Identification and Localization of Cholecystokinin-binding Sites on Rat Pancreatic Plasma Membranes and Acinar Cells: A Biochemical and Autoradiographic Study

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ABSTRACT Using the combined approaches of affinity labeling and light and electron microscopic autoradiography, we investigated the identification and localization of cholecystokinin (CCK)-binding sites on rat pancreatic acinar cells. To define the molecular properties of the CCK-binding site, we incubated rat pancreatic plasma membranes with $^{125}$I-CCK-33 for 15 min at 23°C followed by washing and cross-linking with disuccinimidyl suberate. Specific labeling of a major Mr 85,000 component was revealed as assessed by SDS PAGE under reducing conditions and autoradiography of the dried gels. Components of Mr >200,000, Mr 130,000-140,000, and Mr 55,000 were labeled under maximal cross-linking conditions. The labeling of all components was specifically inhibited by CCK-8 in a dose-dependent manner ($K_d$ ~ 9 nM). The Mr 85,000 component had identical electrophoretic mobilities under reducing and nonreducing conditions indicating that it likely does not contain intramolecular disulfide bonds. The larger labeled species may be cross-linked oligomers of this binding protein or complexes between it and neighboring polypeptides.

For studies on the distribution of CCK-binding sites, pancreatic acini were incubated with $^{125}$I-CCK-33 (0.1 nM) in the absence or presence of CCK-8 (1 μM) for 2 or 15 min at 37°C, washed, and fixed in 2% glutaraldehyde. Quantitative autoradiographic analysis indicated that ~60% of the total grains were located within ±1 HD (1 HD = 100 nm) of the lateral and basal plasmalemma with little or no labeling of the apical plasmalemma. From these data, it was estimated that each acinar cell possesses at least 5,000-10,000 CCK-binding sites on its basolateral plasmalemma. The remaining grains showed no preferential concentration over the cytoplasm or nucleus. Together, these data indicate that CCK interacts with a Mr 85,000 protein located on the basolateral plasmalemma of the pancreatic acinar cell.
using $^{125}$I-CCK-33 (8-12) or $^{129}$I-CCK-8 (13, 14) to characterize the presence of specific, high affinity CCK receptors on isolated pancreatic acini, pancreatic membranes, or brain membranes. Although these studies have revealed significant features of CCK receptors, more definitive cell biology studies delineating both the localization and the structure of the pancreatic acinar cell CCK receptor are currently lacking. A novel method for identifying hormone receptors in intact cells or cell membrane fractions is the technique of affinity labeling (15). This type of approach has been applied for the successful identification of membrane receptors for insulin (16), angiotensin II (17), glucagon (18), MSA (stimulation multiplexing activity; 19, 20), NGF (nerve growth factor; 21), IGF-I and IGF-II (insulinlike growth factors I and II; 20, 22), PDGF (platelet-derived growth factor; 23), and formyl peptide chemotactic receptor (24).

Here we describe the identification of a major affinity labeled component of M, 85,000 in rat pancreatic plasma membranes using $^{125}$I-CCK-33 in conjunction with chemical cross-linking reagents. Labeling of this component was dependent upon the presence of cross-linking reagent and inhibited in the presence of excess unlabeled CCK-8 indicating specific labeling. Light and electron microscopic autoradiographic localization of $^{125}$I-CCK-33 in dispersions of intact pancreatic acini showed that CCK binding was restricted to the basal and lateral domains of the plasmalemma of the acinar cell. These data suggest that, in the pancreas, CCK binds to a M, 85,000 protein located on the basolateral plasmalemma of acinar cells as a first step in the stimulation of secretory protein release. A portion of this work has appeared in abstract form (25).

MATERIALS AND METHODS

Hormones and Antiserum: Synthetic CCK-8 was a gift from Dr. Miguel Ondetti (Squibb Institute for Medical Research, Princeton, NJ). Natural porcine CCK-33 was obtained from Dr. Viktor Mutt (Gastrointestinal Hormone Research Laboratory, Karolinska Institutet, Stockholm, Sweden). Rabbit antiserum raised against CCK-8 (No. 1-48) was a gift from Dr. Graham J. Dockray (University of Liverpool).

Reagents: All chemical cross-linking reagents were obtained from Pierce Chemical Co. (Rockford, IL). Sephadex G-50 (superfine) was from Pharmacia Fine Chemicals (Piscataway, NJ). Soybean trypsin inhibitor and collagenase (EC 3.4.24.3; type I) were obtained from Worthington Diagnostic Systems, Inc. (Freehold, NJ). Monodiodinated Bolton-Hunter reagent (>2,000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Dithiothreitol was from ICN, and 2-mercaptoethanol were from Bio-Rad Laboratories (Richmond, CA). Bovine serum albumin (BSA; fraction V powder) was obtained from Reheis Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

