Articles

Photoaffinity Labeling of Central Cholecystokinin Receptors with High Efficiency†

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ABSTRACT: A new photoreactive tritiated cholecystokinin (CCK) analogue was synthesized which contains the *p*-benzoylbenzoyl moiety linked to an ornithine residue at the N-terminus of the sulfated CCK octapeptide (CCK-8s). *p*-Benzoylbenzoyl-Orn(propionyl)-CCK-8s bound specifically and with high affinity to CCK binding sites in membranes both from pig cerebral cortex and from rat pancreatic membranes. The apparent dissociation constants K_D were 1.2 nM and 0.5 nM, respectively. [^{3}H]-*p*-Benzoylbenzoyl-Orn(propionyl)-CCK-8s was incubated with CCK_B receptor preparations enriched by lectin chromatography and subsequently photoactivated. A polypeptide migrating with an apparent molecular weight M_r of 56 000 in sodium dodecyl sulfate–polyacrylamide gel electrophoresis was specifically labeled. The labeling was suppressed by the CCK_B receptor agonist pentagastrin. The efficiency of incorporation of radioactivity was high, reaching up to 70% of specifically bound radioactivity. After treatment with trifluoromethanesulfonic acid, the molecular weight of the labeled protein decreased to 45 000, indicating that the receptor is glycosylated. Photoaffinity labeling of CCK_A receptors from rat pancreas resulted in the specific labeling of a protein band with M_r of 80 000–100 000. Our synthetic approach should be useful for the design of photoreactive analogues of a variety of peptides. The high efficiency photolabeling of the CCK_B receptor will be valuable for further characterization and purification of this receptor.

Cholecystokinin (CCK),[1] a 33 amino acid peptide originally isolated from pig small intestine (Mutt & Jorpes, 1968), belongs to the CCK/gastrin peptide family found in the gastrointestinal tract as well as in the central nervous system.

The physiological function of intestinal-released CCK includes stimulation of pancreatic secretion, gall bladder contraction, and stimulation of intestinal mobility (Go, 1978). The effects of CCK in the central nervous system are not so well characterized. There have been suggestions that CCK acts as an endogenous satiety factor and that the peptide is involved in the mediation of painful stimuli [for a review, see Woodruff et al. (1991)]. On a molecular level, CCK is likely to act as a cotransmitter in a subpopulation of dopaminergic neurons (Hokfelt et al., 1980; Crawley, 1991).

Specific binding sites for CCK peptides are classified on the basis of their pharmacological properties toward different CCK fragments and nonpeptide antagonists. The peripheral type or CCK_A receptor is mainly located in the pancreas, whereas the central or CCK_B receptor is widely distributed in the central nervous system. CCK_B receptor antagonists with potent anxiolytic activity have been developed (Hughes et al., 1990; Singh et al., 1991). A third type of receptors for CCK peptides is the gastrin receptor in parietal cells which closely resembles the B-type receptor in its binding properties and therefore could be possibly identical with the central CCK_B receptor.

The CCK_A receptor was the object of many investigations [for a review: Rosenzweig and Jamieson (1986)]. Several of them dealt with structural aspects including receptor purification resulting recently in the isolation of receptor cDNA (Wank et al., 1992). Only few such studies have been reported concerning the B-type. The receptor could be shown to be structurally different from the A-type (Sakamoto et al., 1984), and a functional solubilization from pig cerebral cortex has been described (Gut et al., 1989). A cDNA has been cloned from a rat pancreatic acinar carcinoma cell line, which encodes a seven transmembrane protein with CCK_B receptor pharmacology (Wank et al., 1992b). However, additional information is needed, especially about molecular weight and glycosylation, particularly to elucidate the relationship of the CCK_B receptor to the parietal cell gastrin receptor, which was recently characterized by expression cloning (Kopin et al., 1992).

Photoaffinity labeling with photoprobes based on the most active CCK fragment, the sulfated octapeptide CCK-8s, was successfully used to characterize the CCK_A receptor from rat pancreas (Powers et al., 1988; Fourmy et al., 1989), but so far no such work has been reported for the CCK_B receptor, probably due to experimental difficulties resulting from the very low receptor density in the cerebral cortex.

In this work, we describe the synthesis and application of a new CCK-8s-based photoprobe suitable for photoaffinity labeling of central CCK_B receptors with high yield.

EXPERIMENTAL PROCEDURES

Materials. All amino acid derivatives and peptides were from Bachem, Heidelberg. CCK-8s was a kind gift of Dr. B.

