Dual Pathways of Internalization of the Cholecystokinin Receptor

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Abstract. Receptor molecules play a major role in the desensitization of agonist-stimulated cellular responses. For G protein–coupled receptors, rapid desensitization occurs via receptor phosphorylation, sequestration, and internalization, yet the cellular compartments in which these events occur and their interrelationships are unclear. In this work, we focus on the cholecystokinin (CCK) receptor, which has been well characterized with respect to phosphorylation. We have used novel fluorescent and electron-dense CCK receptor ligands and an antibody to probe receptor localization in a CCK receptor-bearing CHO cell line. In the unstimulated state, receptors were diffusely distributed over the plasmalemma. Agonist occupation stimulated endocytosis via both clathrin-dependent and independent pathways. The former was predominant, leading to endosomal and lysosomal compartments, as well as recycling to the plasmalemma. The clathrin-independent processes led to a smooth vesicular compartment adjacent to the plasmalemma resembling caveolae, which did not transport ligand deeper within the cell. Potassium depletion largely eliminated clathrin-dependent endocytosis, while not interfering with agonist-stimulated receptor movement into subplasmalemmal smooth vesicle compartments. These cellular endocytic events can be related to the established cycle of CCK receptor phosphorylation and dephosphorylation, which we have previously described (Klueppelberg, U. G., L. K. Gates, F. S. Gorelick, and L. J. Miller. 1991. *J. Biol. Chem.* 266:2403- 2408; Lutz, M. P., D. I. Pinon, L. K. Gates, S. Shenolikar, and L. J. Miller. 1993. *J. Biol. Chem.* 268:12136-12142). The rapid onset and peak of receptor phosphorylation after agonist occupation correlates best with a plasmalemmal localization, while stimulated receptor phosphatase activity correlates best with receptor residence in intracellular compartments. We postulate that the smooth vesicular compartment adjacent to the plasmalemma functions for the rapid resensitization of the receptor, while the classical clathrin-mediated endocytotic pathway is key for receptor downregulation via lysosomal degradation, as well as less rapid resensitization.

DESENSITIZATION is a ubiquitous phenomenon in which a cell attenuates its response to prolonged or excessive agonist stimulation, and thereby protects itself from damage. An important mechanism for this occurs at the most proximal step in cell signalling, at the level of the receptor itself. Mechanisms of receptor desensitization have been particularly well explored for receptors in the G protein–coupled receptor family, such as the adrenergic receptor (6, 17). Rapid desensitization may occur as a result of receptor phosphorylation interfering with its coupling to a G protein, and by sequestration and internalization of receptors into intracellular compartments. Longer-term desensitization may arise from receptor downregulation with a net loss of binding sites in the cell (6). While the biochemical events occurring at the level of the plasma membrane have been well established, the proximal intracellular pathways and compartments involved in the sequestration and internalization of G protein–coupled receptors are less well understood (54).

Different pathways for internalization of G protein–coupled receptors have been observed. The adrenergic (53, 55), luteinizing hormone (14), and thrombin receptors (19) are internalized by the classical endocytic pathway initiated at clathrin-coated pits, which is best described for LDL, transferrin, and EGF receptors (51). Other studies have demonstrated that adrenergic and muscarinic cholinergic receptors are internalized into smooth vesicles as components of a distinct pathway (43). Both routes of internalization have been described to lead to early endosomes. It is not clear whether distinct endocytic pathways are used by G protein–coupled receptors in different cell types, or whether both pathways may be operating in a given cell type. If the latter is the case, it is not clear what distinct functionality may be present for the different pathways.

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In this work, we have focused on the receptor for cholecystokinin (CCK)[1], a peptide hormone involved in the physiologic stimulation of pancreatic exocrine secretion, gallbladder contraction, gastrointestinal motility, and possibly satiety (36). Type A CCK receptors are present on the pancreatic acinar cell, smooth muscle within the gallbladder muscularis, and at multiple levels of the digestive tract, as well as in the enteric nervous system and on discrete brain nuclei (33). Based on cDNA sequencing, these receptors belong to the family of G protein–coupled receptors, and they are clearly related in structure to the β-adrenergic receptor and rhodopsin (48). Agonist occupation of the CCK receptor activates phospholipase C, leading to inositol phospholipid hydrolysis, generation of diacyl glycerol, activation of protein kinase C, and increased intracellular calcium (12).

The CCK receptor is a particularly interesting target for cell biological assessment of desensitization since extensive data exist relative to its phosphorylation, as well as the kinases and phosphatases acting on it (13, 20, 31, 58). Phosphorylation has been a consistent central theme in desensitization processes (25). Desensitization of cellular responses to CCK stimulation has been demonstrated in pancreatic acinar cells (1, 38), hippocampal neurons (7), chief cells (8), and a pituitary cell line (46). The cellular mechanisms involved, however, have not been well established. In the present study, we report results of morphological characterization of the dynamic processes of sequestration, internalization, and recycling of the CCK type A receptor expressed in a CHO cell line. We demonstrate the compartmentalization of the agonist-occupied CCK receptor into both coated and smooth vesicles near the plasmalemma. These distinct subpopulations of vesicles appear to represent separate and distinct endocytic processes with differing intracellular destinations, which can be manipulated independently in the cell.

Materials and Methods

Reagents

Bovine serum albumin Cohn fraction V was purchased from Intergen Co. (Purchase, NY). Soybean trypsin inhibitor and collagenase were from Worthington Biochemical Corp. (Freehold, NJ). 5-6 carboxytetramethyl rhodamine succinimide was purchased from Molecular Probes, Inc. (Eugene, OR). Sulfosuccinimidyl-4 (*p*-maleimidophenyl) butyrate (SMPB) was purchased from Pierce Chemical Co. (Rockford, IL). Tissue culture supplies were from GIBCO BRL (Gaithersburg, MD), except for plasticware, which was purchased from Falcon Plastics (Becton Dickinson, Oxnard, CA). Cycloheximide was purchased from Sigma Immunochemicals (St. Louis, MO), and EM grade paraformaldehyde was from Electron Microscopy Sciences (Ft. Washington, PA). Fluorescein-conjugated goat anti-rabbit antibody was from Tago, Inc. (Burlingame, CA). Colloidal gold was from Amersham Life Sciences (Fairlawn, NJ). All chemicals were reagent grade.

Biological Activity Assay

The ability of CCK analogues to stimulate dispersed rat pancreatic acini to secrete amylase was used as an indication of biological activity. For this, dispersed acini were prepared by sequential enzymatic and mechanical dissociation of pancreatic tissue from male Sprague-Dawley rats weighing 125–150 g, as described by Lutz et al. (32). Incubations with agonists and amylase assays were performed as we described previously (32, 34). All procedures involving animals were approved by the Mayo Clinic Animal Care and Use Committee.

1. *Abbreviations used in this paper*: CCK, cholecystokinin; CCKR, CCK receptor; SMPB, sulfosuccinimidyl-4(p-maleimidophenyl butyrate).

Receptor-expressing CHO Cell Line

A CHO cell line expressing ~120,000 rat CCK-A receptors per cell (CHO-CCKR) was used in this work (Hadac, E., B. F. Roettger, and L. J. Miller, unpublished observation). Receptors on this cell line demonstrate appropriate binding affinity and specificity, and they are appropriately coupled to intracellular signalling machinery as demonstrated by a CCK-stimulated intracellular calcium response (Hadac, E., B. F. Roettger, and L. J. Miller, unpublished observation). Cells were grown in Ham's F-12 medium in a 37°C humidified incubator containing 5% CO_2, and they were plated on coverslips in 24-well plates 2–3 d before microscopic observation.

