranes (100 μg total) were incubated in 10 mM sodium phosphate, pH 7.4, containing 120 mM NaCl, 5 mM KCl, and 0.16 mM sucrose in the presence or absence of protecting drug. [^{125}I]IACoc (1 μL of 100 nM stock in methanol) was added to a final concentration of 1 nM. The total reaction volume was 100 μL. The samples were incubated on ice for 7.5 min, after which they were exposed to light from a 1 kW mercury vapor lamp for 5 s at a distance of 10 cm. Proteins were separated by SDS−polyacrylamide gel electrophoresis. The gels were stained with Coomassie and dried. Incorporation of [^{125}I]IACoc into the 26 kDa sigma-1 receptor was quantitated using a Molecular Dynamics 445 SI phosphorimager and ImageQuant. Data were normalized to 100% based on the sample with the largest amount of [^{125}I]IACoc incorporation, and the background, determined in the presence of 50 μM haloperidol, was subtracted from the values. Data from two separate experiments were combined and analyzed to determine the percent inhibition, and nonlinear regression was applied to determine the IC_{50} values using the software package GraphPad Prism. The data were fit to both one-site and two-site competitive binding models. The model with the best fit as determined by the software was chosen.

Preparation of Guinea Pig Liver Microsomes. The protocol used is similar to the rat liver microsomal preparation protocol previously reported by Kahoun and Ruoho (*48*). Briefly, guinea pig livers (Rockland Inc.) at 10 mL of buffer/g wet tissue were minced and homogenized by four bursts of 10 s each using a Brinkman polytron on setting 6. Homogenization was performed in ice-cold sodium phosphate (10 mM, pH 7.4) containing 0.32 M sucrose and a cocktail of protease inhibitors [20 mg/mL leupeptin, 5 mg/mL soybean trypsin inhibitor, 100 μM phenylmethanesulfonyl fluoride (PMSF), 100 mM benzamidine, and 1 mM EDTA]. The microsomal membrane suspension was centrifuged for 10 min at 17000*g*, after which the supernatant was centrifuged for 60 min at 105000*g*. The liver microsome pellet was resuspended in homogenization buffer, snap frozen, and stored at −80 °C at a final protein concentration of 10 mg/mL.

Sigma Receptor Constructs. All constructs generated were confirmed by DNA sequencing at the University of Wisconsin Biotechnology Center using the BigDye FS (Perkin-Elmer) protocol. pCDNA3.1(Myc-Sigma-1) contained the cDNA for the GP liver sigma-1 receptor with a c-Myc epitope at the N-terminus. This construct was generated using PCR amplification with *Pfu* polymerase. The forward primer was 5′GGATCCGGATCCGCCACCATGGAACAGAAGCTGATCTCTGAAGAAGATCTG**ATGCAGTGGGCCGTGG**3′, and the reverse primer was 5′CTCGAGCTCG**AGTCAAGGGTCTTGGCCG**3′ (the underlined sequence is the myc epitope, and the bold sequence is the sigma-1 sequence). The PCR-amplified product was gel purified and digested with *Bam*HI and *Xho*I. This reaction was heat inactivated, and the digested product was ligated into *Bam*HI and *Xho*I digested pCDNA3.1(+) (Invitrogen).

C-Terminal truncations in pCDNA3.1(Myc-Sigma-1) were generated by PCR as described above using the forward primer above and the following reverse primers: $\sigma\Delta5$, 5′CTCGAGCTCGAGTCACTCACCACCTACCTC3′; $\sigma\Delta15$, 5′CTCGAGCTCGAGTCACGAGTCTATGCCCGG3′; $\sigma\Delta25$, 5′CTCGAGCTCGAGTCACAGGACTTCCTCACC3′; $\sigma\Delta35$, 5′CTCGAGCTCGAGTCATTTTCACTGGCTGAC3′; and $\sigma\Delta45$, 5′CTCGAGCTCGAGTCATACGGCCGGGGTGTCATC3′. PCR products were cloned into *Bam*HI and *Xho*I double-digested pCDNA3.1(+).

pET2a(ΔNT-Sigma-1-His) contained the cDNA for the N-terminal truncated (amino acids 1−38) GP liver sigma-1 receptor with an N-terminal T7 epitope tag and a C-terminal hexahistidine tag. This protein was overexpressed in *Escherichia coli* and was used as carrier peptide for the N-terminal peptide sequencing of [^{125}I]IACoc-labeled sigma-1 receptor peptides. The construct was generated by PCR using the primers 5′GGATCCGGATCC**ATGGAAGAAATCGCGCAGCTG**3′ (forward) and 5′CTCGAGCTGAGTC**AGTGGTGGTGGTGGTGGTGAGGGTCTTGGCCG**3′ (reverse) and *Pfu* polymerase (Stratagene). Conditions for PCR were 30 cycles of 97 °C for 1 min, 56 °C for 2 min, and 72 °C for 3 min and followed by a 7 min extension at 72 °C. The insert was purified and digested with *Xho*I and *Bam*HI and ligated into *Xho*I/*Bam*HI-digested pET2a (Novagen).