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[1] Abbreviations: BzBz, benzoylbenzoyl; CCK, cholecystokinin; CCK-8ds, unsulfated CCK octapeptide; CCK-8s, sulfated CCK octapeptide; DCC, *N,N'*-dicyclohexylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; FAB-MS, fast atom bombardment mass spectroscopy; Fmoc, 9-fluorenylmethoxycarbonyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; TEAP, triethylammonium phosphate; Tris, tris(hydroxymethyl)aminomethane.

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Penke, University of Medical Chemistry, Szeged, Hungary. *p*-Benzoylbenzoic acid was purchased from Aldrich. SO_3–pyridine complex was from Fluka. [^{3}H]-*N*-Hydroxysuccinimidyl propionate (sp act. 94.3 Ci/mmol, 3.49 TBq/mmol) was from Amersham. Digitonin (water soluble type), trifluoromethanesulfonic acid, and *N*-acetylglucosamine were from Sigma. N-Glycosidase F and octyl glucoside were from Boehringer Mannheim. All chemicals used for electrophoresis were from Bio-Rad.

Analytical Methods

Peptides were separated by reversed-phase high-performance liquid chromatography (HPLC). All separations were carried out on a Varian 5000 liquid chromatograph using a 10-μm LiChrosorb RP 18 column (24 × 0.46 cm). The buffer used was 25 mM triethylammonium phosphate, pH 3.4, with a gradient run from 15 to 75% acetonitrile over 30 min with UV detection at 220 nm. Amino acid analysis was performed on a 420 A derivatizer/130 A separation system (Applied Biosystems). Thin-layer chromatography (TLC) was performed using plates coated with silica gel (Merck, Darmstadt). UV spectra were recorded on a Hitachi U 2000 UV/vis spectrometer. Protein determination was performed using the fluorescamin method with a Jobin Yvon JY 3D spectrofluorometer. Bovine serum albumin was used as a standard protein.

Chemical Synthesis

p-Benzoylbenzoic acid N-Hydroxysuccinimide Ester (**1**). A total of 1.13 g (5 mmol) of *p*-benzoylbenzoic acid and 0.58 g (5 mmol) of *N*-hydroxysuccinimide were suspended in 50 mL of acetonitrile; 1.1 g (5.3 mmol) of DCC was added at room temperature. After being stirred for 15 h at room temperature, the solution was filtered, the solvent was removed in vacuo, and the residue was recrystallized from ethyl acetate. Yield: 580 mg (1.8 mmol, 36%). mp: 207 °C. TLC R_f: 0.35 (hexan/ethyl acetate 1/1).

p-Benzoylbenzoyl-Orn(Fmoc)-OH (**2**). A total of 200 mg (0.564 mmol) H-Orn(Fmoc)-OH of was dissolved in a mixture of 5 mL of 1,4-dioxan and 6.6 mL of a 2% solution of Na_2CO_3 in water; 187 mg (0.564 mmol) of **1** dissolved in 1,4-dioxan was added, and the mixture was stirred for 16 h at room temperature. The solution was concentrated in vacuo, acidified with 1 M HCl, and extracted with ether. The combined organic phases were washed with water and dried over Na_2SO_4 and the solvent was removed in vacuo. The resulting 270 mg of crude product was recrystallized from ether to give 200 mg (0.36 mmol, 63%) of pure **2**. FAB-MS m/z = 563. UV λ_{max} = 342 nm. ϵ = 159 mol^{-1} cm^{-1} (1 mg/mL in acetonitrile).

CCK-8ds (**3**). A total of 25 mg of Boc-CCK-8ds was treated with 2 mL of 50% TFA in CH_2Cl_2 for 15 min at room temperature. The solvent was partially removed in vacuo, and the product was precipitated with ether, washed, and dried. The yield was quantitative, the product was uniform as proved by HPLC, and retention time was 18.9 min.

p-Benzoylbenzoyl-Orn(Fmoc)-CCK-8ds (**4**). A total of 20.0 mg (35.5 μmol) of **2** was dissolved in 1 mL of ethyl acetate. A solution of 4.08 mg (35.5 μmol) of *N*-hydroxysuccinimide in 100 μL of acetonitrile was added. After being cooled to 0 °C, a solution of 7.33 mg of DCC (35.5 μmol) in 100 μL of ethyl acetate was added and the mixture was stirred for 2 h; 800 μL of this solution was mixed with a solution of 22.9 mg (21.5 μmol) of CCK-8ds in 3 mL of DMF and 11.5 μL (86 μmol) of triethylamine. After being stirred for 1.5 h, the solution was concentrated in vacuo and the peptide was precipitated with 4 mL of ether. The precipitate was washed twice with ether and dried in vacuo to give 25 mg (16.3 μmol, 76%) **4**.