Radioiodination of CCK-33: CCK-33 was acylated with $^{125}$I-labeled Bolton-Hunter reagent (26) using a modification of the method of Retief (27) as described by Sankaran et al. (8). Following reaction of 10 μg of CCK-33 in 10 μl of 0.05 M Na borate, pH 10, with 1 μCi of Bolton-Hunter reagent for 24 h at 4°C, 0.6 ml of 0.05 M guanidine HCl in column elution buffer (see below) was added to stop the reaction. The sample was then applied to a column (1.6 x 100 cm) of Sephadex G-50 (superfine) equilibrated and eluted with 0.5 M acetic acid, pH 2.39, containing 0.2% gelatin and 50 μM EDTA (elution buffer). Aliquots of each fraction (5 μl) were scanned for radioactive content in a Beckman 5000 ~-cm) of Sephadex G-50 (superfine) equilibrated and eluted with 0.5 M acetic acid, pH 2.39, containing 0.2% gelatin and 50 μM EDTA, placed in a 95°C H₂O bath for 20 min, and run on 10% polyacrylamide slab gels (11 cm x 95°C) in Tris-glycine buffer. Molecular weight standards used were: myosin (M, 200,000), β-galactosidase (M, 116,500), phosphorylase b (M, 92,500), BSA (M, 66,000), ovalbumin (M, 45,000), and carbonic anhydrase (M, 31,000). "Values reported for affinity labeled membrane proteins were obtained from a plot of log Mₐ vs. the relative mobilities of the standard proteins.

Affinity Labeling Procedure

ANTIBODIES: Immunoprecipitation of $^{125}$I-CCK-33 was carried out with antiserum No. L-48 diluted 1/2,000 in 0.05 M Na phosphate, pH 7.4, containing 0.1% BSA, 1% Na azide, and 0.15 M NaCl. Antiserum and radio-labeled CCK were incubated for 24 h at 4°C in the presence or absence of excess unlabeled CCK-8 in a final 1 ml volume. The samples were then cross-linked for 15 min on ice by the addition of 1 mM DSS (final concentration using a 1:50 dilution) freshly dissolved in DMSO, essentially as described by Pilch and Czech (16). After quenching for 10 min at 4°C with 20 mM Tris, pH 7.4, 50 μl of formamide-anything with the tubes centrifuged (12,000 g) for 10 s in a Brinnimann model 2200 microfuge to pellet the adsorbed immune complexes in preparation for gel electrophoresis.

MATERIALS: Fresh membranes or frozen membranes thawed at room temperature determined by the fluorodensitometry method (31) were homogenized in fresh KRH (NaCl 104 [mM], KCl 5 [mM], MgSO₄ 1.2 [mM], CaCl₂ 2.0 [mM], KH₂PO₄ 1.0 [mM], and HEPES [25 mM]), pH 7.4, containing 0.2% BSA, 0.01% Triton X-100, and 100 mM bacitracin. Incubations were carried out with $^{125}$I-CCK-33 for 15 min at 23°C in the presence or absence of unlabeled CCK-8 as indicated. To terminate the binding reaction, the tubes were placed on ice and 1 ml of ice-cold KRH, pH 7.4, containing 0.2% BSA and 0.01% Triton X-100 was added to each tube. Membranes were then pelleted by centrifugation at 12,000 g for 2 min. The pellets were resuspended in 98 μl of ice-cold KRH, pH 7.4, and 2 μl of a stock solution of DSS freshly dissolved in DMSO was added to obtain the final concentrations indicated. Cross-linking was allowed to proceed for 5 or 15 min at 4°C followed by quenching with 20 mM Tris, pH 7.4, and centrifugation at 12,000 g for 4 min to yield a final pellet for analysis by gel electrophoresis.

Gel Electrophoresis and Autoradiography: Cross-linked mem-brane pellets and S. aureus--adsorbed antibodies were solubilized in sample buffer consisting of 125 μl Trit, pH 6.8, containing 4% SDS, 10 mM EDTA, 15% sucrose, and 0.01% bromphenol blue with or without 0.1 M DTT, placed in a 95°C H₂O bath for 20 min, and run on 1% polyacrylamide slab gels (11 cm x 0.75 or 1.5 mm) containing 2 mM EDTA according to the method described by Laemmli (32). After staining in Coomassie Blue (33), the gels were dried and exposed to x-ray film (Kodak X-Omat) at ~70°C for 2 to 4 weeks. Collodion light intensifying screen. Molecular weight standards used were: myosin (M, 200,000), β-galactosidase (M, 116,500), phosphorylase b (M, 92,500), BSA (M, 66,000), ovalbumin (M, 45,000), and carbonic anhydrase (M, 31,000). Values reported for affinity labeled membrane proteins were obtained from a plot of log Mₐ vs. the relative mobilities of the standard proteins.