Expression of ΔNT-Sigma-1-His. A single colony of BL21(DE3)pLysS *E. coli* containing a vector pET2a(ΔNT-Sigma-1-His) was used to inoculate a 5 mL overnight culture in LB (with 100 μg/μL ampicillin), which was used to inoculate 1 L of LB−ampicillin. The culture was grown at 37 °C with vigorous shaking until the A_{600} reached 0.5 OD. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to the flask to a final concentration of 0.05 mM and the incubation continued for an additional 3 h. *E. coli* were collected by centrifugation for 5 min at 5000*g* at 4 °C. The pellet was resuspended in 100 mL of PBS buffer and centrifuged at 5000*g*, washed twice with PBS buffer (100 mL each), and then frozen in dry ice. The cells lysed upon thawing by the resuspension in 100 mL of PBS, and the particulate fraction was collected by centrifugation at 39000*g* for 30 min. The pellet was solubilized in 100 mL of PBS

buffer containing 8 M urea and disrupted by sonication (Sonifier, Model J17V; Branson Instruments Inc., Danbury, CT) on ice in short bursts (50% power, 10 bursts × 3 s each). The sonicated material was mixed for 1 h at 4 °C and then centrifuged at 50000*g* for 20 min at 4 °C. The supernatant was then isolated by SDS−PAGE as described below.

Isolation of ΔNT-Sigma-1-His. A portion of the supernatant prepared above was solubilized in SDS−PAGE loading buffer and loaded into all 10 wells of a 10-well gel, 12% SDS−polyacrylamide gel. The proteins were separated, and the gel was briefly stained in 0.025% (w/v) Coomassie Blue R-250 in 40% methanol for 5 min. Excess stain was removed with 45% methanol and 10% acetic acid (aldehyde-free) for 10 min. The gel containing the fusion protein at approximately 22 kDa was excised with a razor blade. The gel slices were placed into a 1.5 mL microcentrifuge tube and minced with a metal spatula. Water (0.5 mL) was added to form a slurry. The tube was mixed end-over-end overnight at room temperature. The gel slurry was transferred to a Bio-Rad "spin column" and the water collected by centrifugation at 1600*g* for 5 min. The eluted material was concentrated by lyophilization and stored at −20 °C for digestion with CNBr.

Isolation of [^{125}I]IACoc-Labeled Sigma-1. Preparation and photolabeling of COS-7 cell homogenates were performed as described above. Typically, eight photolabeling reactions were performed, and the samples were purified by electrophoresis on 12% SDS−polyacrylamide gels. The photolabeled sigma-1 receptor was visualized by "wet gel" (unstained) autoradiography, and the autoradiogram was used as a template to excise the specifically labeled sigma-1 receptor from the gel with a razor blade. The gel slices were eluted in water as described above. Typically, greater than 80% of the [^{125}I]IACoc-photolabeled sigma-1 receptor was recovered in the aqueous supernatant.

CNBr Digestion of the Carrier and [^{125}I]IACoc-Labeled Sigma-1 Receptors. The SDS−PAGE-purified carrier sigma-1 receptor (ΔNT-Sigma-1-His) and [^{125}I]IACoc-labeled sigma-1 receptor extracts from COS-7 cell expression were pooled and tumbled with CNBr (0.15 M) in 1 mL of 70% formic acid solution for 15−18 h in room temperature. After the acid was removed by lyophilization, water (1 mL) was added, and the sample was lyophilized twice to ensure complete removal of reactants.

Purification of [^{125}I]IACoc-Labeled Peptides. Following CNBr digestion, the carrier and photolabeled peptides were purified by a 16.5% tricine SDS−PAGE electrophoresis and identified by wet gel autoradiography. The radiolabeled peptides were excised, eluted with 0.3 mL of water by tumbling overnight in a 1.5 mL microcentrifuge tube at room temperature, and collected on an Immobilon PVDF membrane (Bio-Rad) mounted in a Prosorb column (Applied Biosystems Division, Perkin-Elmer, Foster City, CA). The column was washed twice with 0.1 mL of 0.1% trifluoroacetic acid solution (TFA) and sent to Dr. Joe Leykam at the Michigan State University Macromolecular Structure Facility for sequencing and radiosequencing using an automated Edman degradation with an Applied Biosystems 477A gas-phase amino acid sequencer. N-Terminal amino acid sequencing was used to identify the peptide. The radioactive content in each of the cycles was used to identify the precise [^{125}I]IACoc insertion site(s) in the sigma-1 receptor ligand binding site.

Western Blot of N-Terminal Myc Epitope Tagged Sigma-1 Receptors. COS-7 cell homogenates (about 6 μg each) expressing the various truncations were separated on a 15% SDS−PAGE gel and transferred to PVDF paper by the method of Matsudaira (*52*). The PVDF paper was treated with 5 mL of SuperBlock (Pierce) in PBS containing 0.05% Tween-20 (PBST) for 1 h to overnight at 4 °C. The SuperBlock solution was removed. Anti-myc antibody (Covance), which was diluted 1:10000 in PBST containing 1% BSA, was then added and incubated for 1 h at room temperature. The primary antibody solution was removed, and the PVDF membrane was washed four times with PBST. Each time, the PVDF membrane was incubated for 5 min with 10 mL of PBST. Secondary antibody (Bio-Rad, HRP conjugated goat anti-mouse) diluted 1:50000−1:100000 in PBST was added for 30 min. The PVDF membrane was washed four times with 10 mL of PBST for 5 min each time, and the polypeptides were visualized using the Pierce Supersignal West Dura extended duration substrate (ECL) and Amersham hyperfilm with an exposure time of less than 10 s.