p-Benzoylbenzoyl-Orn(NH_2)-CCK-8s (**5**). A total of 20 mg (12.4 μmol) of **4** was dissolved in 200 μL of DMF; 200 μL of pyridine and 80 mg (500 μmol) of SO_3–pyridine complex were added and the slurry was stirred for 4 h at room temperature. Then 1 mL of a concentrated aqueous solution of $NaHCO_3$ was added to destroy an excess of SO_3–pyridine complex. To remove salts, 10 mL of H_2O/acetonitrile/AcOH 90/10/1 was added and the solution was passed over a Sep-Pak C-18 cartridge (Millipore), which was subsequently washed with 25 mM TEAP, pH 3.4, and water. Elution was performed with acetonitrile/water 6/4. The solvent was removed in vacuo, and the crude product was dissolved in 800 μL of DMF. To remove the Fmoc protecting group, 200 μL of piperidine was added and the solution was allowed to stand for 20 min. The solvent was removed in vacuo and the residue was purified using HPLC. Yield: 4.5 mg (3.1 μmol, 25%) of pure peptide. Retention time: 22.7 min. Amino acid analysis: Asp 2.0, Gly 0.74, Tyr 1.05, Met 2.25, Phe 1.05, Orn 0.95. Negative ion FAB-MS: m/z = 1463 [M – H]$^-$, 1383 [M – SO_3 – H]$^-$.

[^{3}*H*]-*p-Benzoylbenzoyl-Orn(propionyl)-CCK-8s* (**6**). A total of 0.22 mg (0.15 μmol) of **5** was dissolved in 50 μL of DMF and 7.7 μL of 1% DIEA in DMF; 0.3 mL of a solution of [^{3}H]-*N*-hydroxysuccinimidyl propionate in toluene (1 mCi/mL) was concentrated in a stream of nitrogen to a volume of about 5 μL. A total of 5 μL of the solution of peptide **5** was added and the mixture was further concentrated to remove the toluene; 3 μL of 1% DIEA in DMF was added and the reaction was allowed to proceed for 30 min at room temperature; 200 μL of acetonitrile/25 mM TEAP 55/45 was added and the mixture was separated using HPLC. Yield: 37 μCi (12%). Retention time: 24.5 min.

To prove the identity of the product, the same reaction was performed using unlabeled *N*-hydroxysuccinimidyl propionate and the product was analyzed with negative ion FAB-MS: m/z = 1519 [M – H]$^-$, 1440 [M – SO_3]$^-$.

Membrane Preparation. Crude membranes from pig brain were prepared as described (Gut et al., 1989). Briefly, brain tissue was homogenized by sequential use of a Polytron and a motor-driven glass–Teflon homogenizer. The buffer used was 10 mM Hepes/NaOH, pH 7.4, 120 mM NaCl, 0.32 M sucrose, 1 mM EGTA, 1 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride, 0.01% bacitracin, 0.002% soy bean trypsin inhibitor. After centrifugation at 1000*g* (10 min), supernatants were centrifuged at 30000*g* (30 min). The pelleted membranes were washed and further enriched by centrifugation in a stepwise sucrose gradient. Enriched synaptosomal membranes were harvested from the layer between solutions with 0.8 M and 1.2 M sucrose.

Rat pancreatic membranes were prepared according to Ijzerman and Melman (1992) by homogenization of whole pancreatic tissue in 100 volumes of buffer (10 mM Hepes/NaOH, pH 7.4, 5 mM $MgCl_2$, 130 mM NaCl) followed by centrifugation at 50000*g* (15 min). The pellet was resuspended in 100 volumes of the above buffer and centrifuged at 50000*g* (15 min). The pelleted membranes were rehomogenized and stored frozen at –70 °C.

Solubilization. Enriched synaptosomal membranes (9 mg/mL) were solubilized with 1.7% digitonin and 10 mM Mg^{2+} as described by Gut et al. (1989).

