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Photoaffinity Labeling of Rat Pancreatic Cholecystokinin Type A Receptor Antagonist Binding Sites Demonstrates the Presence of a Truncated Cholecystokinin Type A Receptor

SANDRINE SILVENTE POIROT, CHANTAL ESCRIEUT, MARLÈNE DUFRESNE, JEAN MARTINEZ, MICHÈLE BOUISSON, NICOLE VAYSSE, and DANIEL FOURMY

Groupe de Recherche de Biologie et Pathologie Digestive, Institut National de la Santé et de la Recherche Médicale, Unité 151, Institut L. Bugnard, Centre Hospitalier Universitaire de Rangueil, 31054 Toulouse Cedex, France (S.S.P., C.E., M.D., M.B., N.V., D.F.), and Centre National de la Recherche Scientifique, Faculté de Pharmacie, Montpellier, France (J.M.)

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SUMMARY

During the past few years, several antagonist ligands for cholecystokinin (CCK) receptors have been discovered, but the mechanism of action of these candidate drugs, as well as the nature of their molecular targets, remains poorly documented. In a previous study, we developed a new antagonist radioligand, ^{125}I-Bolton-Hunter-labeled JMV-179, for the CCK-A receptor (CCK-AR), to analyze CCK antagonist binding sites in pancreatic plasma membranes. We found that ^{125}I-Bolton-Hunter-labeled JMV-179 identified 4 times as many sites as did an agonist radioligand, although agonists were able to interact competitively with the entire population of antagonist sites. In the present work, using biochemical approaches we have identified and characterized CCK antagonist binding sites in pancreatic plasma membranes. We synthesized the photoactivable antagonist probe ^{125}I-azidosalicylic acid (ASA)-JMV-179. The binding of ^{125}I-ASA-JMV-179 to plasma membranes was inhibited by JMV-179 (IC_{50}, 6 ± 2 nM), by (Thr^{28},Ahx^{31})-CCK-25-33 (IC_{50}, 1.2 ± 0.5 nM), and by the nonpeptide CCK-AR antagonist L-364,718 (IC_{50}, 2 ± 1 nM). Photoaffinity labeling using pancreatic membranes or acini demonstrated that ^{125}I-ASA-JMV-179 detected a new 47–50-kDa protein in addition to the 85–100-kDa CCK-AR. The 47–50-kDa protein was not directly detected by a photoactivable agonist, but agonists could inhibit its covalent labeling by ^{125}I-ASA-JMV-179 (IC_{50} for (Thr^{28},Ahx^{31})-CCK-25-33, 15 nM). In competition assays using nonsolubilized or solubilized membranes, this protein displayed binding features of the CCK-AR and was retained on immobilized wheat germ agglutinin, as was the CCK-AR. To further characterize the 47–50-kDa protein, deglycosylation and protease digestions were performed, and the digestion products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protease digestions of both the CCK-AR and the 47–50-kDa protein yielded identical labeled fragments, demonstrating a structural relationship between the two proteins. The CCK-AR, which has three potential sites for *N*-glycosylation on the amino-terminal extracellular domain and one on the second extracytoplasmic loop, was deglycosylated to a 42-kDa peptide. The 47–50-kDa protein was deglycosylated to a 35-kDa peptide. These data, and the localization of the labeled fragments in the amino acid sequence of the receptor, suggest that the 47–50-kDa protein represents a CCK-AR lacking its amino-terminal extracellular domain. This study, with the first photoreactive antagonist probe for the CCK-AR, demonstrates that some of the heterogeneity of CCK antagonist binding sites in pancreatic plasma membranes is due to the presence of a truncated CCK-AR.

CCK is widely distributed in the central and peripheral nervous systems, as well as in the gastrointestinal tract (1). It is thought that CCK and CCK receptors are involved in several pathologies of the central nervous system and the gut (2). Specific membrane receptors with different pharmacological features and associated biological effects have been found in these various tissues (3). In the rodent pancreas, the main action of CCK is to stimulate exocrine enzyme secretion by occupancy of the CCK-AR subtype (4). *In vitro*, CCK-stimulated amylase release from acini follows a biphasic dose-response curve, with a stimulatory phase for concentrations of CCK between 1 and 100–300 pM and an inhibitory phase for concentrations between 100–300 pM and 10 nM (3, 4). The mechanism that governs these two phases of the CCK-induced

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ABBREVIATIONS: CCK, cholecystokinin; CCK-AR, cholecystokinin type A receptor; JMV-179, Tyr(SO_3H)-Nle-Gly-D-Trp-Nle-Asp-2-phenylethyl ester; Ahx, 2-aminohexanoic acid; ASA, azidosalicylic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; STI, soybean trypsin inhibitor; PMSF, phenylmethylsulfonyl fluoride; BH, Bolton-Hunter-labeled; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ASD, azidosalicylamidodithiopropionate.

biological response in pancreatic acini is not clearly understood, although recently several intracellular events, including inositol-1,4,5-trisphosphate formation and intracellular Ca^{2+} mobilization and oscillation, have been identified (5, 6). In addition, the CCK-AR has been characterized as a *N*-glycoprotein of 85–100 kDa by photoaffinity labeling with agonist probes (7–9), and its corresponding cDNA has been cloned (10). The deduced primary structure suggests that the CCK-AR is a member of the superfamily of G protein-coupled receptors and therefore has a structure presumably composed of seven transmembrane domains, three cytoplasmic loops, three exoplasmic loops, an exoplasmic amino-terminal domain, and a cytoplasmic carboxyl-terminal tail.

The pancreas of some species, including dogs (11), calves (12), and humans (13) but not rats, contains a large amount of CCK-B receptors. At present, a wide variety of potent and selective CCK receptor antagonists that can selectively block each CCK receptor subtype are available (2). Some of these, such as the nonpeptide benzodiazepine analogues and JMV-180, a partial agonist (3, 6, 7), have already profoundly facilitated pharmacological and functional studies of the CCK-AR. However, the mechanism of receptor blocking or activation by these candidate drugs remains poorly studied. One approach to study this mechanism would be to identify the antagonist binding site(s) of the receptor. Photoaffinity labeling and site-directed mutagenesis are two powerful techniques that could be used in such an approach.

Recently, we synthesized a radioiodinated derivative of JMV-179 (14, 15), a peptidic antagonist, and characterized the states and populations of the CCK-AR in pancreatic plasma membranes. We demonstrated that the radiolabeled antagonist ^{125}I-BH-JMV-179 identified ≈4 times as many sites as a radiolabeled CCK agonist, even though CCK was able to competitively interact with the entire population of antagonist binding sites (16). Similar data have been previously obtained with the radiolabeled nonpeptide antagonist [^{3}H]L-364,718 (17).

In the present work, we have synthesized a photoactivable analogue of JMV-179, ^{125}I-ASA-JMV-179, to identify and characterize CCK antagonist binding sites on pancreatic plasma membranes. We found that the ^{125}I-ASA-JMV-179-labeled CCK receptor has a component of 85–100 kDa, as well as a distinct component of 47–50 kDa that was not detected by agonist photoreactive probes. Both the pharmacological behavior and the biochemical features of this new 45–50-kDa component suggest that it corresponds to a truncated CCK-AR.