Protein Assays. The protein concentration was determined with the dye reagent from Bio-Rad (Bio-Rad Laboratories) using the microassay protocol (Bradford method). Bovine serum albumin was used as the standard protein.

SDS−Polyacrylamide Gel Electrophoresis. Molecular weight standards were purchased from Sigma Chemical Co., St. Louis, MO. Protein separation was performed according to the method of Laemmli (*53*) using either 12% or 15% polyacrylamide gels or by the method of Schagger and von Jaggow (*54*) with the following proteins as molecular mass markers: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceradehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa), and aprotinin (6.5 kDa).

RESULTS

Synthesis of 3-Substituted Cocaine Derivatives. The synthesis of the 3-substituted analogues (**3**−**8**) is outlined in Scheme 1. (−)-Ecgonine methyl ester (**2**) was obtained from (−)-cocaine (**1**) by refluxing (−)-cocaine in a mixture of methanol and concentrated H_2SO_4 overnight; (−)-ecgonine methyl ester (**2**) was obtained in 95% yield as a yellow oil. 3-Iodo- and *p*-nitrobenzoyl chloride were reacted with (−)-ecgonine methyl ester under solvent-free conditions or by refluxing in toluene in the presence of triethylamine to afford the 3-carboxy analogues (**3** and **4**, respectively). Purification of products was performed by either preparative thin-layer chromatography (TLC) or flash chromatography. (−)-4-Nitrobenzoyl ecgonine methyl ester was reduced to (−)-4-aminobenzoylecgonine methyl ester (**5**) in methanol with Pd/C−H_2 in 90% yield. Reaction of 4-aminobenzoylecgonine methyl ester (**5**) with $Me_4N^+ICl_2^-$ under solid-state conditions (*55*) afforded 4-amino-3-iodobenzoylecgonine methyl ester (**6**) in ∼90% yield after purification of the crude product by preparative thin-layer chromatography (TLC) (toluene−Et_2NH, 4:1). The (−)-4-azidobenzoylecgonine methyl ester (**7**) or 4-azido-3-iodobenzoylecgonine methyl ester (**8**) was

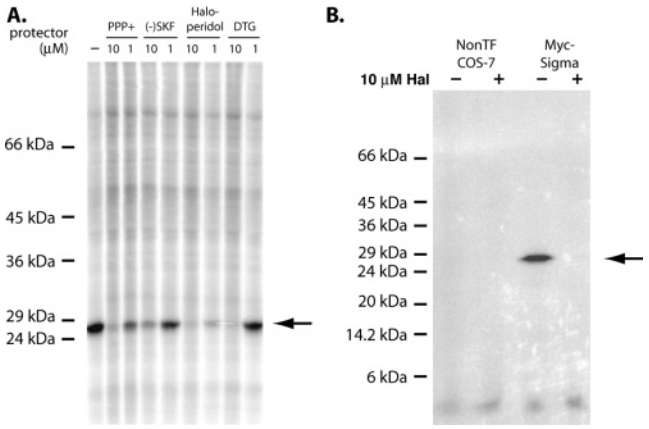


FIGURE 1: Photolabeling of the COS-7-expressed guinea pig sigma-1 receptor using [^{125}I]IACoc and protection with sigma-1 receptor ligands. COS-7 cell homogenates containing sigma-1 receptor (100 μg of total protein) were incubated at 4 °C with and without protectors as indicated above in a volume of 99 μL for 30 min. Then 1 μL of [^{125}I]IACoc (1 nM final concentration) was added, and the mixture was incubated for an additional 7.5 min. The reaction mixtures were photolyzed for 5 s at 4 °C. [^{125}I]IACoc derivatization of the sigma-1 receptor was determined by phosphorimager analysis following separation by SDS−PAGE. The phosphorimager scan of an SDS−polyacrylamide gel is shown in panel A, demonstrating the pharmacology of the COS-7-expressed guinea pig sigma-1 receptor. The arrows indicate the position of the 26 kDa sigma-1 receptor. There is no detectable specific [^{125}I]-IACoc labeling in the nontransfected COS-7 cell membranes (shown in panel B as nonTF).

obtained from the reaction of (−)-4-aminobenzoylecgonine methyl ester (**5**) or (−)-3-iodo-4-aminobenzoylecgonine methyl ester (**6**) with HNO_2 and then NaN_3 in excellent yields. Compounds **2**−**8** were confirmed by ^{1}H and ^{13}C NMR analysis.

The Sigma-1 Receptor Can Be Transiently Expressed in COS-7 Cells. The guinea pig sigma-1 receptor was subcloned into the mammalian expression vector, pCDNA3.1(+). This construct included the ten amino acid myc epitope at the N-terminus of the sigma-1 receptor sequence. The protein was expressed in COS-7 cells and exhibited characteristic sigma-1 pharmacology, as demonstrated by the inhibition of [^{125}I]IACoc photoaffinity labeling by various sigma ligands (Figure 1, panel A). As expected, haloperidol, a high-affinity ligand for the sigma receptor (both sigma-1 and sigma-2), showed the best protection at 1 μM, while the other ligands showed complete, or almost complete, protection at 10 μM. There was no detectable [^{125}I]IACoc photoaffinity labeling in nontransfected COS-7 cell membranes (Figure 1, panel B).