WGA–Sepharose Chromatography. Solubilized proteins were diluted with four volumes of WGA buffer (10 mM Hepes/NaOH, pH 7.4, 120 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 10% (w/v) glycerine, 0.01% bacitracin, 0.002% soy bean trypsin inhibitor) and passed over a 5-mL WGA–Sepharose (Pharmacia) column preequilibrated with WGA buffer containing 0.1% digitonin. After washing with this buffer, bound proteins were eluted with WGA buffer containing 0.1% digitonin and 0.6 M *N*-acetylglucosamine.

Binding Studies. Binding studies were performed by rapid filtration assay as described by Gut et al. (1989). Briefly 50–100 μg of membranes was incubated with radioactive ligand for 45 min at 20 °C. Then, free ligand was separated by filtration over GF/C glass fiber filters (Whatman). Unspecific binding was determined in the presence of 1 μM unlabeled pentagastrin (cortical membranes) or CCK-8s (pancreatic membranes).

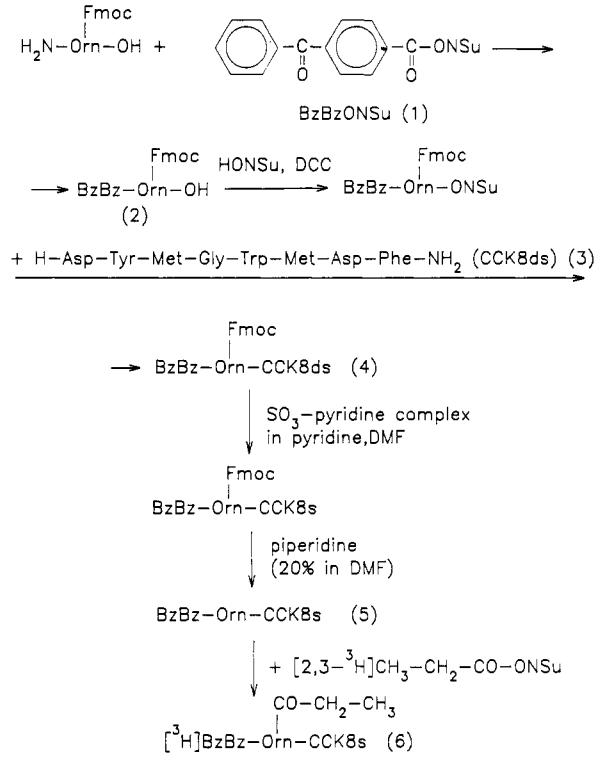
Binding studies with solubilized proteins were performed as described above, with the exception of protein being precipitated by addition of poly(ethylene glycol) and bovine γ-globulin to a final concentration of 7% and 0.075%, respectively, before filtration.

Photoaffinity Labeling. (i) Investigation of the Time Course of Photoactivation of the Benzoylbenzoyl Group. Bovine serum albumin (10 mg/mL) wad dissolved in incubation buffer (10 mM Hepes/NaOH, pH 7.4, 120 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.01% bacitracin, 0.002% soy bean trypsin inhibitor, 0.1 mM bestatin, 0.01 mM phosphoramidon), and 1 nM [^{3}H]-*p*-benzoylbenzoyl-Orn(propionyl)-CCK-8s was added. After 30 min of incubation at room temperature, the mixture was photolyzed. As a source of light, we used a HBO 200 high-pressure mercury lamp (Osram), which was built in a system with a reflector and a focusing lens (E. Leitz, Wetzlar). The sample solution was filled in a quartz cuvette, which was placed in a cooled metal block open to the light source. A glass filter (Schott, Mainz) was used to cut off light with wavelengths shorter than 320 nm. At the beginning and after different periods of irradiation, aliquots were taken and protein-bound ligand was separated from free ligand by chloroform–methanol precipitation (Wessel & Flügge, 1984). The protein pellets were redissolved in 1% SDS, and the extent of covalent incorportion was determined by scintillation counting.

(ii) Labeling of Receptor Protein. WGA-enriched proteins or rat pancreatic membranes were diluted with an equal volume of incubation buffer. Photoreactive ligand was added, and after incubation for 40 min at 20 °C, the mixture was photolyzed using a 200-W mercury lamp at wavelength > 320 nm. Unspecific labeling was determined in the presence of 1 μM pentagastrin or 1 μM CCK-8s. Protein was recovered by chloroform–methanol precipitation or centrifugation and redissolved in SDS–PAGE sample buffer (62.5 mM Tris-HCL, pH 6.8, 5% SDS, 10% glycerine, 5% 2-mercaptoethanol, and 0.001% bromophenol blue). Gel electrophoresis was performed according to Laemmli (1970) with 9–10.5% slab gels. After electrophoresis, gels were cut into 2-mm slices, which were treated with 0.5 mL Lumasolve (Baker) each. Radioactivity was determined by liquid scintillation counting.