Experimental Procedures

Materials. The carboxyl-terminal nonapeptide of CCK, (Thr^{28},Ahx^{31})-CCK-25-33, was synthesized by Luis Moroder (Max Planck Institut für Biochemie, München, Germany). The antagonist JMV-179, an analogue of the carboxyl-terminal heptapeptide of CCK, was synthesized as described in Ref. 15. *N*-Hydroxysuccinimidyl-4-ASA was obtained from Pierce (Amsterdam, Holland). Na^{125}I (specific activity, 137 MBq) was obtained from Amersham International (les Ulis, France). Chemicals used in SDS-PAGE were obtained from Bio-Rad (Richmond, CA). Endo-β-*N*-acetylglucosaminidase F (endoglycosidase F) was prepared from *Flavobacterium meningosepticum* (American Type Culture Collection). *O*-Glycanase was obtained from Genzyme (Boston, MA).

Plasma membrane and acini preparations. Enriched pancreatic plasma membranes were prepared from male rats as described (8). In brief, the excised pancreata were homogenized with a Dounce homogenizer in 10 volumes of 0.3 M sucrose containing 0.1% STI, 1 mM PMSF, and 1 mM 2-mercaptoethanol. After filtration, the sucrose concentration was brought to 1.3 M by addition of 2 M sucrose and the homogenate was overlaid with 0.3 M sucrose and centrifuged for 3 hr at 149,000 × *g*. Membranes at the 0.3–1.3 M interface were collected, washed, and stored at −70° in binding buffer (50 mM HEPES, pH 7, 5 mM $MgCl_2$, 115 mM NaCl, 0.01% STI, 0.1% bacitracin, 1 mM EGTA, 0.1 mM PMSF, 0.2% bovine serum albumin).

Dispersed pancreatic acini were prepared from similar animals by collagenase (75 units/ml) dissociation (5). The basic medium used for isolation was Krebs-Ringer-HEPES medium, pH 7.4, containing 115 mM NaCl, 4.8 mM KCl, 0.5 mM $CaCl_2$, 1.4 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM glucose, 2 mM glutamine, 24.5 mM HEPES, 1% essential amino acids, 1% nonessential amino acids, and 0.2% albumin. After dissociation, the acini were suspended in the same buffer containing 0.01% STI and 0.1% bacitracin.

Solubilization of pancreatic plasma membranes. Pancreatic plasma membranes were solubilized in solubilization buffer (50 mM Tris·HCl, pH 7, 115 mM NaCl, 5 mM EDTA, 1 mM benzamidine, 0.1 mM PMSF, 20%, w/v, glycerol, 3%, w/v, CHAPS) at 4° for 20 min. Nonsolubilized material was pelleted by ultracentrifugation at 400,000 × *g* for 10 min and discarded. The concentration of CHAPS in the supernatant was lowered to 0.1% by chromatography on a PD-10 column (Pharmacia) equilibrated in solubilization buffer containing 0.1% (w/v) CHAPS. Soluble proteins were either used immediately or stored at −80°, without significant loss of binding activity.

Synthesis and purification of iodinated agonist and antagonist ligands. ^{125}I-ASD-(Thr^{28},Ahx^{31})-CCK-25-33 was prepared as described previously (9). ^{125}I-ASA-JMV-179 was prepared using the same protocol as used for ^{125}I-ASD-(Thr^{28},Ahx^{31})-CCK-25-33. Briefly, *N-t*-butoxycarbonyl-JMV-179 was deblocked by trifluoroacetic acid treatment. Coupling of JMV-179 to ASA reagent was performed by mixing 1 μmol of deblocked JMV-179, dissolved in 0.5 ml of 50 mM sodium tetraborate, pH 8.5, with 5 μmol of ASA dissolved in 0.5 ml of dimethylformamide. The reaction was carried out in the dark for 15 hr at 20°, and the mixture was then applied to a C-18 μBondapak column, using 0.125 M triethylammonium phosphate, pH 3.5, plus acetonitrile as eluant (linear gradient from 36% to 60% acetonitrile in 48 min). The analytical run was monitored by recording the absorbance at 280 nm. The fractions corresponding to absorbance peaks were tested for their ability to inhibit the binding of CCK and JMV-179 to pancreatic plasma membranes. Only the molecules contained in a peak with a retention time of 46 min were endowed with binding capability. To avoid photolysis of the ligand during preparative runs, the UV detector was switched off while ASA-JMV-179 was eluted. Finally, ASA-JMV-179 was rechromatographied using 1% formic acid plus acetonitrile as eluant and was lyophilized for storage.

Radioiodination of ASA-JMV-179 was performed at 20° in 0.2 M sodium phosphate buffer, pH 7.5. To 10 μg of peptide in 10 μl of sodium tetraborate were added 50 μl of sodium phosphate buffer and 10 μl of Na^{125}I solution (1 mCi), followed by four additions of 5 μl of a chloramine T solution (0.5 μg/μl) at 10-sec intervals. The reaction was stopped by addition of an excess of a NaI/tyrosine mixture. The labeled components were then separated on a C-18 column as described above. Radioactivity elution was recorded with a radioisotope detector (model 170; Beckman). The specific activity of the probe was 2000 Ci/mmol. The inability of the probe to stimulate amylase release from rat pancreatic acini was controlled.

Binding studies and photoaffinity labeling. In a preliminary test, we checked that the binding of ^{125}I-ASA-JMV-179 to rat pancreatic membranes was saturable, reversible, time dependent, and protein concentration dependent. Nonspecific binding, occurring in the presence of 1 μM unlabeled JMV-179 or (Thr^{28},Ahx^{31})-CCK-25-33, was about 10% of the total binding. Binding conditions were similar to those determined previously for ^{125}I-BH-JMV-179 (16). Briefly, membranes (40 μg of protein) were incubated in the dark in binding buffer containing 200 pM ^{125}I-ASA-JMV-179 or 50 pM ^{125}I-ASD-(Thr^{28},Ahx^{31})-

CCK-25-33, alone or in the presence of unlabeled competitors (as indicated in the figure legends), at 25° for 120 min (steady state conditions). Bound radioligand and free radioligand were separated by centrifugation (10,000 × *g*, 10 min at 4°). In some experiments, membranes were prepared and incubated with or without the following protease inhibitors: 0.05% STI, 1 mM PMSF, 5 μM pepstatin, 2 mM 1,10-phenanthroline, and 0.1 μM α_2-macroglobulin.

Binding of ^{125}I-ASA-JMV-179 to solubilized CCK receptors was carried out at 4° for 15 hr in solubilization buffer containing 0.1% CHAPS (final volume, 0.5 ml), by incubating 200 pM radioligand, in the presence or absence of competitors, with 5 μg of solubilized membrane proteins. Protein-bound radioligand was precipitated at 4° by addition of 0.1 ml of a solution containing 0.4% γ-globulin and 2% potassium iodide, followed by 0.5 ml of a solution of 20% polyethylene glycol. The solution was mixed and then immediately centrifuged at 10,000 × *g* for 10 min. The supernatant was discarded and the radioactivity associated with the pellet was measured. Nonspecific binding was determined in the presence of 1 μM JMV-179.