IC_{50}'s of 3-Substituted Cocaine Derivatives. From previous studies in our laboratory (*48*), it was observed that the addition of an iodine molecule at the 3-position and a 4-azido moiety on the phenyl ring of cocaine increased binding affinity to the sigma-1 receptor by 2−3 orders of magnitude over the parent compound. We determined the IC_{50}'s for several cocaine derivatives using protection of [^{125}I]IACoc photolabeling of the 26 kDa sigma-1 receptor as the assay. Guinea pig liver membranes containing the sigma-1 receptor were incubated with 1 nM [^{125}I]IACoc in the presence of increasing concentrations of ligand. The samples were separated by SDS−PAGE, and the sigma-1 receptor at 26 kDa was identified by a phosphorimager scan of the dried gel (Figure 2A). Included in Figure 2A is a complete gel lane showing [^{125}I]IACoc labeling of guinea pig liver membranes in the presence and absence of 50 μM haloperidol. As can be seen, only the 26 kDa sigma-1 receptor is specifically labeled, as determined by haloperidol protection. Quantitation of the radioactivity in the 26 kDa sigma-1 receptor was determined using the phosphorimager scans and ImageQuant software as described in Experimental Procedures. These data were plotted and fit to both a one-site and a two-site competitive binding model to the IC_{50} (see Figure 2B for representative curves). The best fitting model determined by the Prism software package was chosen. Both cocaine and 4-nitrococaine fit best to a two-site competitive binding model. The data are summarized in Table 1. As can be seen, IACoc (IC_{50} of 64 nM) showed the highest affinity, 100 times better than cocaine (IC_{50} of the highest affinity site, 6800 nM). The rank order of affinities were IACoc (4-azido-3-iodococaine) > 4-nitrococaine > 4-azidococaine > cocaine > 4-aminococaine > 3-iodococaine > 4-amino-3-iodococaine.

Identification of the [^{125}I]IACoc Insertion Site. The photoactive cocaine derivative, [^{125}I]IACoc, was radioiodinated in a carrier-free manner as previously described by Kahoun and Ruoho (*48*) and used to derivatize COS-7 cell membranes expressing the sigma-1 receptor. The expressed sigma-1 receptor displayed a pharmacology consistent with the sigma-1 receptor as demonstrated in Figure 1. [^{125}I]IACoc derivatization of the COS-7-expressed sigma-1 receptor was inhibited by the sigma-1 receptor ligands PPP+, (+)-*N*-allylnormetazocine (SKF 10,047), haloperidol, and DTG. Each ligand demonstrated a concentration-dependent inhibition, in which the extent of sigma-1 receptor derivatization was less in the presence of 10 μM protecting ligand than with 1 μM.

Several samples of COS-7-expressed sigma-1 receptor were derivatized with [^{125}I]IACoc and separated by SDS−PAGE. A wet gel autoradiograph identified the 26 kDa sigma-1 receptor, which was excised with a razor blade. This [^{125}I]IACoc-labeled sigma-1 receptor was then pooled with a sample of *E. coli* expressed sigma-1 receptor, ΔNT-Sigma-1-His, which had been excised and eluted from an SDS−polyacrylamide gel (while the *E. coli* expressed sigma-1 receptor is not functional to bind ligand, it serves as a source of carrier protein and peptide for sequencing). The pooled samples were digested with cyanogen bromide, and the peptides were separated using a tricine gel (*54*) and electroblotted to PVDF membrane. Cyanogen bromide, which cleaves after methionine residues, produced the expected peptides and their predicted molecular masses are shown in Figure 3A. The CNBr peptides are identified on the Coomassie-stained PVDF membrane in Figure 3B. This peptide was excised from the PVDF membrane and subjected to N-terminal sequencing and radiosequencing. The radioactivity released by radiosequencing and the single amino acid sequence that was obtained for the 6 kDa fragment are shown in Figure 3C (a single sequence was obtained). It is evident that the primary insertion site of [^{125}I]IACoc is at aspartate 188 in the carboxyl-terminal region of the sigma-1 receptor in a hydrophobic region that has high sequence homology to fungal and yeast sterol isomerases (*56*). A model of the sigma-1 receptor as presented by Aydar et al. (*7*) is shown in Figure 3D, indicating steroid binding domain-like I and