Receptor-specific Probes

The CCK analogue Gly-[(Nle^{28,31})CCK 26-33] was synthesized as we previously described (42). This fully biologically active peptide was used as the core peptide for both fluorescent and electron-dense receptor probes.

Fluorescent Probe. For light level fluorescence studies, the CCK analogue was derivatized with rhodamine by reacting it with 5-6 carboxytetramethyl rhodamine succinimide. The peptide was dissolved in dimethyl formamide, neutralized with *N,N*-di-isopropylethylamine, and reacted with a twofold molar excess of the activated rhodamine for 2 h at room temperature. The reaction was monitored by HPLC, and when complete, excess unreacted fluorophore was quenched with diethylaminopropyl amine. The Rho-CCK product was purified by HPLC, and it was characterized and quantified by quantitative amino acid analysis.

Electron-dense Probe. For electron microscopy studies, Gly-[(Nle^{28,31})-CCK 26-33] was covalently attached to BSA using a SMPB spacer, and this conjugate was adsorbed to 10 nm colloidal gold (2). First, a 10-fold molar excess of SMPB was reacted for 30 min at room temperature with peptide dissolved in dimethyl sulfoxide. The reaction was then quenched with 0.1 M Tris buffer, pH 7.4, and 10 μg of BSA was added. After 30 min at room temperature, the reaction mixture was dialyzed extensively against distilled water. The product was lyophilized and stored at −20°C. To make the gold probe, the BSA-conjugated peptide was added dropwise to 10-nm colloidal gold, pH 6.5, while continuously stirring at room temperature. After 30 min, the suspension was filtered (0.45 μm) and centrifuged at 19,000 rpm (25,000 *g*) for 1 h at 4°C. The supernatant was aspirated and the pellet was dissolved in PBS (1.5 mM NaH_2PO_4, 8 mM Na_2HPO_4, 0.145 M NaCl, 0.1 mM $MgCl_2$, and 0.08 mM $CaCl_2$, pH 7.4) to yield an OD_{520} of 2.5. A control conjugate was generated in an analogous manner by adsorbing BSA to 10 nM colloidal gold. The colloidal gold reagents were always used within 5 h of synthesis because of reported relative instabilities (2).

Receptor Antibodies. A peptide corresponding to the predicted amino-terminal 15-amino acid residues of the rat pancreatic CCK receptor (MSHSPARQHLVESSR) was synthesized on an octameric multiple antigenic peptide resin (49). Antiserum was raised after subcutaneous injection in New Zealand white rabbits (Cocalico Biologicals, Reamstown, PA). The immunogen elicited an immune response to the peptide as determined by ELISA. The antiserum was purified on an affinity column with the peptide antigen attached to Sulfolink coupling gel (Pierce Chemical Co., Rockford, IL). Antibodies were eluted from the gel with 0.1 M glycine, pH 2.8.

Internalization and Recycling of CCK Receptors

Fluorescent Ligand Uptake. Immediately before labeling, CHO-CCKR cells grown on coverslips in 24-well plates were washed three times with PBS at 37°C and once at 4°C. The cells were then labeled with 10 or 50 nM Rho-CCK during a 1-h incubation at 4°C. After labeling, the coverslips for the 0 time point were washed with iced PBS and immediately placed in freshly prepared fixative (2% paraformaldehyde in PBS, pH 7.4) for 1 h at room temperature. The remaining coverslips were washed with 37°C PBS and incubated at 37°C for times ranging from 1 to 60 min, followed by similar fixation. Coverslips were then washed and mounted on slides. Acid washed specimens were prepared as above, except that the coverslips were washed twice with glycine buffer (50 mM glycine, 150 mM NaCl, pH 3.0) for 10 min at 4°C before fixation. Cells were examined using a Nikon Microphot FXA microscope equipped for epifluorescence (Frank Fryer, Huntley, IL) using a 100-W mercury lamp. Photographs were taken with a 35-mm camera using Hypertech film (Microfluor, Stony Brook, NY).

Indirect Immunofluorescence. CHO-CCKR cells grown on coverslips were washed with 37°C PBS, then incubated at 37°C with 10 nM CCK-8 for times ranging from 0 to 30 min. After rinsing with PBS, these were fixed as described above. Fixed cells were blocked for 1 h in blocking buffer (1% BSA and 0.05 M sodium azide in PBS, pH 7.4), and then exposed to purified

primary antiserum (1:20 for 2 h). Specimens were washed extensively with PBS, and then were incubated for 30 min with fluorescein-conjugated goat anti-rabbit antibody (1:50). Specimens were then washed with PBS and mounted on slides for microscopic observation. Relative intensities of fluorescent labeling were analyzed using Image v1.47 image processing and analysis software (National Institutes of Health, Bethesda, MD).

Receptor Recycling Studies. CHO-CCKR cells grown on coverslips were washed in iced PBS buffer, and they were incubated with 50 nM CCK-8 for 1 h at 4°C. Cycloheximide (150 μg/ml) was added during the final 15 min of this incubation, and it was kept in all subsequent incubations. Cells were washed with 37°C PBS and incubated at 37°C for times ranging from 0 to 60 min. At the appropriate time points, the cells were rinsed with 4°C PBS buffer, before being labeled with 50 nM Rho-CCK at 4°C for 1 h to detect CCK receptors on the cell surface. Specimens were fixed and processed for fluorescence microscopy as described above.

Perturbation of Internalization Processes

Hypertonic Media Studies. The specimens were treated in hypertonic media as described by Daukas and Zigmond (10). Briefly, CHO-CCKR cells grown on coverslips were prepared as described above for studies on uptake of fluorescent ligand, except that cells were pretreated for 10 min in either hypertonic sucrose (0.4 M sucrose in PBS, pH 7.4), or hypertonic NaCl (0.225 M NaCl in PBS, pH 7.4) medium before labeling with 10 nM Rho-CCK. The labeled cells were incubated at 37°C in the respective hypertonic medium for times ranging from 0 to 30 min. The specimens were then fixed and processed for fluorescence microscopy.

Potassium Depletion Studies. Potassium depletion experiments were conducted as described by Larkin et al. (22) with modifications according to Hansen et al. (16). Briefly, CHO-CCKR cells grown on coverslips were rinsed with potassium-free buffer (140 mM NaCl, 20 mM Hepes, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml D-glucose, pH 7.4), then hypotonically shocked by rinsing and incubating in hypotonic buffer (potassium-free buffer diluted 1:1 with distilled H_2O, pH 7.4) for 5 min. Cells were quickly washed three times in potassium-free buffer, then incubated for 15 min at 37°C in potassium-free buffer. Next, the cells were labeled with 50 nM Rho-CCK for times ranging from 1 to 30 min. Controls were conducted with 10 mM KCl added to the incubation buffers. Relative intensities of fluorescence labeling were analyzed using Image v1.47 image processing and analysis software (National Institutes of Health).

Ultrastructural Characterization of Internalization Organelles

CHO-CCKR cells were grown in suspension in serum-free medium. Cells were collected by centrifugation at 1,000 rpm for 5 min, washed with PBS at 37°C, and resuspended in iced PBS. Cells were labeled with the gold-CCK analogue complex (OD_{520} = 2.5) at a 1:12 dilution for 1 h at 4°C. After labeling, the samples were placed in a 37°C shaking water bath, incubated for 5–30 min, and then washed three times in iced PBS. Cell pellets were resuspended in paraformaldehyde-lysine-periodate (PLP) fixative (2% paraformaldehyde, 0.075 M lysine-HCl, 0.01 M sodium metaperiodate, in 0.0375 M NH_2PO_4, pH 7.4) and fixed overnight at 4°C. Specimens were postfixed in 1% osmium tetroxide, stained with 0.5% uranyl acetate, and embedded in Spurr's resin. Sections (0.1-μm) were cut on an ultra microtome and viewed on a transmission electron microscope (CM-10; Philips Electronic Instruments Co., Mahwah, NJ). Control samples were prepared as above, either adding 1 μM CCK-8 to the CCK analogue-coated gold or using the BSA-gold complex at a similar dilution.