Chemical Deglycosylation. A total of 200 μg of photolabeled protein was precipitated using the chloroform–methanol method. The pellet was washed with ether to remove methanol and dried; 20 μL of trifluoromethanesulfonic acid was added at 0 °C. The reaction was stopped by addition of a 12-fold volume of 1 M Tris solution, containing 2% SDS. The proteins were separated from salts by gel filtration using a NAP-5 column (Pharmacia) preequilibrated with SDS–PAGE sample buffer.

Scheme I: Synthesis of [^{3}H]-*p*-Benzoylbenzoyl-Orn(propionyl)-CCK-8s



RESULTS

Synthesis of the Photoreactive Cholecystokinin Analogue. The new CCK-8s-based photoaffinity probe [^{3}H]-*p*-benzoylbenzoyl-Orn(propionyl)-CCK-8s was synthesized according Scheme I. For the introduction of a photoreactive group at the N-terminus of the CCK octapeptide, N^{α}-benzoylbenzoyl-N^{δ}(Fmoc)-L-ornithine was prepared (compound **2**, Scheme I). After coupling to unsulfated CCK octapeptide and sulfation, the Fmoc residue was removed from the side chain of ornithine. The radioactive propionyl residue was introduced by acylation of the δ-amino group of ornithine with the commercially available [^{3}H]-*N*-hydroxysuccinimidyl propionate. The unlabeled precursor peptide (**5**, Scheme I) was characterized by mass spectroscopy and amino acid analysis. To obtain analytical data of the final product (**6**, Scheme I), the propionylation of peptide **5** was also performed using nonradioactive *N*-hydroxysuccinimidyl propionate. All mass spectra of the sulfated peptides showed the expected peaks when recorded in the negative-ion mode, while positive-ion spectra revealed only peaks according to the desulfated ions, probably due to rapid fragmentation.

Enrichment of CCK_B Receptors Using Lectin Columns. The density of CCK_B receptors in membranes derived from

Enzymatic Deglycosylation. A total of 200 μg of photolabeled protein was precipitated as above and the pellet was redissolved in 10 μL of 20 mM NaP_i buffer, pH 7.4, containing 2% SDS. The solution was diluted with the above buffer containing octyl glucoside to a final concentration of 1% octyl glucoside, 0.1% SDS; 4–16 units of N-glycosidase F were added, and the mixture was incubated at 20 °C overnight. Then the samples were mixed with equal volumes of SDS–PAGE sample buffer (5% SDS, 5% 2-mercaptoethanol) and subjected to SDS–PAGE on a 10% slab gel.

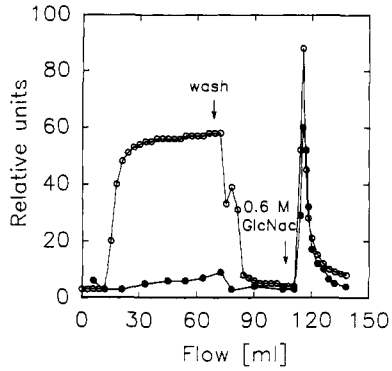


FIGURE 1: Enrichment of pig brain cortical CCK_B receptors by chromatography on wheat germ agglutinin–Sepharose. Ten milliliters of solubilized pig brain cortical membranes was diluted with three volumes of WGA buffer (see Experimental Procedures) and passed three times over a 5-mL WGA–Sepharose column. The column was washed with WGA buffer containing 0.1% digitonin. Bound proteins were then eluted with WGA buffer containing 0.1% digitonin and 0.6 M *N*-acetylglucosamine. Full circles show binding capacity for [^{3}H]propionyl-CCK-8s; open circles show optical density at 276 nm.

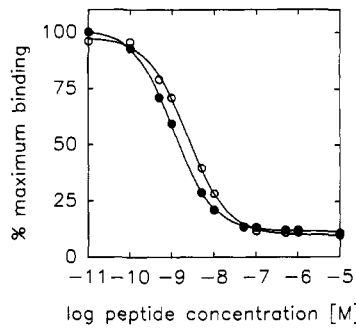


FIGURE 2: Concentration dependencies of CCK-8s and BzBzOrn-(prop)-CCK8s inhibition of [^{3}H]propionyl-CCK-8s binding to pig brain cortical membranes. [^{3}H]Propionyl-CCK-8s (0.5 nM) was incubated for 40 min at 20 °C in the presence of specified concentrations of CCK-8s (●) and BzBzOrn(prop)-CCK-8s (○). Bound [^{3}H]propionyl-CCK-8s was determined as described under Experimental Procedures and is given as a percentage of the binding measured in the absence of competing peptide. The lines through the experimental points represent the computer fit to the data of a single experiment.