Preparation of Rat Pancreatic Membranes: Plasma membranes were prepared from the pancreata of 125-150 g male rats (Charles River Breeding Laboratories, Charles River, MA) using a combination of the methods of Meldolesi et al. (28) and Tartskoff and Jamieson (29). Pancreas and the pancreatic subfractions generated were kept at 4°C at all times. Briefly, the glands were homogenized in 10 vol by weight of ice-cold 0.3 M sucrose containing 0.01% soybean trypsin inhibitor (STI), 1 mM PMSF, and 5 mM 2-mercaptopethanol in a Dounce homogenizer using eight strokes of the loose fitting pestle. The homogenate was filtered through two layers of cheesecloth and centrifuged at 1,200 g for 12 min in a Beckman model J6-B centrifuge (Beckman Instruments, Inc.). The pellet, containing intact nuclei, plasma membrane sheets, unbroken cells, and cell debris was resuspended in 20 ml of 0.3 M sucrose containing 0.01% STI, 1 mM PMSF, and 5 mM 2-mercaptopethanol and homogenized in a Brendler motor-driven pestle at 2,200 rpm. This second homogenate was brought to 1.25 M sucrose by addition of 28.4 ml of 2 M sucrose using gentle magnetic stirring. The sample (12 ml) was then layered over a 1-ml cushion of 2 M sucrose, and overcentrifuged at 10 ml of 1.25 M sucrose and 4 ml of 0.3 M sucrose using a Beckman Type 70 tubes. The gradients were loaded into a Beckman Type 70 rotor and centrifuged for 90 min (149,000 gₘ). Material banding at the 1.2-0.3 M sucrose interface was collected, diluted to a final sucrose concentration of 0.3 M with ice-cold distilled water, and centrifuged for 30 min (149,000 gₘ) using the Type 70 rotor. The washed pellet was homogenized in KRH, pH 7.4, containing 0.2% BSA, 0.01% Triton X-100, and 100 mM bacitracin and stored in aliquots at ~80°C until used in binding (affinity labeling) experiments.
**Analysis of Electron Microscopic Autoradiographs:** For quantitation of the distribution of autoradiographic grains, 18 micrographs were taken randomly from two different grids of each experimental time period at a primary magnification of 2,400 by an observer who was not told of the coding of the grids. Similarly, at least nine micrographs were taken of the corresponding control sets. Each micrograph was photographically enlarged to ×7,800, and autoradiographic grains were scored as follows: grains were assigned to the basal, lateral, or apical domains of the plasmalemma of the acinar cell provided that they were contained in a strip ± 1 HD (1 HD = 100 nm) on either side of the membrane. Grains falling outside this zone were designated as background (i.e., associated with the embedding medium) except in the case of the lateral membranes where all grains falling outside this zone were assigned to the cytoplasmic compartment. The remainder of the autoradiographic grains was assigned either to the cytoplasmic compartment or to the nucleus.

Morphometric analysis of the autoradiographs was carried out using established procedures (36). Briefly, a coherent square lattice was drawn on a plastic transparency and overlaid on the print. Volume density of nuclei, cytoplasm, and embedding medium was determined by scoring intersection points overlying these structures on each print. Surface area of the apical, basal, and lateral domains of the plasmalemma of the acinar cell was determined by counting intersections of membranes with lines of the test grid and multiplying the linear length of membrane by section thickness as determined according to Weibel and Paumgartner (37).

Autoradiographic grains were related to morphometric data as follows: relative grain density over nuclei, cytoplasm, and embedding medium was calculated by dividing the percentage of autoradiographic grains associated with the structure by the percentage volume density that it occupied. Grain density on the three plasmaembranous domains was calculated by dividing actual numbers of autoradiographic grains associated with the domain by its surface area (grains/μm²). An estimation of the receptor site number on the basal/lateral membranes of the acinar cell was derived using the formula given by Fertuck and Salpeter (38). Statistical analyses of data employed one way analysis of variance.

**RESULTS**

**Affinity Labeling of CCK Antibodies**

The application of affinity labeling techniques to the identification of the CCK receptor of necessity requires that both the radio-ligand, ¹²⁵I-CCK-33, and its receptor possess primary amino groups. Specifically, these functional groups must be close (~1.1 nm) to each other in the hormone-receptor complex and amenable to reaction with chemical cross-linking reagents. CCK-33 contains three primary amino groups located at positions Lys₉, or Lys₁₅ (2). To radioiodinate CCK-33, we used the ¹²⁵I-labeled Bolton-Hunter reagent which upon reaction with the peptide acylates only free amino groups with retention of secretagogue activity (8). This type of radioiodination is a necessary requirement, since radioiodination of CCK-33 by conventional oxidative techniques results in a marked reduction of biologic activity (39). Because of this initial derivatization, it was essential to determine whether the ¹²⁵I-CCK-33 so prepared still possessed a free amino group capable of interacting with chemical cross-linking reagents. For this reason, model cross-linking studies were carried out using ¹²⁵I-CCK-33 and antibodies directed against its COOH-terminal octapeptide (CCK-8).