Detection of CCK Receptors in Triton-insoluble Complexes

CHO-CCKR cells grown on flasks were scraped, pelleted, and resuspended in Krebs-Ringer's-Hepes medium (25 mM Hepes, pH 7.4, 1 mM KH_2PO_4, 104 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 0.2% [wt/vol] BSA, and 0.01% soybean trypsin inhibitor). Cellular CCK receptors were then affinity-labeled as we previously described (41). This provided a sensitive method to demonstrate and compare receptor present on the plasma membrane and that which might exist within caveolae. To isolate Triton-insoluble complexes highly enriched in caveolae, the affinity-labeled cell pellet was resuspended in 25 mM MES, pH 6.5, and 0.15 M NaCl, containing 1% Triton X-100, and sonicated for 5 s at setting 7 of a Sonifier cell disruptor (Ultrasonics, Plainview, NY). The Triton-insoluble fraction was then extracted as described by Sargiacomo et al. (46). Both this fraction and an enriched plasma membrane fraction (52) were then solubilized, and component proteins were separated on a 10% SDS-polyacrylamide gel using the method of Laemmli (21). Affinity-labeled receptor was then detected by autoradiography.

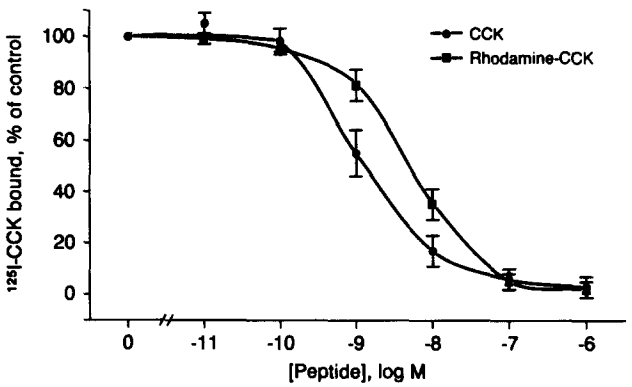

Figure 1. Competition for binding of ^{125}I-D-Tyr-Gly[(Nle^{28,31}) CCK 26-33] to pancreatic membranes by CCK-8 and Rho-CCK. Membranes were incubated with 18–22 pM radioligand plus various concentrations of CCK-8 and Rho-CCK. 100% binding represents specific binding in the absence of competing peptide. Nonspecific binding, determined in the presence of 1 μM CCK-8, represented <15% of total binding. The K_d determined using the LIGAND program (35) was 8.4 ± 2.0 nM for Rho-CCK, and 1.2 ± 0.5 nM for CCK-8. Values represent means ± SEM for three separate experiments performed in duplicate.

Results

Characterization of Receptor-specific Probes

To visualize the sequestration, internalization, and recycling of CCK receptors in receptor-bearing cells, we prepared and characterized receptor-specific probes.

Fluorescent Ligand Characterization. To validate the Rho-CCK conjugate as a physiologic ligand, we studied its binding properties and biological activity. Like native

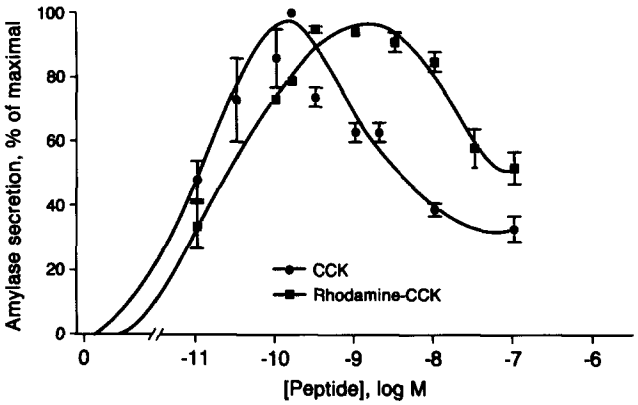

Figure 2. Amylase secretion in response to Rho-CCK and CCK-8 in dispersed rat pancreatic acini. Various concentrations of peptide were incubated with acini (3–4 × 10^6 cells) for 30 min at 37°C, and amylase release was assayed as we described (34). Values represent the means ± SEM of three separate experiments performed in duplicate.

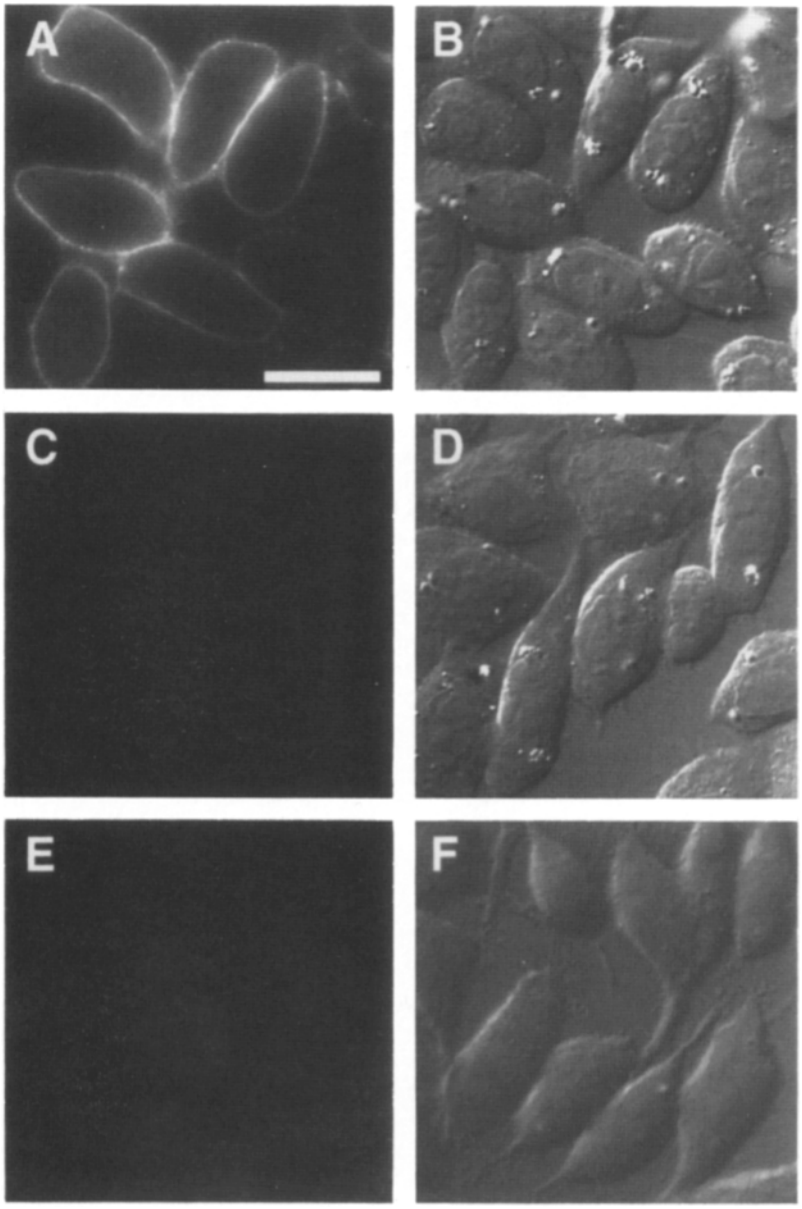

Figure 3. Specific binding of Rho-CCK to CHO-CCKR cells at 4°C. Shown are (*A*) Fluorescent and (*B*) DIC images of CHO-CCKR cells labeled for 1 h at 4°C in 50 nM Rho-CCK, (*C*) fluorescent and (*D*) DIC images of CHO-CCKR cells labeled under identical conditions in the presence of 100-fold excess of CCK-8, and (*E*) fluorescent and (*F*) DIC images of untransfected CHO cells labeled under identical conditions to *A* and *B*. Images are representative of four separate experiments. Bar, 20 μm.