For photoaffinity labeling, pellets of solubilized membrane proteins or pellets of labeled plasma membranes resuspended in 5 mM HEPES buffer, pH 7, were exposed to a 125-W mercury lamp at 4° for 5 min. Labeled proteins were recovered by lyophilization or centrifugation, separated by SDS-PAGE on a 10% polyacrylamide gel, and visualized by autoradiography. To determine the relative pseudo-affinities of competitors for proteins specifically labeled by ^{125}I-ASA-JMV-179, the autoradiographic films were scanned by image analysis using a BIOCOM apparatus (France).

Affinity chromatography on immobilized wheat germ agglutinin. Photoaffinity-labeled membranes were solubilized for 2 hr at 4° using 5% Nonidet P-40 in binding buffer (2 ml/mg of protein). The soluble fraction was diluted 10-fold and incubated with agarose-wheat germ agglutinin (2 ml of gel, corresponding to 4 mg of lectin) at 4° for 15 hr. The column was then extensively washed with 25 mM HEPES, pH 7.5, containing 0.5% Nonidet P-40. For elution, 0.5 M *N*-acetyl-β-D-glucosamine was applied to the gel. Eluted radioactive fractions were concentrated by ultraconcentration for SDS-PAGE analysis.

Enzymatic proteolysis and deglycosylation of the components photoaffinity labeled by ^{125}I-ASA-JMV-179. Affinity-labeled membrane proteins were separated by SDS-PAGE. The wet gel was then frozen at −80° and exposed to an autoradiography film for several hours. The labeled 85–100- and 47–50-kDa bands were excised and the proteins were electroeluted for 24 hr at 4° in 10 mM Tris-acetate buffer, pH 8.6, containing 0.1% Nonidet P-40 and 0.1% β-mercaptoethanol. The electroeluted proteins were carboxymethylated using iodoacetic acid and were then subjected to proteolysis with endoproteinase Glu-C (protein/enzyme ratio, 10:1) and with endoproteinase Glu-C followed by trypsin (ratio, 20:1), as described (18). Proteins were deglycosylated using endo-β-*N*-acetylglycosaminidase F or *O*-glycanase in 20 mM sodium phosphate, pH 6.1, 1% Nonidet P-40, 0.1% SDS, at 37° for 15 hr. The samples were then analyzed by SDS-PAGE on a 15% polyacrylamide gel, followed by autoradiography.

Determination of the molecular masses of labeled peptides. The apparent relative molecular masses of the labeled components were calculated using high molecular weight, prestained proteins (Bio-Rad), i.e., phosphorylase B (M_r 111,000), bovine serum albumin (M_r 84,000), ovalbumin (M_r 47,000), carbonic anhydrase (M_r 33,000), STI (M_r 24,000), and lysozyme (M_r 16,000), and low molecular weight, prestained proteins (BRL, Bethesda, MD), i.e., ovalbumin (M_r 43,000), α-chymotrypsinogen (M_r 25,700), α-lactoglobulin (M_r 18,400), lysozyme (M_r 14,300), bovine trypsin inhibitor (M_r 6,200), and insulin (M_r 3,000).

Results

Antagonist activity and binding characteristics of ^{125}I-ASA-JMV-179. In a previous study, we extensively characterized the binding of the new antagonist ligand ^{125}I-BH-JMV-179 to plasma membranes and showed that ^{125}I-BH-JMV-179 detects 4-fold more binding sites than do agonist radioligands, although CCK agonists can compete for all of the antagonist binding sites (16). We synthetized the photoreactive antagonist analogue ^{125}I-ASA-JMV-179 to label and characterize the CCK antagonist binding sites detected in pancreatic plasma membranes.

In agreement with a previous report using the same biological model and species (15), the photoreactive probe ^{125}I-ASA-JMV-179 did not stimulate amylase release from rat pancreatic acini (data not shown). The binding of ^{125}I-ASA-JMV-179 to rat pancreatic membranes was saturable, reversible, time dependent, and protein concentration dependent (data not shown) and was inhibited in a concentration-dependent manner by JMV-179 (IC_{50}, 6 ± 2 nM), by (Thr^{28},Ahx^{31})-CCK-25-33 (IC_{50}, 1.2 ± 0.5 nM), and by the CCK-AR antagonist L-364,718 (IC_{50}, 2 ± 1 nM). L-365,260, a selective antagonist of the CCK-B receptor, also inhibited the binding, but with low potency (Fig. 1).

These data indicate that CCK-related molecules inhibit ^{125}I-ASA-JMV-179 binding with the same potency as that with which they inhibit ^{125}I-BH-JMV-179 binding to rat pancreatic plasma membranes (16). The probe ^{125}I-ASA-JMV-179 was, therefore, used to affinity label JMV-179 binding sites in pancreatic plasma membranes and acini.

Photoaffinity labeling of the CCK-AR antagonist binding sites by ^{125}I-ASA-JMV-179. Photoaffinity labeling of pancreatic plasma membranes using the antagonist photoreactive probe ^{125}I-ASA-JMV-179 resulted in identification of two distinct components, migrating as broad bands at 85–100 kDa and 47–50 kDa (Fig. 2). The amount of radioactive material recovered in the two labeled bands represented about 5% of ^{125}I-ASA-JMV-179 specific binding, and the proportion of ra-

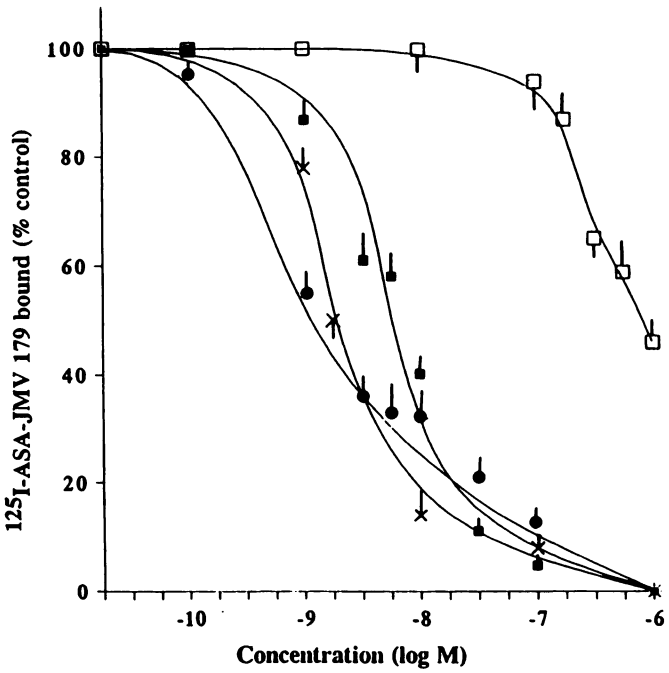

Fig. 1. Competition by JMV-179 (■), (±) L-364,718 (×), (Thr^{28},Ahx^{31})-CCK-25-33 (●), and L-365,260 (□) with ^{125}I-ASA-JMV-179 for binding to rat pancreatic plasma membranes. Membranes were incubated at 25° for 2 hr with the radioligand alone or in the presence of various concentrations of competitors. One hundred percent binding represents specific binding in the absence of competitor peptides. Values are the mean of three separate determinations performed in duplicate.