As illustrated in Fig. 1, lane a, labeling of the heavy and light chains of anti-CCK IgG was obtained using the homobifunctional cross-linker DSS. This labeling was specific as inclusion of unlabelled CCK-8 in the reaction abolished the labeling pattern (Fig. 1, lane b). If either DSS (Fig. 1, lane c) or antibodies (Fig. 1, lanes c and d) were omitted from the reaction, labeling of the heavy and light chains was not obtained. High molecular weight material just entering the resolving gel (Fig. 1) likely represents aggregates of affinity labeled antibodies cross-linked to themselves and/or to other serum proteins. These results indicate that at least one free amino group on ¹²⁵I-CCK-33 is available for reaction with DSS. A variety of other homo- and heterobifunctional cross-linking agents were tested and gave identical results (S. A. Rosenzweig, unpublished results).

**Localization of ¹²⁵I-CCK-33 in Pancreatic Acinar Cells**

As indicated above, ¹²⁵I-CCK-33 contains at least one primary amino group available for potential cross-linking to cells with glucaraldehyde. It was therefore of interest to determine the initial site of interaction of CCK with its receptor on rat pancreatic acinar cells by light and electron microscopic autoradiography. Fig. 2a is a representative light microscopic autoradiograph of pancreatic acini incubated for 15 min at 37°C with ¹²⁵I-CCK-33 prior to glucaraldehyde fixation. It was found that the autoradiographic grains primarily delineated the basal and lateral boundaries of the acinar cells with some grains located over the cytoplasm at this time. Grains did not appear to be concentrated over the apical zones of the acinar cells although the centroacinar lumen is accessible to tracers as large as wheat germ ferritin (34). Fig. 2b demonstrates the low level of nonspecific labeling obtained in a similar preparation incubated with unlabeled CCK-8 in the presence of ¹²⁵I-CCK-33. Similar results were obtained at the light microscopic level with acini labeled with ¹²⁵I-CCK-33 for 2 min at 37°C.

**FIGURE 1** Autoradiograph of affinity-labeled anti-CCK antibodies. Rabbit anti-CCK antiserum was incubated with 0.07 nM ¹²⁵I-CCK-33 for 24 h at 4°C followed by cross-linking with 1 mM DSS. Following quenching with 20 mM Tris, pH 7.4, antibodies were recovered by addition of heat-inactivated, formalin-fixed Staphylococcus aureus cells and centrifugation. The pellets were processed for electrophoresis under reducing conditions as described in the text. Samples run in lanes c and d contained buffer only; lane e, control without DSS. (H) Heavy chain of IgG. (L) Light chain of IgG. (Ab) Anti-CCK antibody.
FIGURE 2  (a) A representative light microscopic autoradiograph of rat pancreatic acini labeled for 15 min at 37°C with ~0.1 nM $^{125}$I-CCK-33. Note that the autoradiographic grains delineate primarily the basal and lateral boundaries of the acinar cells (arrows) with some label present over the cytoplasm at this time. Label does not appear to be concentrated over the apical zones of the acinar cells (arrowheads).  (b) A control preparation showing the low level of nonspecific labeling when incubations were carried out in the presence of 1 nM CCK-8. Bars, 10 μm. × 700.

| Experimental condition | Grains/μm² or % total grains (in parentheses) | Relative grain density % or % total grains (in parentheses) | No. of grains counted |
|------------------------|-----------------------------------------------|-------------------------------------------------|---------------------|
|                         | Apical PM Lateral PM Basal PM Nucleus Cytoplasm Embedding medium |                                                                 |                     |
| 2 min, 37°C $^{125}$I-CCK-33 | 0 ± 0 (0 ± 0) 1.10 ± 0.05 2.57 ± 0.08 0.12 ± 0.02 0.45 ± 0.02 0.33 ± 0.02 | 4 (3.0 ± 0.9) 12.1 ± 0.4 (48.4 ± 1.0) 0.8 ± 0.1 (34.0 ± 1.3) (4.7 ± 0.3) | 678                 |
| 2 min, 37°C $^{125}$I-CCK-33 + 1 μM CCK-8 | 0 ± 0 (0 ± 0) 0.02 ± 0.01 0 ± 0 0.88 ± 0.19 0.98 ± 0.05 0.44 ± 0.10 | (7.3 ± 1.4) (76.8 ± 3.6) (13.1 ± 3.7) | 26                  |
| 15 min, 37°C $^{125}$I-CCK-33 | 0.11 ± 0.02 (0.1 ± 0.1) 2.32 ± 0.07 2.46 ± 0.11 0.72 ± 0.13 0.45 ± 0.01 0.65 ± 0.17 | (25.5 ± 0.6) (34.8 ± 0.6) (1.5 ± 0.1) (30.0 ± 0.8) (8.1 ± 0.6) | 645                 |
| 15 min, 37°C $^{125}$I-CCK-33 + 1 μM CCK-8 | 0 ± 0 (0 ± 0) 0.04 ± 0.01 0.08 ± 0.01 0.44 ± 0.08 0.78 ± 0.03 0.42 ± 0.07 | (4.4 ± 0.9) (5.8 ± 0.7) (6.7 ± 1.1) (58.1 ± 2.8) (15.0 ± 2.2) | 53                  |

Data are based on grain counts and morphometric analysis of 17 and 9 micrographs, respectively, for the experimental and control conditions at the 2-min time-point and 18 and 12 micrographs, respectively, for the experimental and control conditions at the 15-min time-point. Results are expressed as means ± SEM.