CCK-8, Rho-CCK inhibited binding of the well established CCK receptor radioligand (^{125}I-D-Tyr-Gly[(Nle^{28,31})CCK 26-33] (42) to rat pancreatic membranes in a concentration-dependent manner (Fig. 1). Rho-CCK bound to CCK receptors in a high affinity and specific manner. Rho-CCK was also observed to be a fully efficacious acinar cell secretagogue, expressing the biphasic concentration-response curve typical of native CCK, although being slightly less potent (Fig. 2).

The location of CCK receptors on receptor-bearing CHO cells (CHO-CCKR cells) was evaluated using epifluorescence microscopy after Rho-CCK binding. Cells incubated at 4°C in the presence of Rho-CCK displayed a predominantly diffuse fluorescence pattern on the cell surface with occasional punctate regions (Fig. 3, *A* and *B*). Competition with 100-fold excess unlabeled CCK-8 completely displaced this signal (Fig. 3, *C–D*). The Rho-CCK signal was unaffected when binding was conducted in the presence of structurally unrelated acinar cell peptide ligands (data not shown). Untransfected CHO cells incubated with Rho-CCK under similar conditions were not fluorescently labeled (Fig. 3, *E* and *F*).

CCK Receptor Antibody Characterization. The CCK receptor antiserum specifically labeled CCK receptors on CHO-CCKR cells (Fig. 4). The receptor-specific polyclonal antibody detected CCK receptors expressed in the plasmalemma, as shown by membrane labeling in nonpermeabilized cells (Fig. 4 *A*). The labeling was present diffusely over the surface of the cell with some punctate localization. This immunolabeling was not affected by the occupation of the receptor with native hormone (data not shown). Plasma membrane–associated fluorescence was not observed in untransfected native CHO cells that were immunolabeled under the same conditions (Fig. 4 *B*). The fluorescent signal was also

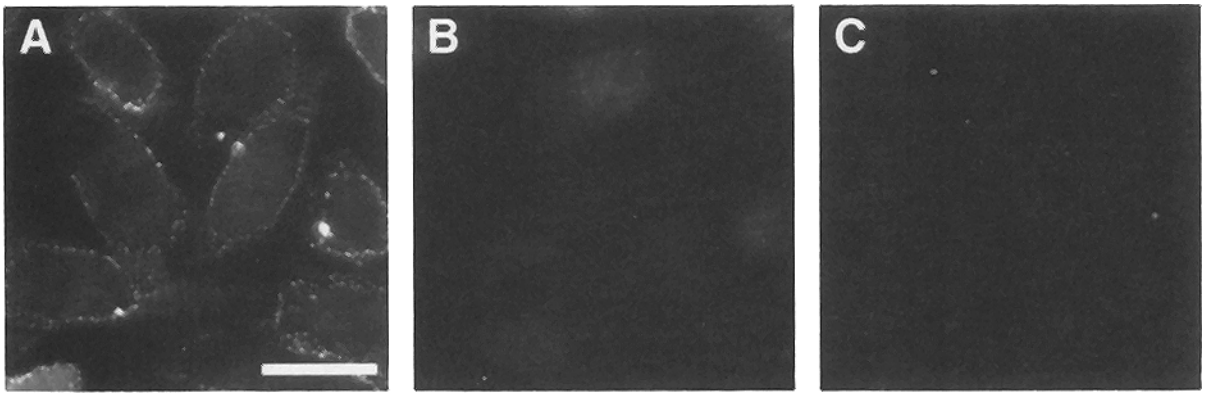

Figure 4. In situ detection of rat pancreatic CCK receptors expressed in CHO-CCKR cells by immunofluorescence. Cells were fixed in 2% paraformaldehyde then labeled with the NH_2-terminally directed CCK receptor primary antibody and fluorescein-conjugated goat anti-rabbit secondary antibody. Receptors were diffusely expressed on the plasma membrane of CHO-CCKR cells (*A*), whereas membrane labeling was not apparent on nontransfected CHO cells (*B*). The membrane immunofluorescence was fully saturable with 1 μM peptide antigen during the primary incubation (*C*). Images are representative of five separate experiments. All photographs were taken with identical exposure times. Bar, 20 μm.

eliminated on CHO-CCKR cells by carrying out the primary incubation in the presence of a competing concentration (1 μM) of the peptide immunogen (Fig. 4 *C*).

Internalization and Recycling of CCK Receptors After Agonist Occupation

We observed the binding and internalization of Rho-CCK in CHO-CCKR cells using epifluorescence microscopy. In these experiments, acidic glycine washes were used to dissociate all fluorescent ligand that was present on the cell surface after various manipulations. When cell surface receptors were occupied with fluorescent ligand by binding at 4°C, cells were diffusely labeled on the plasma membrane (Fig. 5 *A*), and this fluorescent signal was essentially eliminated by treatment with acidic glycine (Fig. 5 *E*). Within 1 min of incubation at 37°C, punctate areas of fluorescence formed at or near the plasma membrane (Fig. 5 *B*), and these were resistant to dissociation in acidic glycine (Fig. 5 *F*). By 5 min of incubation at 37°C, the fluorescent signal had moved to compartments deep within the cell (Fig. 5, *C* and *G*). After 30 min, the fluorescence localization was predominantly perinuclear, but some punctate fluorescence remained near the plasma membrane in an acid-resistant compartment (Fig. 5, *D* and *H*).

Reduction in the number of CCK receptors on the surface of the cells after agonist occupation and incubation at 37°C was demonstrated by indirect immunofluorescence microscopy using a receptor-specific antibody to the CCK receptor. The cells were observed to have substantial specific immunolabeling of the receptor on the plasmalemma at time 0 (Fig. 6 *A*). However, cells that were incubated with agonist at 37°C for 30 min showed a marked reduction in surface immunolabeling of the receptor (Fig. 6 *B*) to a level only 49% of that detected on control cells (Fig. 6 *C*).

To investigate whether CCK receptors recycle to the surface after internalization, we used Rho-CCK as a probe for surface receptor after exposing the cells to nonfluorescent native hormone. In these studies, new receptor synthesis was blocked with cycloheximide to permit observation of CCK receptors targeted back to the plasma membrane in a recycling endocytic pathway. Cell surface receptors available to bind Rho-CCK, indicated by fluorescence on the plasma membrane, were observed to decrease steadily during the first 30 min of incubation of the cells at 37°C, after receptors were saturated with nonfluorescent CCK (Fig. 7 *A–C*). Like the immunolabeling described above, this reflects clearance of agonist-coupled receptors away from the plasma membrane by sequestration and internalization. After incubation of the cells for 60 min at 37°C, surface fluorescence increased (Fig. 7 *D*) as receptors returned to the plasma membrane and were available to bind fluorescent ligand.

Perturbation of Internalization Processes

Hypertonic Medium. Treatment of cells with hypertonic medium eliminates receptor-mediated endocytosis, but not fluid-phase uptake (10, 18). To determine whether CCK receptors internalized via receptor-mediated endocytosis, we treated the CHO-CCKR cells with hypertonic buffer. This treatment did not interfere with Rho-CCK binding to surface receptor (Fig. 8 *A*). However, the fluorescent signal in hypertonically treated cells remained at or near the level of the plasma membrane even after 30 min at 37°C (Fig. 8 *B*), a time by which control cells internalized ligand to the perinuclear region (Fig. 8 *D*). Further confirming this observation, we obtained similar results when cells were treated with hypertonic salt buffer (data not shown).