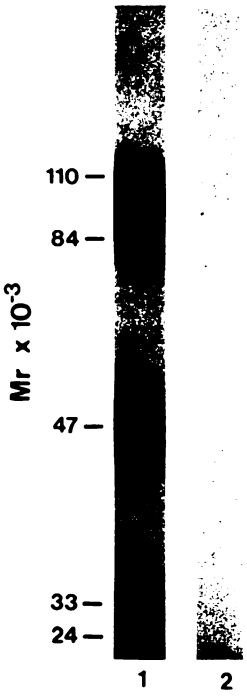

Fig. 2. PAGE-autoradiography of pancreatic membranes photoaffinity labeled by the antagonist probe ^{125}I-ASA-JMV-179. ^{125}I-ASA-JMV-179 was incubated with membranes for 2 hr at 25°, alone (*lane 1*) or in the presence of 1 μM unlabeled ligand (*lane 2*). Photolysis was performed under a UV source for 5 min.

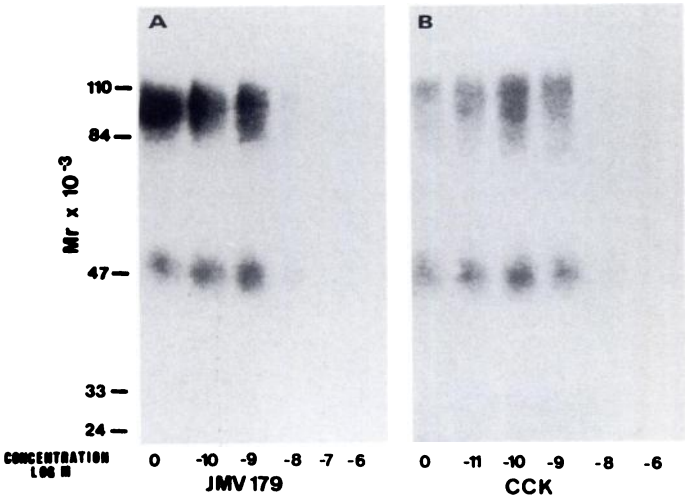

Fig. 3. Inhibition of the photoaffinity labeling of ^{125}I-ASA-JMV-179 binding sites by JMV-179 (A) and (Thr^{28},Ahx^{31})-CCK-25-33 (B). Membranes were incubated with the photoreactive antagonist probe in the absence or in the presence of increasing concentrations of peptides and were then photolyzed. This figure is representative of three experiments performed on different membrane preparations. From scans of the autoradiographs, pseudo-affinities of CCK and JMV-179 for the 85–100-kDa and 47–50-kDa components were determined.

dioactive material migrating at 47–50 kDa was about 50% of that migrating at 85–100 kDa. The labeling of these two components was fully abolished when the binding of the antagonist probe was performed in the presence of a saturating concentration (1 μM) of JMV-179 (Fig. 2, *lane 2*).

We further evaluated the binding specificity and affinity of CCK-related ligands for the two components covalently labeled by ^{125}I-ASA-JMV-179. As shown in Fig. 3, the intensity of the two bands at 85–100 kDa and 47–50-kDa decreased in a concentration-dependent manner in the presence of the antagonist JMV-179 (Fig. 3A) or the agonist (Thr^{28},Ahx^{31})-CCK-25-33 (Fig. 3B). Quantitative densitometric scanning of these two specific bands gave IC_{50} values for (Thr^{28},Ahx^{31})-CCK-25-33 of 5 nM and 15 nM for the 85–100- and 45–50-kDa components, respectively. For JMV-179, an IC_{50} of 5 nM was estimated for both.

When photoaffinity labeling was performed on solubilized membranes, the same two components of 85–100 kDa and 47–50 kDa were observed (Fig. 4). CCK at 1 μM only weakly diminished the labeling, indicating that after solubilization both proteins lose their ability to bind agonists with high affinity (Fig. 4, *lane 2*). The CCK-B receptor antagonist L-365,260 at 1 μM only slightly decreased the intensity of labeling (Fig. 4, *lane 3*), whereas, at the same concentration, the CCK-AR antagonists L-364,718 and JMV-179 both fully abolished the labeling of the two proteins (Fig. 4, *lanes 4* and *5*). In contrast, molecules not related to CCK, such as bombesin, vasoactive intestinal peptide, secretin, somatostatin, and insulin, had no effect on the ^{125}I-ASA-JMV-179 photoaffinity labeling pattern (data not shown). Taken together, these results demonstrate that the labeling of both the 85–100-kDa and 47–50-kDa components by the antagonist probe ^{125}I-ASA-JMV-179 is specific and is consistent with the pharmacological features of the rat CCK-AR.

Structural relationship between the components photoaffinity labeled by ^{125}I-ASA-JMV-179 and the CCK-AR. We examined whether the 85–100-kDa and 47–50-kDa components affinity labeled by ^{125}I-ASA-JMV-179 are structurally related to the pancreatic CCK-AR by testing their ability to be recognized by a lectin and by performing deglycosylation and proteolytic fragmentation of the two labeled entities after separation by preparative SDS-PAGE.

Using photoreactive agonists, we (8, 9) and others (7) have previously identified the pancreatic plasma membrane CCK-

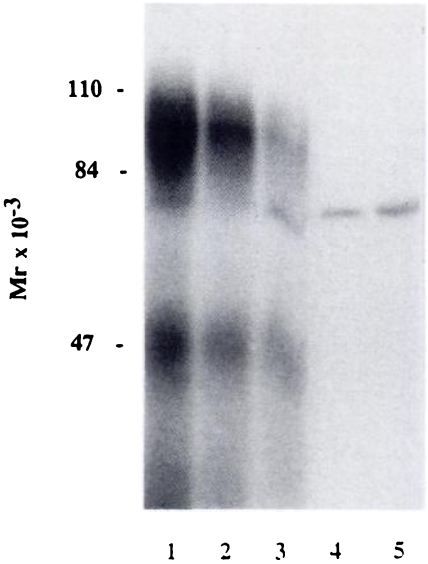

Fig. 4. Photoaffinity labeling of ^{125}I-ASA-JMV-179 binding sites in CHAPS-solubilized plasma membranes. Plasma membranes were solubilized with 3% CHAPS and incubated with ^{125}I-ASA-JMV-179, alone (*lane 1*) or together with 1 μM (Thr^{28},Nle^{31})-CCK-25-33 (*lane 2*), 1 μM L-365,260 (*lane 3*), 1 μM L-364,718 (*lane 4*), or 1 μM JMV-179 (*lane 5*), before photolysis and SDS-PAGE analysis.

AR as a single glycoprotein migrating as a broad band at 85–100 kDa. Moreover, photoaffinity-labeled CCK-AR was fully retained on immobilized wheat germ agglutinin and subsequently eluted by competitor sugars (7, 9, 18). Similarly, the two components of 85–100 kDa and 47–50 kDa that were specifically identified by the new antagonist probe ^{125}I-ASA-JMV-179 were both retained on wheat germ agglutinin and then eluted using a competitor sugar (Fig. 5).

The 85–100-kDa component labeled by ^{125}I-ASA-JMV-179 was deglycosylated to a 42-kDa protein (Fig. 6A, *lane 1*), corresponding to the CCK-AR peptide core previously identified by agonist photoreactive probes (7, 18). Endoglycosidase F digestion of the 47–50-kDa component generated a 35-kDa peptide core (Fig. 6B, *lane 1*), whereas *O*-glycanase treatment had no effect on either the 85–100-kDa component or the 47–50-kDa component (data not shown). Both components, therefore, are glycosylated exclusively with *N*-linked oligosaccharides and differ from each other in their peptide core by about 7 kDa.