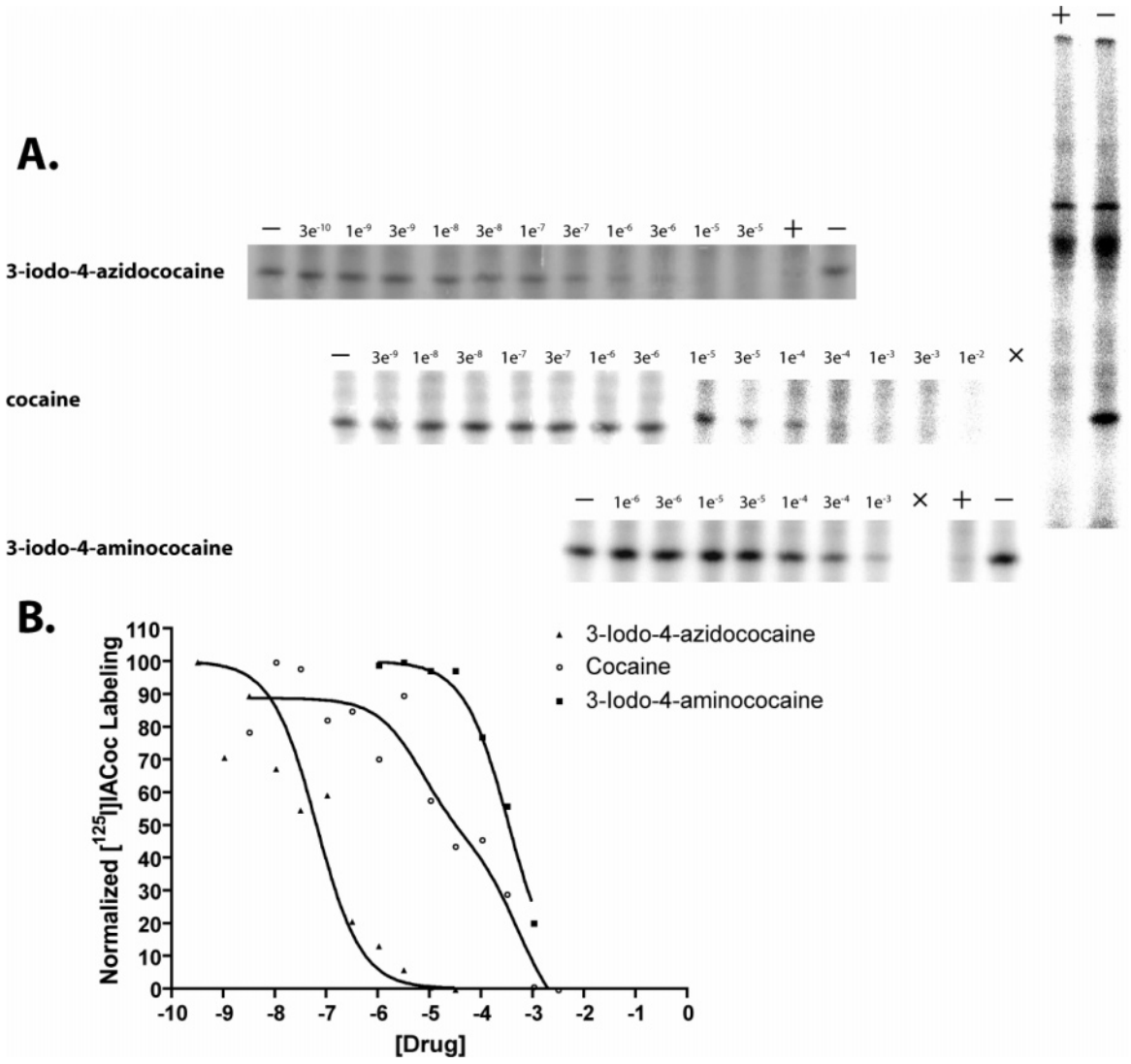


FIGURE 2: Inhibition of [^{125}I]IACoc photolabeling of the 26 kDa sigma-1 receptor by the cocaine derivatives. Approximately 100 μg of guinea pig liver homogenates was used in each lane. The homogenates were incubated at 4 °C with and without protectors as indicated above in a volume of 99 μL for 30 min. Then 1 μL of [^{125}I]IACoc was added, and the mixture was incubated for 7.5 min. The reaction mixtures were photolyzed for 5 s at 4 °C. [^{125}I]IACoc derivatization of the 26 kDa sigma-1 receptor was determined by phosphorimager analysis following SDS−PAGE (A). Controls were determined in the absence (−) and presence (+) of 50 μM haloperidol (shown as complete lanes for the cocaine data). Two separate gels are shown for the cocaine data. The scans have been aligned by the concentration of protecting ligand present. The data were plotted using GraphPad Prism. (B) Shown are representative data for cocaine (○), IACoc (▲), and 4-amino-3-iodococaine (■). The data were normalized to the largest amount of [^{125}I]IACoc incorporation, and the background, as determined in the presence of 50 μM haloperidol, was subtracted. 3-Iodo-4-azidococaine (IACoc) and 3-iodo-4-aminococaine fit best to a one-site competitive binding model, whereas cocaine fit best to a two-site competitive binding model.

II and the location of [^{125}I]IACoc-derivatized aspartate 188. Alignment of the sigma-1 amino acid sequence from several species and the yeast C7−C8 sterol isomerase (Erg2) shows high homology to the two regions, termed steroid binding domains, of Erg2 (Figure 3E). We have named these regions steroid binding domain-like I and II (SBDL I and SBDL II) in the sigma-1 receptor. Aspartate 188 is located in the SBDL II sequence (in bold type in Figure 3E).