Potassium Depletion. The results of the hypertonic treatment experiments suggested that CCK receptors may cluster in clathrin-coated pits, and that they may be internalized by clathrin-coated pits and vesicles. To explore this possibility, CHO-CCKR cells were subjected to potassium depletion, a treatment shown to block clathrin-dependent receptor-mediated endocytosis. This treatment has been shown to interfere with clathrin assembly into patches on the plasmalemma, thereby reducing the number and size of clathrin-coated pits on the plasmalemma (22–24). Potassium depletion did not interfere with fluorescent ligand binding to surface receptors, but this treatment virtually eliminated

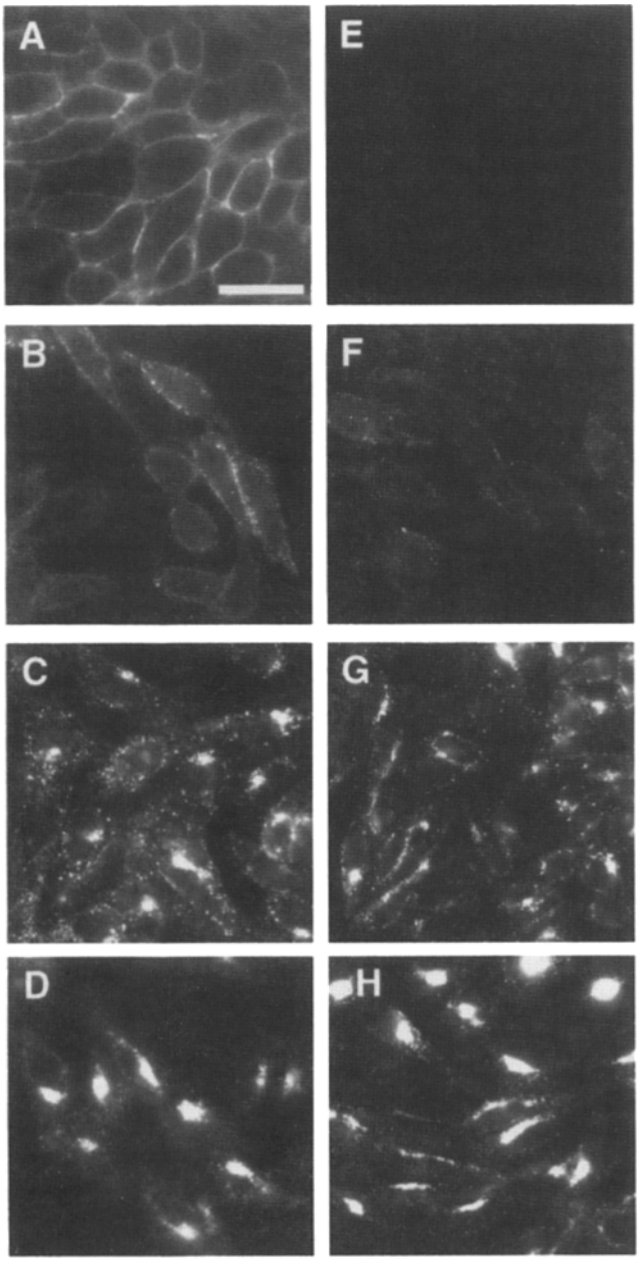

Figure 5. Internalization time course of Rho-CCK by CHO-CCKR cells. Cells were preincubated at 4°C for 1 h in 50 nM Rho-CCK, then washed with 37°C PBS and incubated at 37°C. Cells were fixed at 0, 1, 5, and 30 min either without (*A–D*) or with (*E–H*) acid washing before fixation. The cells were labeled predominantly in a diffuse pattern at the end of the preincubation period (*A*), and this fluorescent label was eliminated by acid washing (*E*). Within 1 min, the fluorescent label had changed to a punctate appearance (*B*), and a portion of the fluorescence could not be eliminated by acid washing (*F*). By 5 min, the fluorescent label was predominantly in small cytoplasmic vesicles with some label in a perinuclear location (*C*), and this label was essentially resistant to acid washing (*G*). After 30 min of incubation at 37°C, most of the fluorescent label had concentrated in the perinuclear region of the cell, but some punctate fluorescence remained near the plasma membrane (*D*) in an acid-resistant compartment (*H*). Images are representative of four separate experiments. Bar, 30 μm.

transport deep within the cell. A punctate pattern of labeling near the plasma membrane was observed as early as 1 min after addition of Rho-CCK at 37°C (Fig. 9 *A*). This pattern remained at this location after 5 min (Fig. 9 *B*) and after 30 min of incubation (Fig. 9 *C*). Control cells were also labeled with a punctate fluorescence pattern on the plasma membrane at 1 min (Fig. 9 *D*), but the predominant labeling was observed at the level of punctate structures deep within the cell by 5 min (Fig. 9 *E*). After 30 min, most of the fluorescent signal was perinuclear, with a distinct small component persisting near the plasma membrane with a punctate distribution (Fig. 9 *F*).

To determine whether the Rho-CCK labeling in the potassium-depleted cells was accessible to the extracellular medium and dissociable from receptors, specimens were washed with acidic glycine. This treatment did not eliminate the punctate fluorescent labeling pattern, suggesting that Rho-CCK was located in a vesicular compartment distinct from the clathrin pathway (Fig. 10). In a similar manner, cells pretreated with hypertonic media and labeled with Rho-CCK also retained punctate fluorescent labeling over the cell surface after acidic glycine washes (data not shown).

Ultrastructural Characterization of Internalization Organelles in CHO-CCKR Cells

The binding and internalization of ligand in CHO-CCKR cells was studied at the level of the electron microscope using the gold-CCK analogue probe. Incubation conditions with gold-CCK were analogous to those with the fluorescent Rho-CCK. After binding at 4°C, all of the colloidal gold was localized to the cell surface. 97% of this was adjacent to noninvaginated areas of the membrane, while 3% was in coated pits (n = 255).

During the internalization time course at 37°C, colloidal gold was clearly observed near the plasma membrane in coated pits or vesicles (Fig. 11, *A* and *B*) and in tubulovesicular endosomal compartments (Fig. 11, *C* and *D*). After 5 min at 37°C, the percentage of gold observed adjacent to noninvaginated regions of the plasma membrane decreased to 14%; 8% of the gold was in coated pits, 56% of the gold was within structures typical for the clathrin-mediated endocytosis pathway, and the remaining 22% of the gold was in noncoated invaginations of the plasma membrane resembling caveolae (n = 559). Gold was observed deep within the cell in perinuclear vesicular compartments resembling lysosomes at later time points (Fig. 11, *E* and *F*). Gold was not observed in any intracellular structures in control samples incubated at 37°C with gold-CCK in the presence of 1 μM CCK-8, or in cells incubated with BSA adsorbed to gold (data not shown).

A significant proportion of the gold (22%) was observed in noncoated plasma membrane invaginations resembling caveolae (Fig. 12, *A* and *B*). Coated pits not containing gold were also readily apparent in the same area of the plasma membrane, showing that the sample preparation was adequate to identify coated structures. Gold localization to noncoated structures was substantially enriched by maintaining lower intracellular levels of potassium in the cells during incubation with gold-CCK. The ligand-coated gold in these samples was localized exclusively to noncoated structures and noncoated pits near the plasma membrane (Fig. 12 *C*).