We then performed proteolytic digestions of both the 85–100-kDa and 47–50-kDa components. Identical labeled fragments were generated from the two labeled proteins (Fig. 6). Endoproteinase Glu-C yielded a main fragment of 34 kDa (Fig. 6, *lanes 2*) and a minor fragment of 12 kDa. Subsequent endoglycosidase F treatment produced a peptide of 22 kDa with no change in the minor 12-kDa fragment (Fig. 6, *lanes 3*). Digestion of the labeled 85–100-kDa and 47–50-kDa components by endoproteinase Glu-C followed by digestion with trypsin yielded a major labeled fragment of 12 kDa (Fig. 6, *lanes 4*), the size of which was not further affected by endoglycosidase deglycosylation (Fig. 6, *lanes 5*), showing that the 12-kDa fragment does not contain any carbohydrate chains. In a previous study (18), we showed that fragmentation, by the aforementioned enzymes, of the CCK-AR affinity labeled by the agonist probe ^{125}I-ASD-(Thr^{28},Ahx^{31})-CCK-25-33 produced a pattern of labeled fragments identical to those found in the present study.

This set of data demonstrates that the 85–100-kDa protein photoaffinity labeled by the antagonist probe ^{125}I-ASA-JMV-179 is, in fact, the CCK-AR. Indeed, the 85–100-kDa component behaves pharmacologically as the membrane CCK-AR, fails to bind (after solubilization) an agonist but not an antagonist of the CCK-AR (19, 20), and yields peptides identical to those of the CCK-AR after treatment with three different enzymes. On the other hand, the identical patterns of labeled fragments resulting from the 47–50-kDa protein or from the CCK-AR provide conclusive support for a structural relationship between the two labeled proteins. Additionnally, the fact that the 47–50-kDa protein differs from the CCK-AR in its peptide core, while behaving pharmacologically like the CCK-AR, strongly suggests that the 47–50-kDa binding protein corresponds to a truncated form of the CCK-AR.

Because previous studies documented that cleaved forms of receptors endowed with ligand-binding capability could result from *in vitro* proteolysis of the intact receptor (21–23), we examined the possibility that the 47–50-kDa truncated CCK-AR labeled only by the antagonist probe could be generated during the experiments by proteolytic cleavage of the intact CCK-AR at 85–100 kDa. To test this hypothesis, we performed the following experiments. Firstly, membranes photoaffinity labeled by the agonist ^{125}I-ASD-(Thr^{28},Nle^{31})-CCK-25-33 were incubated for an additional 60 min at 37° in the presence of fresh untreated membranes (Fig. 7, *lanes 1* and *2*). Secondly, pancreatic membranes were preincubated for 60 min at 37° before binding and photolysis using ^{125}I-ASA-JMV-179 (Fig. 7, *lanes 3* and *4*). Thirdly, photoaffinity labeling using the antagonist probe was performed on plasma membranes prepared in the absence or presence of several protease inhibitors at high concentrations (Fig. 7, *lanes 5-8*). Fourthly, for comparison, photoaffinity labeling using ^{125}I-ASA-JMV-179 was carried out on isolated pancreatic acini (Fig. 7, *lanes 9* and *10*). Whatever the conditions tested, very similar photoaffinity labeling patterns were obtained. The 47–50-kDa component was detected only using the antagonist probe. In addition, experiments fa-

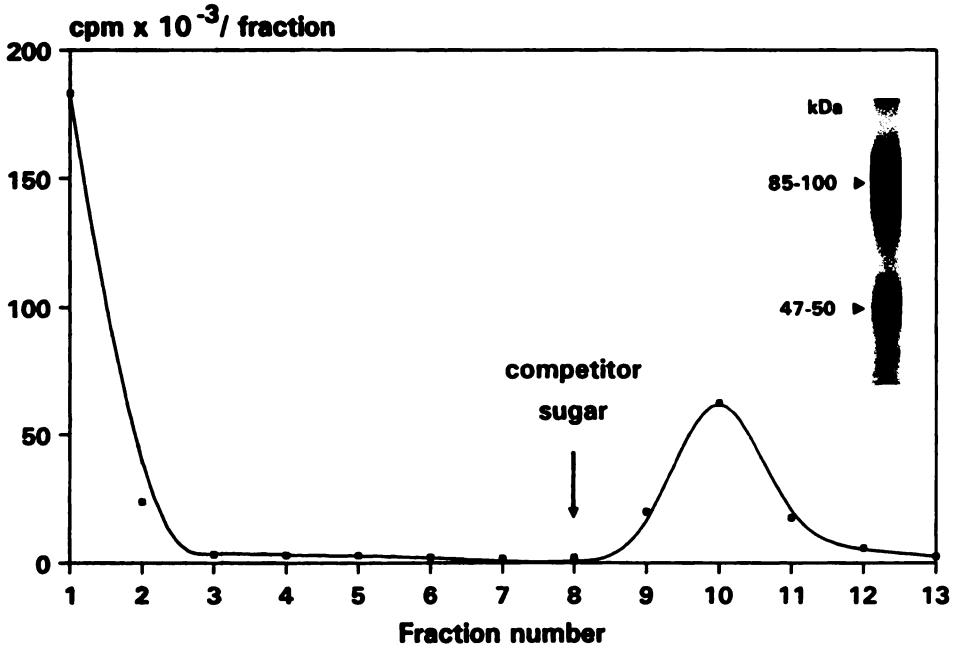

Fig. 5. Chromatography of ^{125}I-ASA-JMV-179 binding sites on immobilized wheat germ agglutinin. Membranes were photoaffinity labeled with the antagonist probe ^{125}I-ASA-JMV-179, solubilized using Nonidet P-40, and incubated with the affinity gel. After washing, adsorbed material was eluted by addition of *N*-acetyl-β-D-glucosamine and was then analyzed by SDS-PAGE. *Inset*, SDS-PAGE analysis of an aliquot of the radioactive peak (fractions 9–11), showing that both the 85–100-kDa and 47–50-kDa components were retained by the lectin.