pig cerebral cortex is only in the range of 30 fmol/mg of protein. Therefore, enriched receptor preparations for the use in photoaffinity labeling experiments were isolated by lectin chromatography. Among the lectins tested for binding to CCK_B receptors (concanavalin A, peanut lectin, Ulex europeaus A I lectin, and wheat germ lectin), only the last one was able to bind the receptor. In a typical experiment (Figure 1) 10% of total protein and 60% of specific binding activity were eluted from the column with 0.6 M *N*-acetylglucosamine. This resulted in a 6-fold enrichment of CCK_B receptor protein as compared to solubilized membranes. The binding capacity for [^{3}H]propionyl-CCK-8s was in the range of 150–250 fmol/mg of protein.

Binding Studies. Competitive binding and saturation binding experiments showed that *p*-benzoylbenzoyl-Orn-(propionyl)-CCK-8s (**6**) binds with high affinity to both membrane-bound and solubilized CCK_B receptors from pig cerebral cortex and to CCK_A receptors in rat pancreatic membranes. In competitive binding experiments with [^{3}H]-propionyl-CCK-8s (Figure 2), the photoreactive unlabeled ligand (**6**) and the parent peptide CCK-8s bound with roughly equinanomolar affinity (i.e., with K_D of 1.2 nM and 0.6 nM, respectively) to pig brain membranes. After detergent solubilization and enrichment of the pig cerebral CCK_B receptor by lectin chromatography, saturation binding experiments with [^{3}H]propionylated photoreactive ligand (**6**) and [^{3}H]propionyl-CCK-8s resulted in nearly identical equilibrium binding parameters (Figure 3 and Table I). The affinity of *p*-benzoylbenzoyl-Orn(propionyl)-CCK-8s to CCK_A receptors of rat pancreatic membranes was likewise very high; an apparent dissociation constant K_D of 0.5 nM was determined.

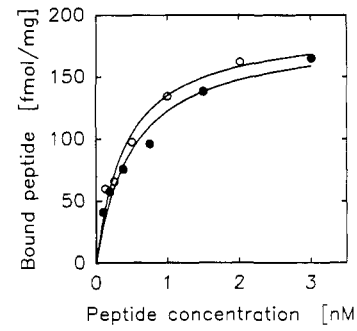


FIGURE 3: Saturation binding of [^{3}H]propionyl-CCK-8s (○) and [^{3}H]BzBzOrn(prop)-CCK-8s (●) to WGA-enriched solubilized pig brain cortical membranes. Increasing concentrations of radioactive peptides were incubated in the dark with WGA-enriched solubilized pig brain cortical membranes. Binding was measured as described in Experimental Procedures, and values were corrected for unspecific binding measured in the presence of 1 μM pentagastrin.

Table I: Binding of BzBzOrn(prop) CCK-8s to CCK Receptors

receptor preparation	K_D (nM)
pig brain membranes	1.2 ± 0.3 (n = 2)[a]
WGA-enriched, solubilized pig brain membranes	0.5 ± 0.2 (n = 3)[b]
rat pancreatic membranes	0.5 ± 0.2 (n = 3)[b]

[a] Calculated from competition binding data. [b] Calculated from saturation binding data.

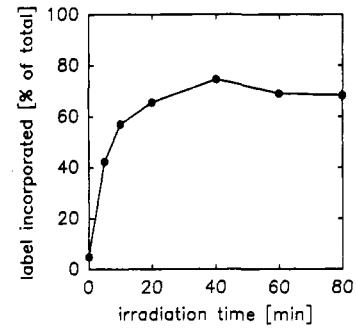


FIGURE 4: Time course of photoactivation of [^{3}H]BzBzOrn(prop)-CCK-8s. Albumin (10 mg/mL) was incubated with 1 nM [^{3}H]-BzBzOrn(prop)-CCK-8s and irradiated using a mercury lamp at wavelength > 320 nm. Ligand which was covalently incorporated into protein was separated by precipitation. The plot shows the extent of covalent incorporation of radioactive ligand as a function of the time of irradiation.