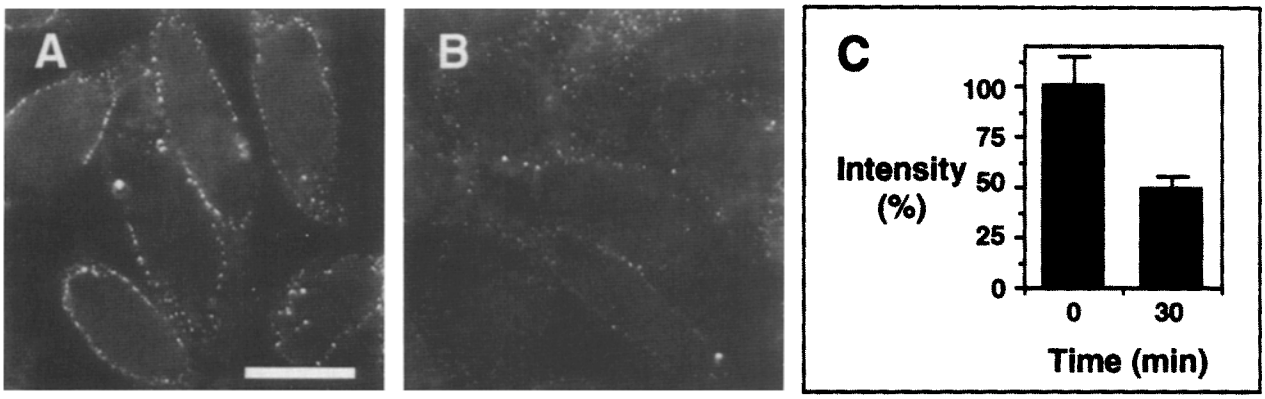

Figure 6. Agonist-occupied receptor disappearance from the plasma membrane after incubation at 37°C. Cells were fixed before or 30 min after incubation with 10 nM CCK-8 at 37°C. They were then labeled with the CCK-receptor specific antibody and fluorescein-conjugated goat anti-rabbit secondary antibody. Before exposure to hormone, receptors were readily detected on the membrane, as shown by a widespread fluorescent signal (*A*). After 30 min at 37°C, the quantity of receptor at the surface and accessible to antibody was reduced, as shown by a decreased fluorescent signal (*B*). This decrease represented 49 ± 7% of the initial signal (*C*), as quantified using Image software (National Institutes of Health). Identical exposure times were used for photographic images. Images are representative of three separate experiments. Bar, 20 μm.

These observations suggest that a minor population of agonist-occupied receptors localize to caveolae-like structures. Quantitation of fluorescence demonstrated that ≤40% of bound ligand could enter this compartment.

Detection of CCK Receptors in Triton-insoluble Complexes

The electron microscopy studies indicated that CCK receptors localize to noncoated structures resembling caveolae. To further study this possibility, caveolae-rich, Triton-insoluble complexes were isolated using the method of Sargiacomo et al. (45) from cells with their CCK receptors affinity labeled. As shown in Fig. 13, CCK receptors migrating similarly on an SDS gel with plasma membrane receptors were detected in the isolated Triton-insoluble complexes.

Discussion

In many CCK receptor-bearing cells, responses to the pep-

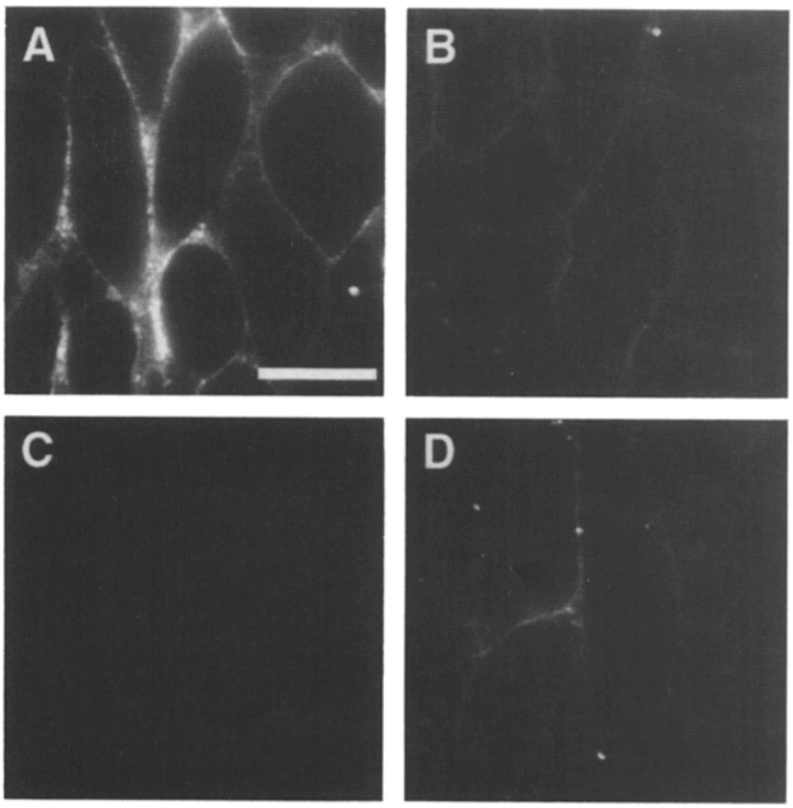

Figure 7. Visualization of receptor reappearance on the surface of CHO-CCKR cells using Rho-CCK as an indicator after receptor sequestration and internalization stimulated by nonfluorescent native hormone. Cells were preincubated in 50 nM CCK-8 for 1 h at 4°C, then warmed to 37°C. After further incubation at 37°C for noted times, cells were cooled to 4°C, and surface receptors were labeled with Rho-CCK. Cycloheximide was present during all manipulations to eliminate new receptor synthesis. Cells not warmed to 37°C displayed a typical pattern of diffuse surface fluorescence (*A*). This fluorescent labeling decreased somewhat after 15 min of incubation at 37°C (*B*), and it was markedly reduced after 30 min (*C*). The fluorescent signal increased after a 60-min incubation with hormone at 37°C, indicating that a higher concentration of ligand-binding receptors were present at the surface of the cell (*D*). Photographs were taken with identical exposure times. Images are representative of three separate experiments. Bar, 25 μm.