* Morphometric analysis of the micrographs forming the data base for Table I indicates that the apical plasmalemma accounts for 4% of the surface area of the total acinar cell plasmalemma, with the basal and lateral plasmalemmal domains accounting for 40% and 56%, respectively, of the remainder. The nucleus represents 9% of the total acinar cell volume, similar to that reported for the guinea pig pancreas in situ (40). The nucleus, cytoplasm, and embedding medium comprise 7.7%, 75.2%, and 17.6% of the surface area, respectively, of all micrographs analyzed.

† Relative grain densities are not significantly different from each other at the 99% confidence levels as tested using one-way analysis of variance.

TABLE I

| Distribution of Autoradiographic Grains |
|----------------------------------------|
| Experimental condition |
| | Grains/μm² or % total grains (in parentheses) | Relative grain density % or % total grains (in parentheses) | No. of grains counted |
|----------------------------------------|-------------------------------------------------|---------------------|
| 2 min, 37°C $^{125}$I-CCK-33 | 0 ± 0 (0 ± 0) 1.10 ± 0.05 2.57 ± 0.08 0.12 ± 0.02 0.45 ± 0.02 0.33 ± 0.02 | 4 (3.0 ± 0.9) 12.1 ± 0.4 (48.4 ± 1.0) 0.8 ± 0.1 (34.0 ± 1.3) (4.7 ± 0.3) | 678                 |
| 2 min, 37°C $^{125}$I-CCK-33 + 1 μM CCK-8 | 0 ± 0 (0 ± 0) 0.02 ± 0.01 0 ± 0 0.88 ± 0.19 0.98 ± 0.05 0.44 ± 0.10 | (7.3 ± 1.4) (76.8 ± 3.6) (13.1 ± 3.7) | 26                  |
| 15 min, 37°C $^{125}$I-CCK-33 | 0.11 ± 0.02 (0.1 ± 0.1) 2.32 ± 0.07 2.46 ± 0.11 0.72 ± 0.13 0.45 ± 0.01 0.65 ± 0.17 | (25.5 ± 0.6) (34.8 ± 0.6) (1.5 ± 0.1) (30.0 ± 0.8) (8.1 ± 0.6) | 645                 |
| 15 min, 37°C $^{125}$I-CCK-33 + 1 μM CCK-8 | 0 ± 0 (0 ± 0) 0.04 ± 0.01 0.08 ± 0.01 0.44 ± 0.08 0.78 ± 0.03 0.42 ± 0.07 | (4.4 ± 0.9) (5.8 ± 0.7) (6.7 ± 1.1) (58.1 ± 2.8) (15.0 ± 2.2) | 53                  |

Data are based on grain counts and morphometric analysis of 17 and 9 micrographs, respectively, for the experimental and control conditions at the 2-min time-point and 18 and 12 micrographs, respectively, for the experimental and control conditions at the 15-min time-point. Results are expressed as means ± SEM.

* Morphometric analysis of the micrographs forming the data base for Table I indicates that the apical plasmalemma accounts for 4% of the surface area of the total acinar cell plasmalemma, with the basal and lateral plasmalemmal domains accounting for 40% and 56%, respectively, of the remainder. The nucleus represents 9% of the total acinar cell volume, similar to that reported for the guinea pig pancreas in situ (40). The nucleus, cytoplasm, and embedding medium comprise 7.7%, 75.2%, and 17.6% of the surface area, respectively, of all micrographs analyzed.

† Relative grain densities are not significantly different from each other at the 99% confidence levels as tested using one-way analysis of variance.
FIGURE 3  (a) An electron microscopic autoradiograph of a rat pancreatic acinus labeled with ~0.1 nM $^{125}$I-CCK-33 for 15 min at 37°C. The majority of the autoradiographic grains in this micrograph is localized to the lateral plasmalemma (apposed arrows) and the basal plasmalemma (arrows). (b) The corresponding control preparation incubated with $^{125}$I-CCK-33 in the presence of 1 mM unlabeled CCK-8. (L) Acinar lumen. Bars, 2 μm. × 8,000.
percentage of total grains counted, it is clear that at both time points ~60% of the autoradiographic grains were localized within ±1 HD of the lateral and basal plasmalemma, the remainder being randomly distributed over the cytoplasm (~30%), the nucleus (1–2%), or the embedding medium (5–8%). When expressed as grains/μm² of plasmalemma, it is evident that the grains over the basolateral plasmalemma were highly concentrated relative to those over the apical plasmalemma.

When we assume a specific radioactivity of the labeled CCK-33 of 500–1,000 Ci/mmol, we can estimate, using the formula given by Fertuck and Salpeter (38), that each acinar cell possesses, at a minimum, 5,000–10,000 CCK-binding sites using a subsaturating concentration of hormone (0.1 nM). This calculation also assumes that the combined basal and lateral plasmalemma of the rat pancreatic acinar cell is ~580 μm², similar to that of the guinea pig pancreatic acinar cell (40). Our estimates of the number of receptor sites for CCK in the rat pancreatic acinar cell are consistent with the estimates of receptor number published by Jensen et al. (10) for the guinea pig pancreatic acinar cell but are at variance with those of Sankaran et al. (9) whose data indicate that the rat pancreatic acinar cell possesses approximately 650 high affinity and 100,000 low affinity binding sites per cell. The discrepancy between our data and those of the latter authors is presently not understood.