Requirement of the C-Terminus for [^{125}I]IACoc Labeling. Carboxyl-terminal deletion mutants of the sigma-1 receptor were constructed to determine the contribution of the C-terminus to binding of ligands. Deletion mutants in which 5, 15, 25, 35, and 45 amino acids were removed from the C-terminus were generated and expressed in COS-7 cells. These constructs contained an N-terminal myc epitope and are depicted in Figure 4A. Aspartate 188 (38 residues from the C-terminus) would be removed by Δ45 but retained by the other constructs. As can be seen from the anti-myc Western blot (Figure 4B), all of the constructs are expressed in COS-7 cells; however, removal of 15 residues from the C-terminus was sufficient to abrogate [^{125}I]IACoc labeling of the sigma-1 receptor binding site.

DISCUSSION

(1) Cocaine Binding to the Sigma-1 Receptor Is Enhanced by Generation of a Phenyl Ring Dipole. Several cocaine-related tropane analogues have been previously synthesized (*57*−*66*), leading to high-affinity and selective cocaine receptor ligands and providing information about the structure/activity relationship of cocaine-related tropane derivatives. Cocaine contains an 8-azabicyclo[3.2.1]octane framework and is one of the eight possible stereoisomers of methyl

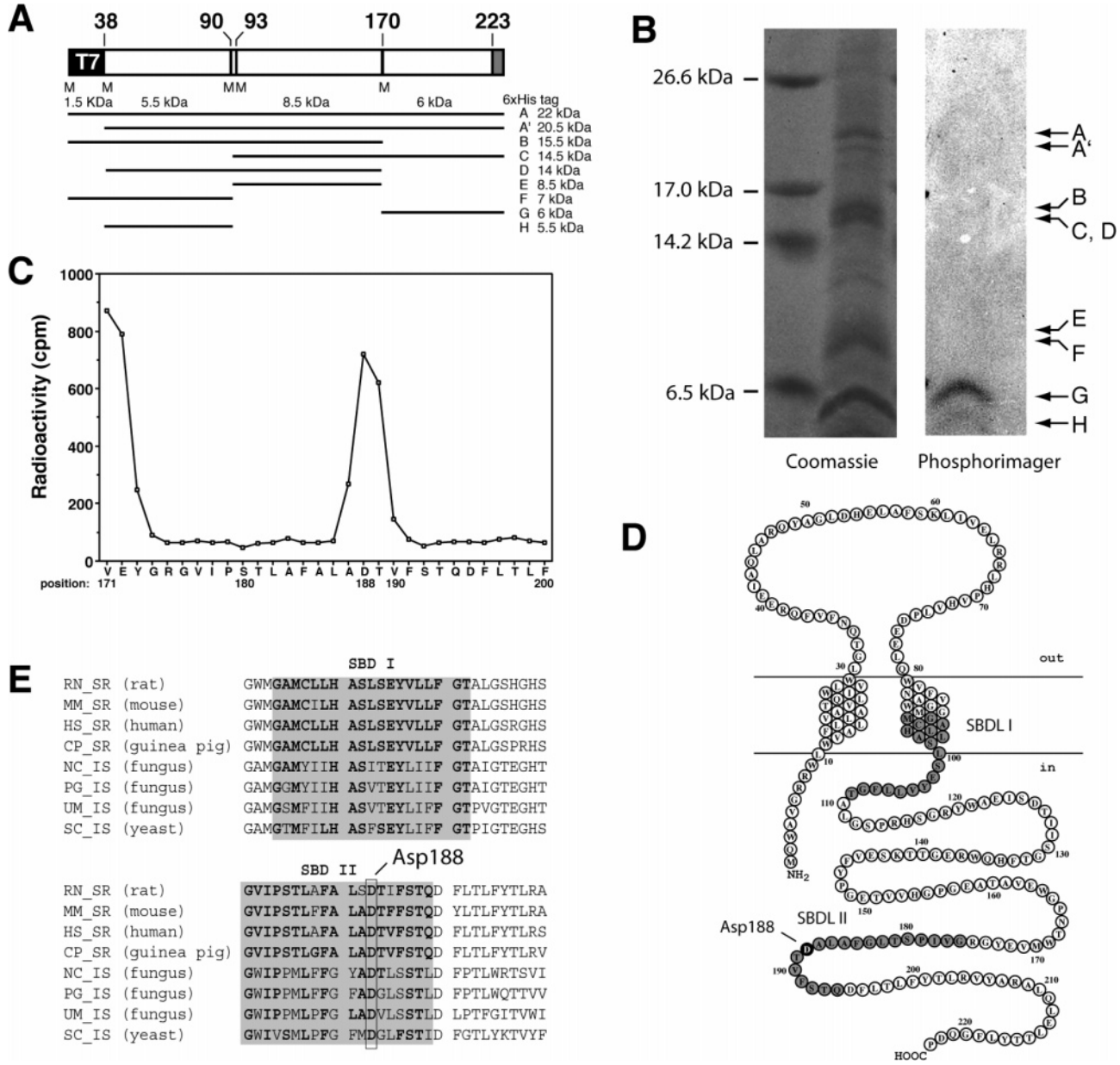


FIGURE 3: Identification of the [^{125}I]IACoc binding site. (A) Sigma-1 receptor cleavage by CNBr. The positions of methionine residues are indicated. The numbers refer to amino acid numbers in the wild-type guinea pig liver sigma-1 receptor. Below are shown the expected peptides and their predicted molecular mass based on incomplete digestion with CNBr. (B) A Coomassie-stained 16.5% tricine gel of purified T7-Δsigma-1-His pooled with [^{125}I]IACoc-labeled sigma-1-His from COS-7 cells and the corresponding phosphorimager scan showing [^{125}I]IACoc-labeled peptides are shown. Peptide G (C-terminal peptide) was specifically labeled by [^{125}I]IACoc and confirmed by sequencing. (C) The N-terminal amino acid sequence and radioiodine release from the 6 kDa CNBr peptide. The primary radioactive peak corresponds to aspartate 188, which is shown in a model of the sigma-1 receptor (panel D, dark residue). SBDL I and SBDL II are shown as shaded residues in the model and in the sequence alignment (E). Identical residues are in bold type. The model is based on data from Aydar et al. (*7*).

3-(benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate (*66*). In addition to the synthesis of this azabicyclo ring system, a major hurdle to its synthesis has been control of stereochemistry, both of enantiomeric integrity and of the thermodynamically unstable axial carboxylate function. Most of the nonracemic cocaine analogues were synthesized by derivatization of natural cocaine (*60*−*65*), while others have been obtained by resolution or separation of racemic or diastereomeric reaction mixtures (*57*−*59*, *66*).