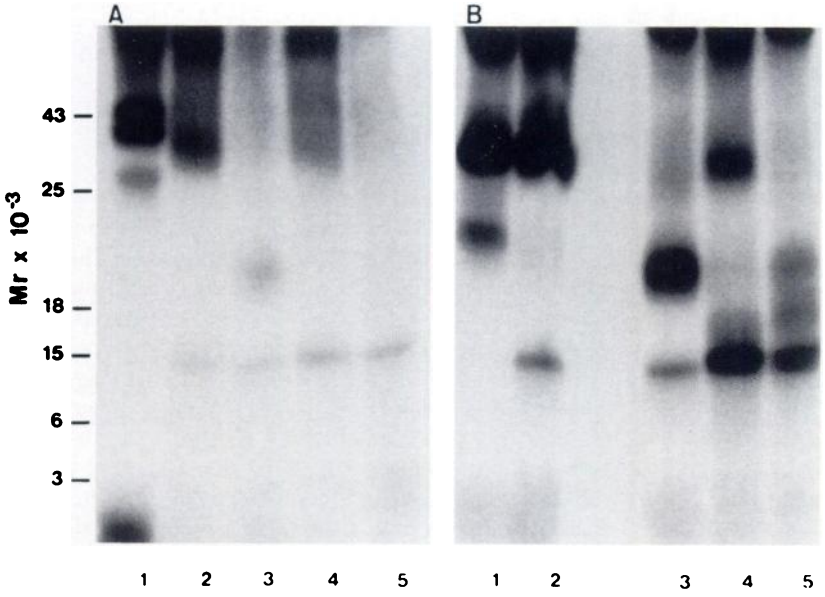

Fig. 6. Endoglycosidase F and protease digestion of the 85–100-kDa and 47–50-kDa components photoaffinity labeled by ^{125}I-ASA-JMV-179. After preparative photoaffinity labeling of pancreatic membranes and SDS-PAGE separation, radioactive material corresponding to the 85–100-kDa and 47–50-kDa components was electroeluted, reduced and carboxymethylated, and then subjected to enzyme treatment. A, The 85–100-kDa labeled component was digested with endoglycosidase F (*lane 1*), endoproteinase Glu-C (*lane 2*), endoproteinase Glu-C followed by endoglycosidase F (*lane 3*), endoproteinase Glu-C followed by trypsin (*lane 4*), or endoproteinase Glu-C followed by trypsin and then endoglycosidase F (*lane 5*). B, The 47–50-kDa labeled component was subjected to the same treatment as indicated for A.

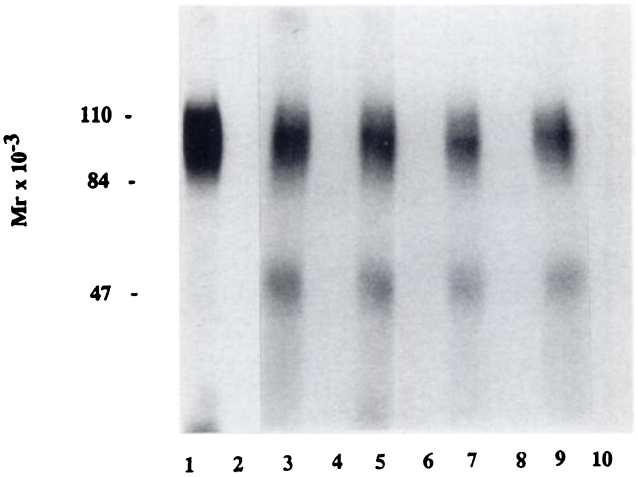

Fig. 7. Demonstration of the absence of proteolytic degradation of the photoaffinity-labeled CCK-AR during membrane preparation and photoaffinity labeling. Some membranes prepared under standard conditions were photoaffinity labeled using ^{125}I-ASD-(Thr^{28},Ahx^{33})-CCK-25-33 and then incubated for an additional 60 min at 37° in the presence of fresh untreated membranes (*lanes 1* and *2*). Other membranes prepared under standard conditions were preincubated for 60 min at 37° before labeling using ^{125}I-ASA-JMV-179 (*lanes 3* and *4*). In another experiment, photoaffinity labeling by ^{125}I-ASA-JMV-179 was performed with membranes prepared in the absence (*lanes 5* and *6*) or the presence (*lanes 7* and *8*) of several protease inhibitors at high concentrations. Finally, isolated pancreatic acini were photoaffinity labeled with the antagonist probe (*lanes 9* and *10*). *Lanes 2*, *4*, *6*, *8*, and *10*, assays performed in the presence of 1 μM unlabeled peptide.

voring proteolysis did not result in an increase in the 47–50-kDa component and a parallel decrease in the 85–100-kDa component. Finally, the 47–50-kDa component was also present in membranes prepared in the presence of high concentrations of protease inhibitors and in pancreatic acinar cells. Therefore, the 47–50-kDa component does not appear to result from CCK-AR proteolysis that could occur during *in vitro* experiments but, rather, exists in pancreatic tissue.

Localization of the labeled fragments in the CCK-AR polypeptide chain. Because the amino acid sequence of the rat pancreatic CCK-AR has been determined by cDNA sequencing (10), we attempted to localize in the primary structure the labeled fragments obtained by endoglycosidase F and protease digestions of both the CCK-AR and the 47–50-kDa truncated CCK-AR (Fig. 8). To do so, we used the PC-Gene program to determine cleavage sites within the receptor protein and the fragments that could be generated using our digestion protocols. The localization of the labeled fragments was made possible only by assuming that asparagines corresponding to consensus sites for *N*-glycosylation, at positions 25, 28, and 39 on the amino-terminal extracellular domain and at position 205 on the second extracellular loop (10), bear carbohydrate chains of identical mass. In fact, experimental data suggest that this is the case. Indeed, limited deglycosylation of affinity-labeled pancreatic CCK-AR using endoglycosidase F demonstrated the presence of four *N*-linked carbohydrate chains, each accounting for about 12 kDa (7). Moreover, the present study shows that both the 34-kDa endoproteinase Glu-C fragment and the 47–50-kDa truncated CCK-AR have their masses decreased by 12 kDa, without any intermediate component, after endoglycosidase F treatment.

Thus, the 34-kDa fragment generated by endoproteinase Glu-C cleavage of the intact CCK-AR has a peptide core of 22 kDa and most likely bears a single *N*-linked carbohydrate chain. It was designated F_{22}. Peptides delimited by endoproteinase Glu-C cleavage sites and containing carbohydrate chains linked via Asn^{39} or Asn^{205} could fulfill the first requirement. Only two peptides, corresponding to sequences Trp^{54}-Glu^{250} and Arg^{154}-Asp^{355}, have masses very close to 22 kDa and include a single consensus glycosylation site, Asn^{205}. The possibility that the sequence Arg^{154}-Asp^{355} could correspond to the final endoproteinase Glu-C product F_{22}, which would then be converted to the final tryptic product F_{12}, has been ruled out for the following reasons. This sequence contains seven endoproteinase Glu-C cleavage sites, six of which are highly probable sites due to their location in an hydrophilic environment, and 23 tryptic cleavage sites, 17 of which are located carboxyl-terminally to Asn^{205}. In contrast, the sequence Trp^{54}-Glu^{250} bears four potential endoproteinase Glu-C cleavage sites, at positions 102, 121, 153, and 218. Only the site at position 153 is located in an