When the autoradiographic grains over the cytoplasm and nucleus of the acinar cell are expressed as relative grain density (i.e., percentage of autoradiographic grains divided by the respective volume density as a percentage), it is clear that at either time point the relative grain density never exceeded unity, indicating an absence of concentration of label over these compartments. In fact, the relative grain densities over these compartments and over the embedding medium did not differ significantly from each other. The distribution of autoradiographic grains over the control preparations showed no particular pattern and is noteworthy for the low level of labeling of the plasmalemma consistent with the low levels of nonspecific binding (~10%) of CCK-33 to acini in the presence of the potent competitive inhibitor, CCK-8 (data not shown).

Finally, we should mention that our autoradiographic studies showed no apparent specific binding sites for ¹²⁵I-CCK-33 to centroacinar or ductular cells nor were binding sites detectable on endocrine islet cells that occasionally contaminate our acinar cell preparation. Acinar cells that form a halo around endocrine islets bound CCK-33 in amounts qualitatively similar to nonperiinsular acinar cells.

**Affinity Labeling of Pancreatic Plasma Membranes**

Fig. 4 shows an autoradiograph of an SDS gel in which pancreatic membranes cross-linked to ¹²⁵I-CCK-33 with DSS were run under reducing or nonreducing conditions. A major component of M₉, 85,000 and minor components of M₉, 130,000–140,000 and M₉, 55,000 were seen to be labeled by the radioligand (Fig. 4, lane a). Species of M₉ >200,000 were also heavily labeled and likely consist of cross-linked heteropolymers of the CCK-binding site and other membrane proteins or of homopolymers of the M₉, 85,000 CCK-binding site. When incubations were carried out in the presence of excess unlabeled CCK-8 (Fig. 4, lane b), labeling of membrane proteins was abolished indicating that they were specifically labeled. The labeling of membrane proteins by ¹²⁵I-CCK-33 was unaffected by the presence of micromolar concentrations of insulin, secretin, or bombesin (S. A. Rosenzweig, unpublished results).

When cross-linked membranes were electrophoresed under nonreducing conditions (Fig. 4, lane c), the major M₉, 85,000 component observed under reducing conditions (Fig. 4, lane a) showed no shift in apparent molecular weight. This suggests that it does not contain intramolecular disulfide bonds for otherwise its apparent molecular weight should decrease under nonreducing conditions (33). An increase in the radioactivity associated with the M₉, 130–140,000 region was noted in gels run under nonreducing conditions, suggesting that the M₉, 85,000 species may be part of larger disulfide-linked complexes in the intact membrane. It should be noted that preparation of membranes in the absence of 2-mercaptoethanol did not alter the pattern of affinity-labeled proteins (data not shown), indicating that we were not reducing disulfide bonds in native binding sites using our standard isolation procedure.

The effect of varying the concentration of DSS on the affinity labeling of rat pancreatic plasma membranes is shown in Fig. 5. When DSS was omitted from the affinity labeling reaction, there were no labeled proteins detectable in the autoradiograph (Fig. 5). Maximal cross-linking of ¹²⁵I-CCK-33 to membrane proteins was obtained with 50 μM DSS. The efficiency of cross-linking decreased at DSS concentrations >50 μM. At these higher concentrations, DSS may be working
FIGURE 5 Effect of varying the DSS concentration on $^{125}$I-CCK-33 cross-linking to membranes. $^{125}$I-CCK-33 (0.25 nM) was incubated with pancreatic plasma membranes as described. Peptide bound to membranes was cross-linked to its binding sites with 0.01 to 0.5 mM DSS. The final pellets obtained were analyzed by electrophoresis on a 10% polyacrylamide SDS slab gel under reducing conditions. Shown in the left panel is the Coomassie Blue-staining pattern of the gel. The right panel shows the autoradiograph of the same gel. The arrow indicates the position of the $M_r$ 85,000 protein.

as a monofunctional cross-linker due to reagent excess in the presence of a limited number of available NH$_2$ groups.

Fig. 6 shows the effect of increasing the concentration of unlabeled CCK-8 on the affinity labeling of membranes. The upper panel shows the effect of increasing the concentration of CCK-8 on total binding of $^{125}$I-CCK-33 to membranes including both cross-linked and noncross-linked $^{125}$I-CCK-33. As <5% of the peptide is released from binding sites during cross-linking and subsequent washing, the total membrane-associated counts are representative of $^{125}$I-CCK-33 bound prior to cross-linking. 50% inhibition of binding was attained with 9 nM CCK-8. The middle panel of Fig. 6 shows that as the concentration of CCK-8 was increased, there was a commensurate decrease in the affinity labeling of specific membrane proteins. Quantitation of the affinity-labeled $M_r$ 85,000 protein itself was assessed by cutting bands of this molecular weight region from the gel and counting them. The results indicate that the concentration of CCK-8 causing half-maximal inhibition of affinity labeling of the $M_r$ 85,000 protein was 9 nM (Fig. 6, lower panel). The curves shown in Fig. 6 are superimposable suggesting that the binding of $^{125}$I-CCK-33 to membranes is a valid reflection of its interaction with the $M_r$ 85,000 protein.