Photoaffinity Labeling. At first we examined the photoactivation of the benzoylbenzoyl group. Therefore, the time course of covalent incorporation of the radioactive photolabile ligand into a large excess of albumin was measured. As it is shown in Figure 4, complete activation was accomplished after an irradiation time of 40 min, which was then chosen for further labeling experiments. It must be noted that these results were strongly dependent on the intensity of the light source. Therefore, the optimal irradiation time for benzoylbenzoyl ligands should be individually determined for the available source of light using the method described in Experimental Procedures.

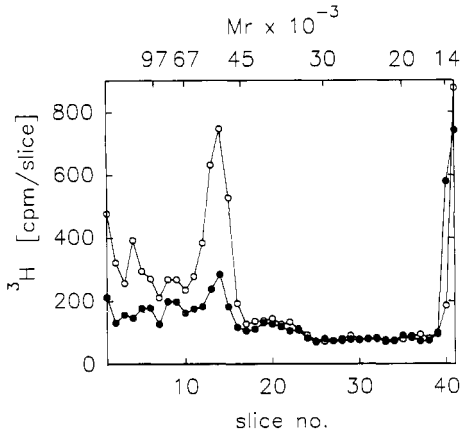


FIGURE 5: Photoaffinity labeling of WGA-enriched solubilized pig brain cortical membranes. Protein (100 μg) was incubated with 1.8 nM [^{3}H]BzBzOrn(prop)-CCK-8s for 40 min at 20 °C and photolyzed at 4 °C for 40 min using a mercury lamp at wavelength > 320 nm. Labeled protein was precipitated, dissolved in SDS–PAGE sample buffer, and subjected to SDS–PAGE. Gels were cut into 2-mm slices, and radioactivity was determined by liquid scintillation counting. The curves show the distribution of radioactivity in the absence (○) and presence (●) of 1 μM pentagastrin.

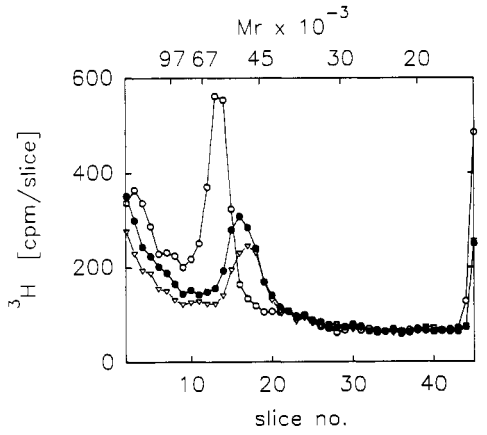


FIGURE 6: Deglycosylation of photoaffinity labeled CCK_B receptors from WGA-enriched solubilized pig brain cortical membranes. Labeled proteins were precipitated, dried, and treated with anhydrous trifluoromethanesulfonic acid for 0 min (○), 5 min (●), or 30 min (▽). After neutralization and removal of salts, proteins were subjected to SDS–PAGE. A shift of the labeled band from M_r of 56 000 to M_r of 45 000 is clearly seen.

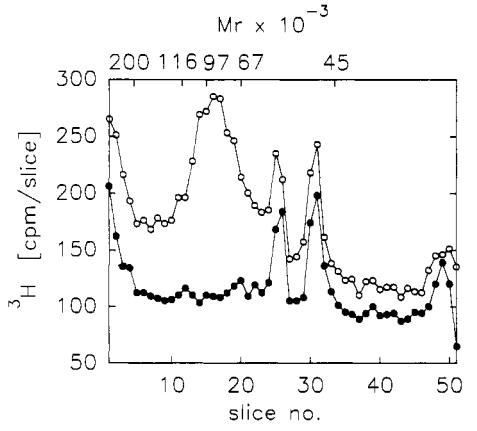


FIGURE 7: Photoaffinity labeling of CCK_A receptors from rat pancreatic membranes. Membranes (300 μg) were incubated with 1.8 nM [^{3}H]BzBzOrn(prop)-CCK-8s in the absence (○) and presence (●) of 1 μM CCK-8s for 40 min at 20 °C and photolyzed at 25 °C for 40 min. Membranes were collected by centrifugation, dissolved in SDS–PAGE sample buffer, and resolved by SDS–PAGE. Besides two unspecifically labeled proteins, a band with M_r of 80 000–100 000 is specifically labeled.