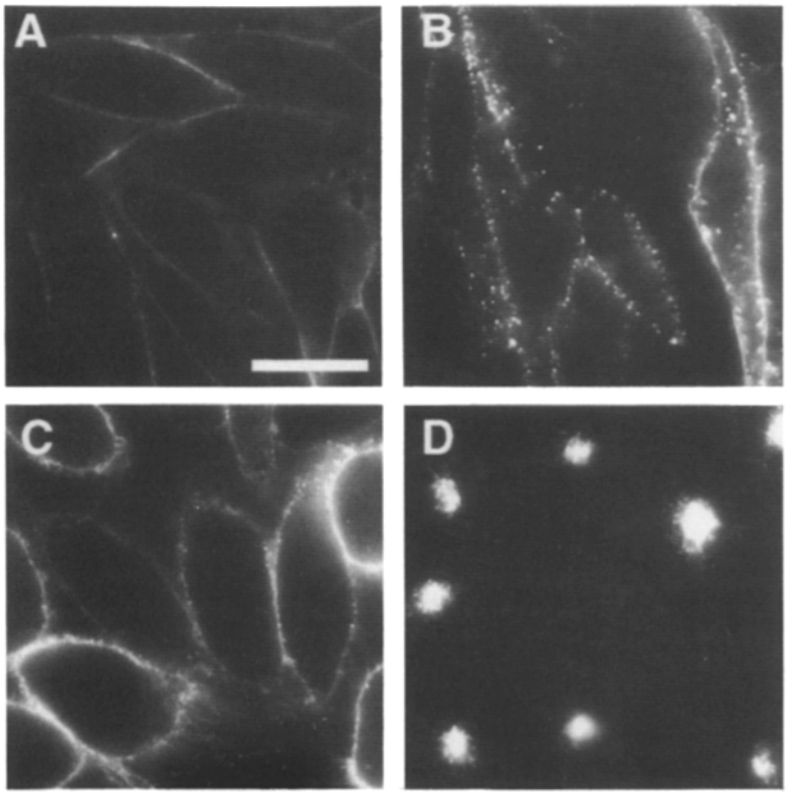

Figure 8. Inhibition of Rho-CCK internalization by incubation in hypertonic media. Cells were pretreated with hypertonic sucrose for 10 min, then preincubated with 10 nM Rho-CCK for 1 h at 4°C. Cells were then washed with 37°C hypertonic sucrose and incubated at 37°C for 0 and 30 min before being fixed. Control cells were treated identically, except that no sucrose was added to the incubation buffer. Hypertonically treated cells were diffusely labeled on the plasma membrane after the preincubation period (*A*), and the fluorescent label remained membrane associated after 30 min at 37°C (*B*). Control cells were characterized by a diffuse plasmalemmal label at the end of the preincubation period (*C*) and the typical perinuclear staining pattern after 30 min of incubation at 37°C (*D*). Images are representative of three separate experiments. Bar, 20 μm.

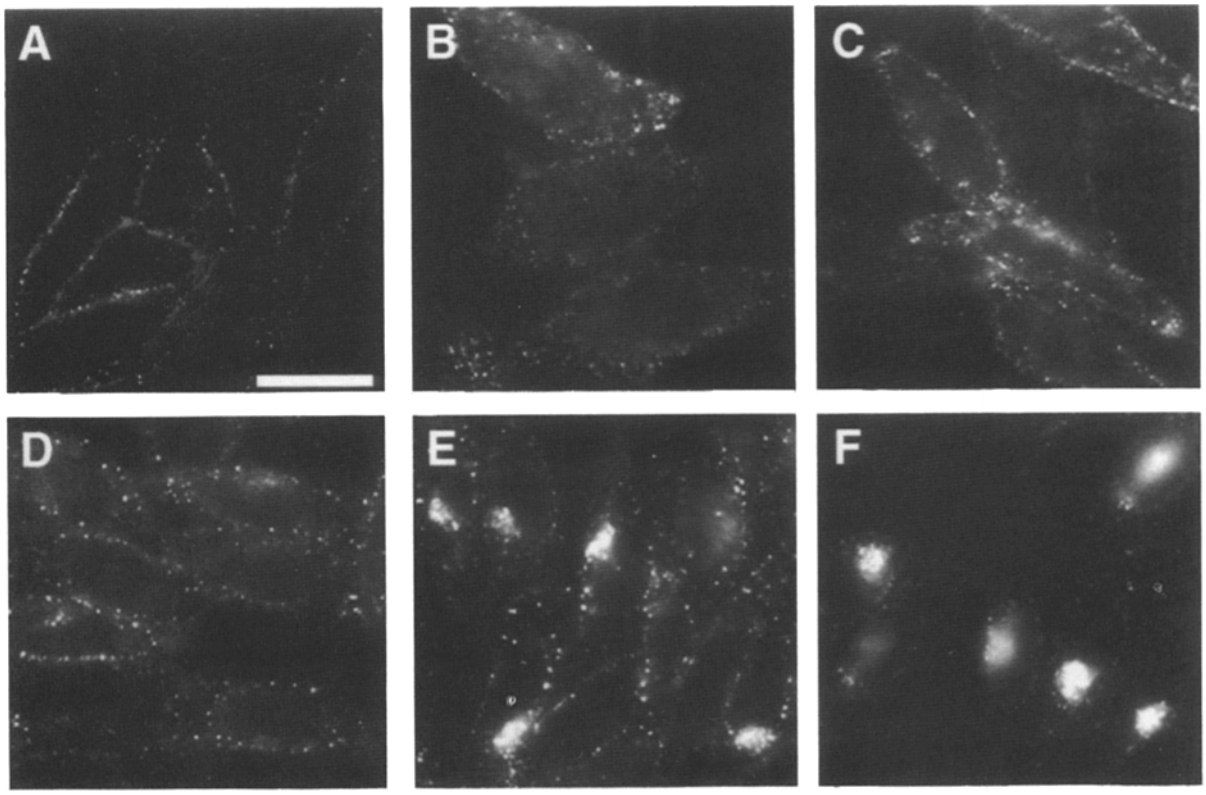

Figure 9. Inhibition of Rho-CCK internalization by lowering intracellular levels of potassium. CHO-CCKR cells grown on coverslips were hypotonically shocked in potassium-free hypotonic buffer, then incubated 15 min in potassium-free buffer. Cells were labeled at 37°C with 50 nM Rho-CCK and fixed at 1, 5, and 30 min. After 1 min, the fluorescent label on the membrane was punctate (*A*). The punctuate membrane label persisted at 5 (*B*) and 30 min (*C*). Control cells displayed a punctate pattern on the membrane at 1 min (*D*), with punctate intracellular label at 5 min (*E*), and a dense accumulation of label near the nucleus after 30 min (*F*). Images are representative of three separate experiments. Bar, 20 μm.

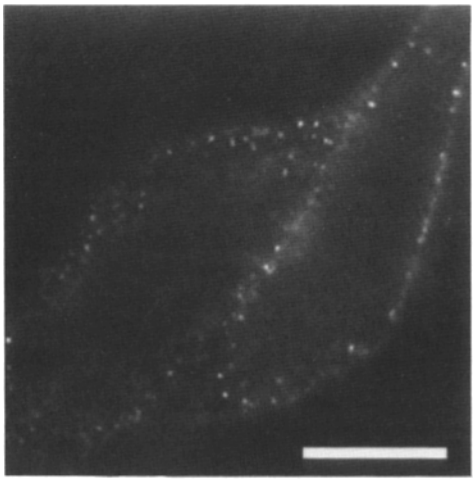

Figure 10. Sequestration of Rho-CCK in potassium-depleted CHO-CCKR cells. CHO-CCKR cells were hypotonically shocked with potassium-free buffer then incubated 15 min in potassium-free buffer to decrease intracellular levels of potassium. Cells were then labeled with 50 nM Rho-CCK for 1 min, washed with acidic glycine, and fixed. All incubations were done at 37°C. The ligand–receptor complexes become rapidly resistant to dissociation in acidic glycine, as shown by persistent fluorescent labeling after 1 min at 37°C. The image is representative of three separate experiments. Bar, 10 μm.

tide hormone become attenuated by processes of desensitization (1, 7, 8, 38, 46). Prominent among the earliest and most physiologically relevant cellular processes of desensitization are events that occur at the level of the receptor molecule, the most proximal point in the signaling cascade (17, 26, 30). Receptor phosphorylation has been implicated as a key signal for these processes (17, 26, 30). This has been demonstrated to result in the uncoupling of receptors from their proximal effector molecules, and it has been implicated as a possible signal for the cellular trafficking of the receptor through sequestered, internalized, and recycling compartments. Despite this proposed relationship, there are remarkably little direct data correlating receptor phosphorylation with its transit through distinct cellular compartments. Most insights into receptor phosphorylation have been indirect, based on the mutagenesis of consensus sites for receptor phosphorylation (27). Furthermore, the intracellular compartments reported to be traversed by members of the extensive family of guanine nucleotide–coupled receptors have been varied, inconsistent, and not well characterized.