Previously, we synthesized the cocaine photoaffinity label, [^{125}I]-3-iodo-4-azidobenzoylmethylecgonine ([^{125}I]IACoc) and characterized it as a high-affinity photoaffinity label for the sigma-1 receptor in rat liver microsomal membranes (*48*). The unique feature of this photoprobe, which differed from cocaine only by addition of a 3-position iodine atom and a 4-position azido moiety on the benzoyl ring, was the fact that the affinity for the sigma-1 receptor binding site increased by 2−3 orders of magnitude compared to the parent compound cocaine. We have investigated the basis for this increase in affinity by synthesizing all combinations of the iodo and azido groups on the phenyl ring, as well as the precursor 4-nitro- and 4-aminobenzoyl derivatives.

To analyze the affinity of the various derivatives, we assayed [^{125}I]IACoc incorporation into the 26 kDa sigma-1

Table 1: IC_{50}'s of Cocaine Derivatives[a]

compound	log IC_{50} site 1	IC_{50} site 1 (μM)	log IC_{50} site 2	IC_{50} site 2 (μM)
3-iodo-4-azidococaine	−7.2	0.064		
4-nitrococaine	−7.0	0.110	−5.3	4.7
4-azidococaine	−5.9	1.3		
cocaine	−5.2	6.8	−3.3	440
4-aminococaine	−4.0	90		
3-iodococaine	−3.8	170		
3-iodo−4-aminococaine	−3.4	350		

[a] IC_{50}'s were determined by inhibition of [^{125}I]IACoc photoaffinity labeling of the 26 kDa sigma-1 receptor. Data from two separate experiments were fit to both a one-site and a two-site competitive binding model. Best fitting models based on the Prism software are shown.

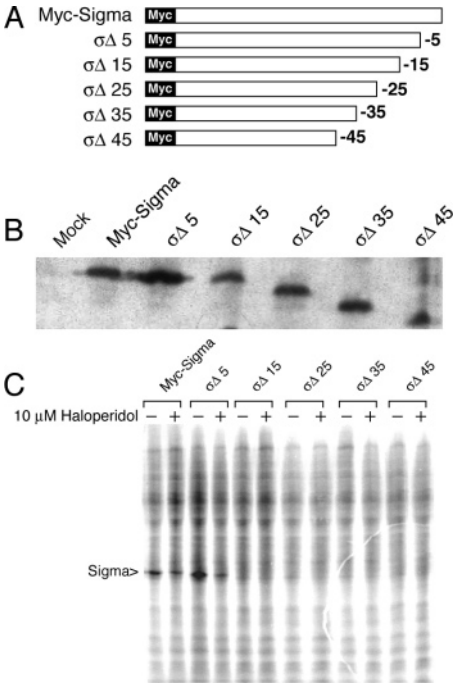


FIGURE 4: Western blot and [^{125}I]IACoc photolabeling of C-terminal truncated sigma-1 receptors. (A) Diagram of the full-length and truncated Myc-Sigma-1 constructs. (B) Western blotting of the above constructs using anti-Myc antibody. All of the constructs were well expressed as indicated by Western blotting. (C) Photolabeling of the full-length and truncated Myc-Sigma-1 constructs. Phosphorimager scan of the same gel. Full-length Myc-Sigma-1 and Δ5 showed protectable labeling using 1 μM haloperidol as protector. The other C-terminal truncations were not labeled by [^{125}I]IACoc.

receptor in the presence of various concentrations of protecting ligands. This approach allows the measurement of the affinity specifically for the labeled sigma-1 receptor, in a native membrane environment in the absence of detergents. On the basis of previous results from our laboratory (*48*), the binding of [^{125}I]ACoc to rat liver membranes occurs very rapidly with an association rate constant (k_{obs}) of 0.29 min^{-1}. The short incubation times with the ligands are adequate for the system to reach equilibrium. An assumption made here is that a constant fraction of the bound [^{125}I]IACoc molecules covalently react in the binding site. Therefore, at any given concentration of competing inhibitor, the amount of incorporated [^{125}I]IACoc reflects the amount of label bound to the site at equilibrium.