To find the best conditions for specific labeling of the CCK_B receptor, we examined the labeling of cortical membranes, digitonin-solubilized membranes, and solubilized membranes which had been enriched for the CCK_B receptor by lectin chromatography. While in membranes several proteins were unspecifically labeled, we found that in solubilized membranes and especially in WGA-enriched material specific labeling was predominant. Labeling of WGA-enriched solubilized membranes with 1.8 nM [^{3}H]-*p*-benzoylbenzoyl-Orn(propionyl)-CCK-8s showed a single peak at M_r of 56 000 ± 3000 (n = 6) after separation of labeled proteins with SDS–PAGE (Figure 5). Labeling was suppressed in the presence of 1 μM of the CCK_B agonist pentagastrin, indicating specific labeling of CCK_B receptors. Efficiency of specific incorporation of radioactivity in the M_r = 56 000 band was very high, 30–40% for solubilized receptor, reaching up to 70% of maximum specific binding for receptor enriched by lectin chromatography.

After chemical deglycosylation with trifluoromethanesulfonic acid, the receptor peak shifted to M_r of 45 000 ± 2000 (n = 2) (Figure 6). The loss in radioactivity may be explained by the observation that membrane aggregates were not completely dissolved during chemical deglycosylation. When N-glycosidase F was used for deglycosylation, the peak at M_r of 56 000 also completely disappeared, indicating that N-glycosidic linkage is predominant (results not shown).

Photoaffinity labeling of rat pancreatic membranes using [^{3}H]-*p*-benzoylbenzoyl-Orn(propionyl)-CCK-8s revealed specific labeling of a protein with M_r of 80 000–100 000 (Figure 7). Efficiency of labeling was about 15% of total receptor protein.

DISCUSSION

So far, only few studies were undertaken to elucidate the properties of CCK_B receptors. Sakamoto et al. (1984) used cross-linking of ^{125}I-CCK-33 with short-wavelength ultraviolet light and labeled a M_r = 51 000 protein in mouse brain membrane. In experiments with pancreatic membranes, use of such "long" CCK-33-based probes resulted in a labeling pattern different from that found with "short" CCK-8s-based probes (Sakamoto et al., 1984; Powers et al., 1988, 1991). As the latter have their activable group near or in the hormone binding site of the receptor, they are more likely to label the receptor protein than long probes; their reactive groups are located in the amino-terminal region of the 33 amino acid peptide and might react with receptor-associated proteins.

To obtain more information about the CCK_B receptor, we synthesized a CCK-8s-based tritiated photoprobe with the *p*-benzoylbenzoic acid moiety as the photoactivable group. Previous investigations (Williams & Coleman, 1982; Kauer et al., 1986; Boyd et al., 1991) have shown that the benzophenone group allows photoaffinity labeling with extraordinary high efficiency of covalent incorporation of radioactivity into proteins, which would be important in view of the low density of CCK_B receptors in the brain. An additional advantage of this group is the mild conditions of irradiation at wavelengths longer than 320 nm, which are necessary for photoactivation. Our synthetic approach—the introduction of the photoreactive residue at the α-amino group of ornithine and of the tritium-labeled propionyl residue at the δ-amino group at the last stage of synthesis—will be applicable to all peptide hormones with α- or ϵ-amino groups.

Using this photoprobe, we found a molecular weight M_r of 56 000 for the CCK_B receptor. The specificity of labeling

was demonstrated by the suppression of labeling by an excess of the CCK_B receptor agonist pentagastrin. The difference from M_r of 51 000 previously reported (Sakamoto et al., 1984) may be caused by different glycosylation of the receptor in different species. After chemical deglycosylation, we found a molecular weight M_r of 45 000, a result which is quite in accordance with the molecular weight of numerous G-protein coupled receptors with seven transmembrane domains, especially the recently cloned receptors with CCK_A and CCK_B receptor subtype pharmacology (Wank et al.,1992a,b, Kopin et al., 1992).

As *p*-benzoylbenzoyl-Orn(propionyl)-CCK-8s bound with equal high affinity to the CCK_A receptor of rat pancreatic membranes, we used it for labeling in this tissue as well. The labeled protein was identical to that labeled previously by others (Powers et al., 1988; Fourmy et al., 1989) using short CCK-8s-based photoprobes.

A remarkable property of the photoprobe used in our studies is the high efficiency of covalent incorporation in the receptor. We reached covalent labeling of up to 70% of the CCK_B-type binding sites, which allowed labeling of receptor preparations containing not more than 80 fmol of receptor/mg of protein. This high degree of labeling will be of particular value for purification of affinity-labeled fragments of the receptor, a favored chemical method for localizing the ligand binding site of the receptor.

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