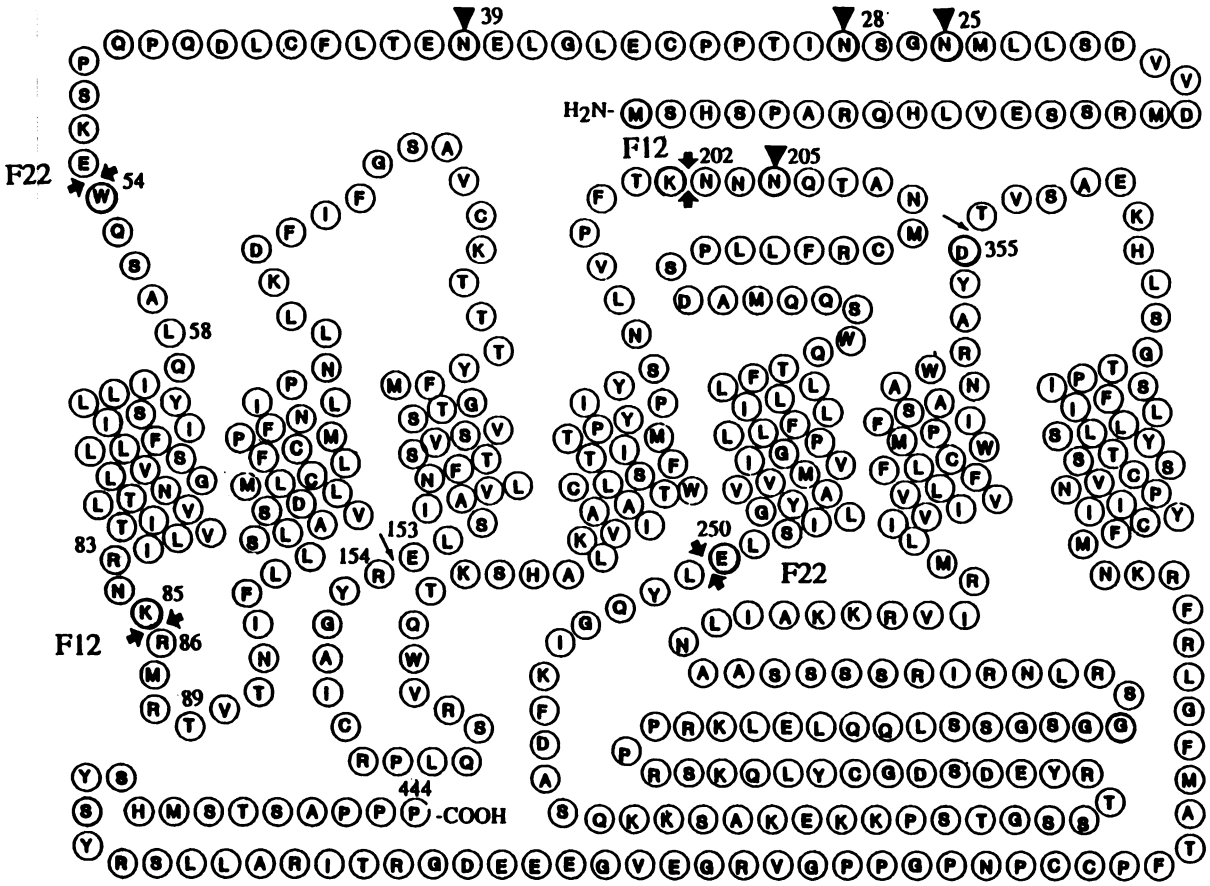

Fig. 8. Amino acid sequence of the CCK-AR and localization of the labeled fragments generated by endoproteinase Glu-C and tryptic digestion of the receptor photoaffinity labeled with the ^{125}I-ASA-JMV-179 probe. The sequence of the rat pancreatic CCK-AR is from Ref. 10. Amino acids are denoted by the single-letter code. The receptor is represented according to the topography of G protein-coupled receptors, with seven transmembrane domains, three cytoplasmic loops, three exoplasmic loops, an exoplasmic amino-terminal domain, and a cytoplasmic carboxyl-terminal tail. ▼, Glycosylation sites at residues 25, 28, 39, and 205. Only those cleavage positions for endoproteinase Glu-C and trypsin that can yield fragments having a size compatible with the experimental data are shown (↓, ⬇). F_{22} generated by endoproteinase Glu-C treatment followed by endoglycosidase F deglycosylation corresponds to the sequence Trp^{54}-Glu^{250}, and fragment F_{12} generated by digestion with endoproteinase Glu-C followed by trypsin corresponds to the sequence Arg^{86}-Lys^{202}. Experimental data also suggest that the 47–50-kDa component identified only by the antagonist photoreactive probe ^{125}I-ASA-JMV-179 corresponds to the receptor lacking its amino-terminal extracellular domain.

hydrophilic environment. Cleavage at this site should generate peptides of 11.2 and 10.8 kDa. In fact, we observed that the endoproteinase Glu-C digestion of the intact receptor generated a minor labeled 12-kDa fragment, in addition to the main 34-kDa fragment (Fig. 6A, *lane 2*). This minor fragment should result from slow digestion at position 153.

After digestion with trypsin, a single nonglycosylated peptide of 12 kDa, designated F_{12}, was produced. This peptide is generated from the 34-kDa fragment (data not shown). The absence of carbohydrate side chains on the 12-kDa tryptic fragment places this peptide carboxyl-terminally to Asn^{205} within the sequence Trp^{54}-Glu^{250}. The endoproteinase Glu-C fragment F_{22} delimited by Trp^{54}-Glu^{250} contains 12 potential tryptic cleavage sites, four of which are located in the hydrophilic sequence Arg^{83}-Thr^{89}. In this region, cleavage between Lys^{85} and Arg^{86} is most probable, due to the preference of trypsin for the doublet Lys-Arg. Therefore, the labeled fragment F_{12} generated by successive digestions of the intact CCK-AR using endoproteinase Glu-C and trypsin can be defined as peptide Arg^{86}-Lys^{202} (Fig. 8). This peptide includes three transmembrane domains, parts of the first intracellular loop and of the second extracellular loop, and the entire first extracellular loop.

The mass of the 47–50-kDa truncated CCK-AR labeled only by the antagonist probe was decreased to 35 kDa by deglycosylation, indicating the presence of a single carbohydrate side chain of 12 kDa. The same approach as described above identified Asn^{205} as the only glycosylation site in this truncated receptor and consequently defined the missing sequence as that bearing the other three glycosylation sites, namely the amino-terminal extracellular tail. This proposal agrees well with the two peptide cores, for the CCK-AR and its truncated form, differing by about 7 kDa. Because identical fragments were produced by protease digestion of the truncated and intact CCK-AR, we identified the peptide resulting from endoproteinase Glu-C cleavage of the truncated CCK-AR as the sequence Trp^{54}-Glu^{250} and that resulting from endoproteinase Glu-C/trypsin cleavage as the sequence Arg^{86}-Lys^{202}.

Discussion

In a previous study, we found that our new peptide antagonist radioligand, ^{125}I-BH-JMV-179, detected a larger number of binding sites than did the agonist radioligand ^{125}I-BH-(Thr^{28},Ahx^{31})-CCK-25-33 in pancreatic plasma membranes, although (Thr^{28},Ahx^{31})-CCK-25-33 could interact competitively

with the entire population of antagonist binding sites when this peptide was used as a competitor for ^{125}I-BH-JMV-179 binding (16). These observations, together with the identification of two discrete binding components by analysis of the binding kinetics of ^{125}I-BH-JMV-179, led us to conclude that the antagonist ligand was able to reveal several populations of binding sites, one of which could not directly bind agonist ligands (16).