The reproducibility of affinity labeling of the $M_r$ 85,000 component was investigated by using several different hetero- and homobifunctional cross-linking reagents. When ethylene glycol bis(succinimidyl succinate) (EGS), a homobifunctional reagent having a structure similar to DSS, was used in cross-linking experiments, a labeling pattern similar to that produced by DSS was obtained (Fig. 7). Dimethyl suberimidate (DMS), a homobifunctional imidoester (41, 42), was found to be a very inefficient cross-linker in these experiments, although its specificity for reaction (i.e., free amino groups) is similar to DSS. Nonetheless, it did effect the specific labeling of a $M_r$ 85,000 component in rat pancreatic plasma membranes (Fig. 7). Similarly, when the heterobifunctional photoaffinity cross-linking reagent N-hydroxysuccinimidy1-4-azidobenzoate (HSAB; 43) was employed, the $M_r$ 85,000 component was the major membrane species to be specifically labeled.

DISCUSSION

Pancreatic plasma membranes contain a protein of apparent $M_r$ 85,000 that is specifically labeled by $^{125}$I-CCK-33 in conjunction with chemical cross-linking reagents. It should be noted that this molecular weight estimation includes the mass of the covalently attached $^{125}$I-CCK-33 ($M_r$ 4,210). Assuming a stoichiometry of 1:1, the mass of the native membrane binding protein is ~81,000 daltons. Our results further indicate that this protein lacks intramolecular disulfide bonds since it did not exhibit a shift in its electrophoretic mobility in gels run under reducing or nonreducing conditions.
FIGURE 6 Competitive inhibition by CCK-8 on $^{125}$I-CCK-33 binding and cross-linking to pancreatic plasma membranes. Equal aliquots of rat pancreatic plasma membranes were incubated with $^{125}$I-CCK-33 (0.25 nM) and 0–100 nM CCK-8. The upper panel shows total membrane-associated $^{125}$I-CCK-33 following binding and cross-linking with DSS (50 μM). The middle panel shows the autoradiographic pattern of affinity-labeled proteins derived from membranes shown in the upper panel and run under reducing conditions (0.1 M DTT). The lower panel illustrates the radioactivity covalently associated with the Mr 85,000 protein obtained by cutting the bands from the gel (middle panel) and counting them in a γ-counter. The arrow indicates the Mr 85,000 protein.

Additional affinity labeled components of Mr 55,000, Mr 130,000–140,000, and Mr >200,000 were also observed. The Mr 55,000 component may consist of either a proteolytically cleaved form of the Mr 85,000 protein or another membrane protein close enough to the hormone-receptor complex to become cross-linked to receptor-bound $^{125}$I-CCK-33 by DSS. The Mr 130,000–140,000 component and the Mr >200,000 component may comprise a mixture of heteropolymers of membrane proteins that are closely apposed to the CCK receptor such that they become cross-linked to the affinity labeled binding site by DSS or consist of homopolymers of the Mr 85,000 species. Because the labeling intensity of the Mr 130,000–140,000 species was seen to increase in samples run under conditions.
nonreducing conditions, it likely may represent a disulfide linked complex of the \( M_t \), 85,000 component with another membrane protein. Whether proteins with \( M_t \) >85,000 are directly involved in hormone recognition, binding, or action or represent different functional states of the receptor (44) is not known.

Using ultraviolet irradiation as a means of cross-linking \( ^{125}\text{I}-\text{CCK-33} \) to its binding sites, Svoboda et al. (45) recently described the specific labeling of a \( M_t \), 76,000 polypeptide in rat pancreatic plasma membranes. Given the variability of molecular weight determination by SDS gel electrophoresis, it is likely that the \( M_t \), 85,000 component we describe in the present paper is similar if not identical to this \( M_t \), 76,000 protein. These investigators (45) further suggest that this component is part of larger molecular weight disulfide-linked complexes (\( M_t \), 96,000 and \( M_t \) >200,000) in native membranes as it is not detected in gels run under nonreducing conditions. The difference between our results and those of Svoboda et al. (45) may be due to the different methodologies used to identify CCK-binding sites.