The results show that inhibition of [^{125}I]IACoc photolabeling of the 26 kDa guinea pig liver microsomal sigma-1 receptor occurred most efficiently with 4-nitrococaine, 4-azidococaine, and 3-iodo-4-azidococaine (see Figure 2 and Table 1). These results are consistent with the fact that generation of a dipole in the phenyl moiety of cocaine by introducing electron-withdrawing substituents on the phenyl ring enhances the affinity of the cocaine derivatives for the sigma-1 binding site. This also implies that the binding site in the region of the phenyl ring of cocaine contains a source of electrons that could be in the form of the π system of aromatic amino acid side chains, such as phenylalanine, tyrosine, or tryptophan, or other electron-rich sources, such as ionized aspartate or glutamate residues. These data further predict that substitution of dipole-generating phenylalkyl substitutions on the nitrogen atom of sigma-1 ligands that preserve the important lone pair electrons of the nitrogen atom for sigma-1 binding (*67*) will greatly enhance the affinity for the sigma-1 binding site.

(2) Identification of the Interaction Site of the Benzoyl Moiety of Cocaine with the Sigma-1 Binding Site. Photoaffinity labeling is a useful tool for the identification of portions of a ligand binding site. This approach was used to identify a region of the sigma-1 receptor that interacts with the benzoyl moiety of cocaine. Figure 1 shows the highly selective covalent labeling of the overexpressed guinea pig sigma-1 receptor with [^{125}I]IACoc in COS-7 cell membranes. Appropriate protection of photolabeling at 26 kDa was demonstrated for the sigma-1 ligands, PPP^+, SKF 10,047, haloperidol, and ditolylguanidine (DTG).

Using a cyanogen bromide cleavage strategy and *in situ* derivatization of the sigma-1 receptor binding site in the membrane, the denatured [^{125}I]IACoc-photolabeled sigma-1 receptor was then pooled with an SDS−PAGE purified *E. coli* expressed sigma-1 receptor, which was used as a source of carrier protein in order to obtain sequence information. Since the overexpressed sigma-1 receptor was the major protein from *E. coli*, and the CNBr fragment from the C-terminus was identical to the CNBr fragment obtained from the overexpressed sigma-1 receptor, it was reasoned that this approach would be useful for obtaining sequence and radiosequence information. Upon CNBr cleavage, a 6 kDa radiolabeled peptide fragment was identified, from which, when sequenced by conventional and radiosequencing approaches, a single sequence was obtained, which demonstrated that the majority of the radiolabel was inserted into aspartate 188 (Figure 3C). Incomplete extraction of the derivatized aspartate by the butyl chloride/ethyl acetate extraction protocol that was used during the microsequencing was shown to account for the overlap of the radioactivity with the threonine 189 position (data not shown). The radioactivity observed in the first two cycles is due to a leaching of peptide that occurs from the PVDF paper during the initial cycles of sequencing. We have observed this when sequencing other peptides.

The discovery of the interaction of Asp188 with the benzoyl moiety is highly significant since this region of the sigma-1 receptor aligns favorably with the sequence of the steroid binding domain II (SBD II) of the yeast and fungal sterol isomerases, as shown in Figure 3D,E (*68*). These data indicate that the sigma-1 receptor interacts with cocaine (and perhaps other ligands for the sigma-1 receptor that contain

equivalent aromatic substitutions), at least in part, at the SBDL II region and is consistent with deletion of residues 119−149 (exon 3), which reduced radioligand binding to the sigma-1 receptor (*69*). A report from Seth et al. demonstrated that the aspartate 126 and glutamate 172 in the sigma-1 receptor are obligatory for ligand binding (*70*), which, in part, supports our data for aspartate 188 lying in, or close to, the binding site. Furthermore, perturbation of the C-terminus disrupts this interaction, as shown by Figure 4. When systematic truncation of the sigma-1 receptor was performed and the truncated receptors were expressed in COS-7 cells, photolabeling of the sigma-1 receptor by [^{125}I]IACoc was still observed when only 5 amino acids (residues 219−223) were removed from the C-terminus; however, labeling was abolished when 15 amino acid residues (residues 209−223) were removed from the C-terminus, suggesting that important structural determinants that support[^{125}I]IACoc binding and derivatization exist in the C-terminal portion of the sigma-1 receptor.

In summary, these data demonstrate that the interaction of cocaine derivatives with the sigma-1 receptor are profoundly affected by generation of a dipole in the phenyl ring of cocaine with a reduction of the π electron density in the ring. Consistent with this dipole generation, the interaction site of the phenyl ring of cocaine with the sigma-1 receptor has been identified to be close to or in direct interaction with Asp188 in the putative SBDL II binding domain in the third hydrophobic sequence in the C-terminus of the receptor. High-affinity binding of [^{125}I]IACoc to the sigma-1 receptor is further dependent on structural features of the C-terminus that are abrogated when greater than five residues are removed from the C-terminus.

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