In this work, we synthesized a photoactivable analogue of ^{125}I-BH-JMV-179, i.e., ^{125}I-ASA-JMV-179, to search for a molecular basis for the antagonist binding site heterogeneity in plasma membranes. The main binding characteristics of the new probe indicate that it behaves identically to the radioligand ^{125}I-BH-JMV-179. The efficiency of covalent labeling of ^{125}I-ASA-JMV-179 binding sites was comparable to that achieved with the agonist ^{125}I-ASD-(Thr^{28},Ahx^{31})-CCK-25-33, which also bears an aryl azide as the photoreactive moiety at the peptide amino-terminal end. Using ^{125}I-ASA-JMV-179 for photoaffinity labeling of plasma membranes, we detected, in addition to the CCK-AR at 85–100 kDa, a component of 47–50 kDa that was not detected by the agonist probe. However, the labeling of the 47–50-kDa component was inhibited by (Thr^{28},Ahx^{31})-CCK-25-33, thus demonstrating that this component could also selectively bind CCK agonists and antagonists of the CCK-AR. On the other hand, lectin recognition, deglycosylation, and protease fragmentation demonstrated that the 47–50-kDa component is structurally related to the CCK-AR but differs in the mass of its peptide core. Therefore, both biochemical and pharmacological data strongly suggest that the 47–50-kDa component represents a truncated form of the CCK-AR. Attempts to localize the labeled peptides in the primary structure of the CCK-AR led us to define the 47–50-kDa protein identified by the probe ^{125}I-ASA-JMV-179 as a CCK-AR, most likely lacking its amino-terminal extracellular region. Identification of this truncated form of the CCK-AR, coexisting in plasma membranes with the intact CCK-AR, is therefore in line with our initial hypothesis of CCK antagonist binding site heterogeneity in plasma membranes. Furthermore, the observation that the truncated form of the CCK-AR binds CCK only in competition assays can be compared with previous binding data suggesting the presence in plasma membranes of a population of CCK antagonist binding sites that can bind CCK agonist only in competition experiments. Accordingly, the existence of a truncated form of the CCK-AR in pancreatic plasma membranes should contribute partly or completely to the additional binding sites detected using antagonist radioligands. The question of whether only the truncated CCK-AR accounts for the additional sites revealed by the antagonist ligand cannot be clarified by the present study, although photoaffinity labeling data suggest that the truncated protein represents almost 50% of the intact receptor.

The inability of the photoactivable agonist to detect the truncated CCK-AR, whereas the unlabeled CCK agonist inhibits antagonist radioligand binding to that truncated receptor, cannot reflect different behaviors of the two ligand forms because of the incorporation of the labeled and photoactivable moieties at the amino terminus of CCK. Indeed, previous structure-activity relationship studies on CCK from different groups have demonstrated that such modifications do not affect binding and biological properties of the peptides (3). On the other hand, photoaffinity labeling using an intrinsic agonist probe having its photoreactive moiety in the carboxyl-terminal part of the peptide also failed to detect the truncated CCK-AR (8). More plausible is the explanation that the photoreactive agonist failed to detect the truncated receptor because of the much lower affinity of the probe for the truncated receptor than for the intact receptor. This hypothesis, which is apparently inconsistent with results from quantitative gel scanning indicating that the nonlabeled agonist can compete with similar potencies with the photoreactive antagonist ^{125}I-ASA-JMV-179 for binding to the intact CCK-AR and the truncated CCK-AR, would indicate that the truncated receptor recognizes agonists with a lower affinity than that of the antagonist-truncated receptor complex. This conclusion raises the question of the importance of the missing region of the truncated CCK-AR, presumably the amino-terminal extracellular part of the CCK-AR, for direct binding of agonists but not antagonists. As hypotheses, the amino-terminal region of the receptor could contribute to binding of agonist either because it interacts transiently with agonist ligands before they bind to a site located in the embedded part of the receptor or because it contains amino acids directly involved in the binding site. Alternatively, the amino-terminal region of the receptor could play a role in transition of the receptor from a resting state to a high affinity state and/or in stabilization of the corresponding ternary complex, which includes G protein(s). In this case, the agonist probes would not identify the truncated CCK-AR because this receptor form fails to correctly couple to G protein(s), whereas the antagonist probe, which binds to coupled CCK-AR and uncoupled CCK-AR with very similar affinities (16), detects both intact and truncated CCK-AR. Our previous finding (16) that the sulfate group on the tyrosine residue of CCK peptides is of much less importance for antagonist affinity than for agonist affinity leads us to consider that the negative charge of this sulfate group could interact with an amino acid located in the amino-terminal region of the CCK-AR.

Some recent reports dealing with the identification of ligand binding sites of G protein-coupled receptors have documented involvement of regions other than transmembrane segments, namely extracellular regions. For instance, for the endothelin type A receptor it has been shown by expression of chimeric and truncated receptors (24, 25) that the first extracellular loop (B loop) and a part of the amino-terminal region in close proximity to the first transmembrane segment are required for agonist binding. The lutropin receptor has a high affinity, ligand-binding domain in its large amino-terminal extracellular region and a low affinity site, able to induce cAMP synthesis, located in the embedded part of the receptor (26). In the neurokinin type 1 receptor, the extracellular loops seem to contain amino acids involved in the binding site for agonist peptides (27). On the other hand, a nonpeptide antagonist of this receptor, CP-96,345, interacts with amino acids, especially a histidine, located on the external side of the sixth transmembrane domain (28).

The mechanism that generates the truncated CCK-AR in pancreatic membranes and acini remains unknown. As a first hypothesis, alternative splicing or incorrect splicing of the CCK-AR gene could explain appearance of the truncated CCK-AR, although it is not yet known whether the rat CCK-AR gene contains sequences that are able to direct alternative splicing. Nevertheless, it has been recently documented that the CCK-B receptor gene contains introns and an alternative donor site in exon 4 that are responsible for generation of two

different mRNA transcripts (29). For another neuropeptide receptor, the pituitary adenylate cyclase activating polypeptide receptor, five splice variants have been described (30). Interestingly, these variants differ in the sequence of their third cytoplasmic loop, which is involved in the coupling with G proteins. As a second hypothesis, a mechanism involving proteolysis of the CCK-AR at the acinar cell surface in pancreatic tissue is also plausible.

Concerning the potential function of the truncated CCK-AR and assuming that the truncated CCK-AR really exists in pancreatic tissue and can bind CCK with only low affinity, biological or pharmacological responses observed with high doses of CCK agonists, such as experimental pancreatitis and inhibition of the supramaximal secretory response of pancreatic acini (3), could be mediated via the truncated CCK-AR. On the other hand, the recent findings that muscarinic and adrenergic receptors are composed of two independently folding structural domains, namely, transmembrane segments I-V and transmembrane segments VI-VII, that are able to interact functionally with each other at the molecular level provided evidence for intermolecular cross-talk between G protein-coupled receptors (31). Accordingly, one can envisage that the truncated CCK-AR could be secondarily activated, under normal conditions, through the intact CCK-AR.

To summarize, the current work, using the first CCK-AR antagonist photoreactive probe, demonstrates the presence of a truncated CCK-AR in pancreatic acini and membranes and therefore provides the first structural basis for an heterogeneity of CCK-AR antagonist binding sites in pancreatic preparations. Obviously, these data do not demonstrate the involvement of the truncated CCK-AR either in the biphasic CCK-induced secretory response of pancreatic acini or in other biological responses. At present, further investigations using anti-CCK-AR antibodies are necessary to definitively establish that the missing sequence in the truncated CCK-AR corresponds to the amino-terminal extracellular region of the CCK-AR. Furthermore, expression of the truncated CCK-AR in eukaryotic cells is necessary to study in detail the role of the missing region of the receptor and to determine whether CCK agonists at high concentrations can induce formation of intracellular second messengers through occupation of that truncated receptor.

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Send reprint requests to: Daniel Fourmy, INSERM U151, CHU de Rangueil, 31054 Toulouse Cedex, France.