Previous reports on the CCK receptor in rat pancreatic acini have described the existence of two classes of binding sites. These two classes are distinguishable by dissociation constants of 64 pM (high affinity sites) and 21 nM (low affinity sites) based on Scatchard analysis (9). In guinea pig pancreatic acini, binding assays suggest a \( K_d \) of 0.6 nM using CCK-8 as competitor (10). Studies employing crude rat pancreatic membranes have revealed a single class of CCK-binding sites with a dissociation constant of 1.35 nM (46) or 0.5 nM (11). In the competitive displacement experiment shown in Fig. 6, a \( K_d \) value of \~9 nM was obtained. This value most closely corresponds to the low affinity CCK-binding site reported in rat pancreatic acini (9), suggesting that the \( M_t \), 85,000 component may be the low affinity binding site. The dissociation constant obtained in the present study is about one order of magnitude larger than that of the single class of receptors described in isolated pancreatic membranes (11). This apparent discrepancy can be explained in part by the higher concentration of \( ^{125}\text{I}-\text{CCK-33} \) used in the present experiments (250 pM) compared to that used by others in acini (2.5 pM [9]; 25 pM [10]) or membranes (15–50 pM; see references 11, 46). The use of larger amounts of \( ^{125}\text{I}-\text{CCK-33} \) was necessary in order to obtain a signal detectable by autoradiography of the dried gels. It is not possible to ascertain from our studies, however, whether the high and low affinity sites represent distinct molecular entities or whether they correspond to two different conformational states of a single protein. The latter case would yield similar affinity-labeled component(s) when analyzed by gel electrophoresis.

Light and electron microscopic autoradiographic localization of \( ^{125}\text{I}-\text{CCK-33} \) in intact pancreatic acini indicated that CCK bound to the basal and lateral plasmalemma of the acinar cell with few, if any, autoradiographic grains being associated with the apical plasmalemma. Recently, after the work reported here was completed, Williams et al. (47) also showed by electron microscopic autoradiography that CCK receptors are localized to the basolateral plasmalemma of mouse pancreatic acini.

While it could be argued that the autoradiographic grains associated with the plasmalemma may be derived from degradation products of \( ^{125}\text{I}-\text{CCK-33} \), the studies of Sankaran et al. (9) and Williams et al. (47), who used CCK-33 iodinated in the same manner as was done by us, indicate that no \~5% of the peptide should be hydrolyzed over a 15-min incubation at 37°C. Additionally, as the iodinated sites on CCK-33 are limited to the biologically inactive NH\(_2\)-terminus of the peptide, then binding of \( ^{125}\text{I} \)-labeled degradation products should not be competed for by CCK-8 in control preparations. Finally, qualitatively similar localization of label to the basolateral plasmalemma was observed in acini incubated for 2 or 15 min at 4°C where degradation of \( ^{125}\text{I}-\text{CCK-33} \) should be minimal (data not shown).

The localization of CCK receptor sites to the basolateral plasmalemma of the pancreatic acinar cell is consistent with the assumption that in this highly polarized epithelial cell, secretagogues arrive from the circulation in situ to interact with this plasmalemmal domain and there initiate the chain of events leading to exocytosis at the apical pole—events separated in space and time. A similar distribution of insulin receptors on the rat pancreatic acinar cell has been reported by Bergeron et al. (48) and by Goldfine et al. (49) in situ and in vitro, respectively. Receptors for peptide hormones and other ligands have also been demonstrated on the sinusoidal membrane of the hepatocyte (50–52) which is topologically equivalent to the basolateral plasmalemma of the pancreatic acinar cell. Our studies point out again the biochemically unique properties of the apical and basolateral domains of polarized epithelial cells (53–55) including the pancreatic acinar cell where we have shown qualitative differences in the distribution of cell surface glycoconjugates between these two domains (56).

Although our studies were designed to determine the initial site of interaction of CCK with its receptor(s) on the acinar cell, we were surprised that no statistically significant increase in relative grain density was detected over the cytoplasm or nucleus of the acinar cell compared to that of the embedding medium at the 15-min experimental time point since radio-labeled peptide hormones have been reported to be internalized in times \~15 min in other systems (57). Similarly, Williams et al. (47) have presented evidence which shows that, with the exception of a modest accumulation of label in multivesicular bodies, there is no specific concentration of label over intracellular organelles in mouse pancreatic acini exposed for 30 min at 37°C to \( ^{125}\text{I}-\text{CCK-33} \). A possibility to explain our results is that \( ^{125}\text{I}-\text{CCK-33} \) is indeed internalized but as the radio-label is located on the biologically inactive amino terminal of the molecule, it may be proteolytically removed leaving the bio- logically active, nonradiolabeled COOH-terminus.

In conclusion, our data suggest that the initial site of interaction of CCK-33 (and likely of CCK-8) with the pancreatic acinar cell is the basolateral plasmalemma and that the minimum molecular weight for the CCK-binding site is approximately 81,000.

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Note Added in Proof: Sakamoto et al. (Sakamoto, C., J. A. Williams, K. Y. Wong, and I. D. Goldfine, 1983, The CCK receptor on pancreatic plasma membranes: binding characteristics and covalent cross-linking, FEBS (Fed. Eur. Biochem. Soc.) Lett., 151:63–66) have recently re-

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ported that the \( M_i \) of CCK binding sites in mouse pancreatic plasma membranes is \(-80,000\).

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