In this work, we focus on the type A CCK receptor, one member of the G protein–coupled receptor family that has been extensively studied in regard to its phosphorylation (13, 20, 31, 58). The agonist-stimulated phosphorylation of this receptor molecule has been directly demonstrated in its native cellular environment, both in response to its occupation with agonist ligands and in response to other acinar cell secretagogues acting through distinct receptors to activate protein kinase C (20, 58). The stoichiometry of phosphorylation and the distinct sites of phosphorylation have been defined (39). Multiple cellular kinases rapidly phosphorylate this receptor in an agonist concentration–dependent manner (13). Furthermore, in a novel and important recent observation, a type 2A–like protein phosphatase dephosphorylates this receptor in an agonist-stimulated manner (31). Thus, CCK occupation of its receptor initiates intracellular activity cascades that include the activation of kinases, which phosphorylate this receptor, and a phosphatase, which promptly reverses this process. Since previous work has suggested that receptor dephosphorylation may be a key signal for the resensitization of G protein–coupled receptors (57), insights into the mechanism whereby the cell accomplishes this become particularly important.

Observations in this report focus on a recently established cell line that expresses a large number of type A CCK receptors, whose occupation with native agonist has been shown to elicit appropriate intracellular signaling events (Hadac, E., B. F. Roettger, and L. J. Miller, unpublished observations). This report uses several new and unique CCK receptor probes. An antibody was developed which specifically recognizes an extracellular epitope on this receptor. Novel fluorescent and electron-dense agonist probes were synthesized as well. The fluorescent analogue of CCK was shown to represent a fully efficacious agonist acting at the pancreatic acinar cell receptor to elicit an intracellular calcium response, as well as a complete concentration–response curve that includes both the concentration-dependent stimulation of amylase secretion and supramaximal inhibition of secretion.

Like many receptors that undergo internalization (50), the CCK receptor contains a tyrosine-containing turn sequence motif (NPIIY) that is predicted to reside near the cytosolic surface of its seventh transmembrane domain. While such a motif leads to the constitutive internalization of molecules, such as the transferrin receptor (51), in the G protein–coupled receptors previously studied in which this is present, sequestration is dependent on occupation with agonist (5, 53, 55). Here, too, our data demonstrate that in the basal state, the CCK receptor is diffusely distributed over the plasmelammal surface of the CCK receptor–expressing CHO cell line, and that occupation of receptors by native agonist stimulates internalization of ligand–receptor complexes. Furthermore, while most of the occupied receptors are promptly cleared from the cell surface upon agonist binding, receptor antibody is able to detect unoccupied receptors that continue to reside on the surface despite agonist-activated signaling cascades initiated within the cell.

Consistent with recent work (53–55) focusing on the β_2-adrenergic and thrombin receptors, the predominant path for CCK receptor internalization represents the classical endocytic pathway, initiated at the level of entry into clathrin-coated pits and vesicles. Once internalized, receptors are sorted out of endosomes, either to be recycled to the plasmalemma or to be degraded in lysosomes. The localization of ligand-occupied CCK receptor to the clathrin-mediated endocytic pathway was fully expected, in light of earlier autoradiographic studies in pancreatic acinar cells, in which grains were associated with an occasional lysosome (56). In that work, however, the majority of grains were associated with the endoplasmic reticulum and not with any distinct vesicular or endosomal population.

Of particular interest in the current report is the recognition of an additional minor pathway mediated by noncoated pits and vesicles that remain near the plasmalemma. While smooth vesicles containing G protein–coupled receptors have previously been observed (43), their identity and fate

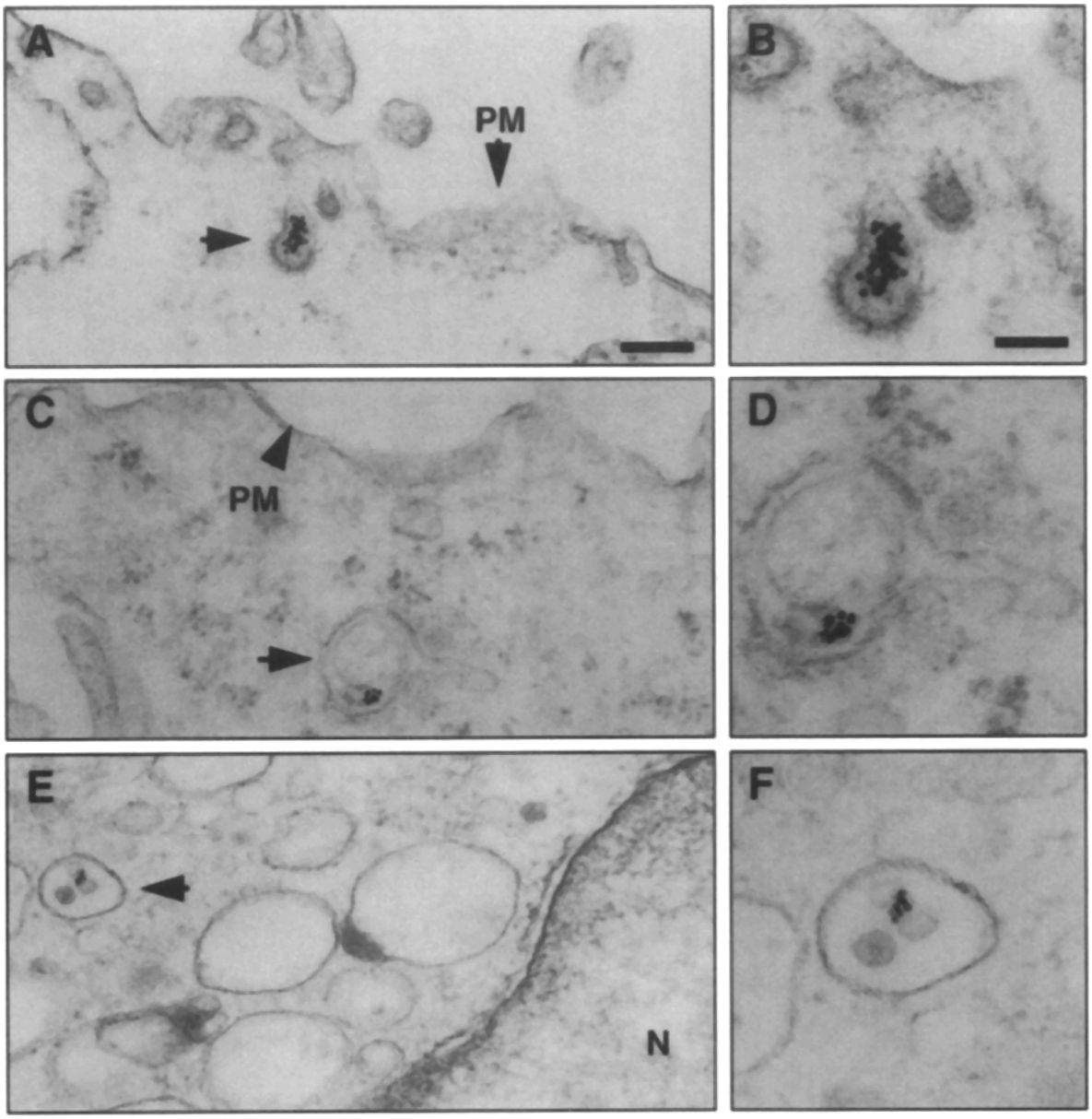

Figure 11. Localization of gold-CCK in CHO-CCKR cells. Cells were preincubated with gold-CCK for 1 h at 4°C. Cells were warmed to 37°C and fixed after 5 and 30 min, then processed for electron microscopy. After 5 min of incubation at 37°C, gold label has clustered near the plasma membrane (*A* and *C*). A higher magnification of these structures shows that gold localized to coated pits (*B*) and tubulovesicular endosomes (*D*). After 30 min of incubation, the gold label was observed near the nucleus (*E*) in multivesicular bodies (*F*). Bar, 200 μm. Inset bar, 100 μm.

are somewhat unclear. The work of Raposo (43) suggests that the ultimate fate of the smooth vesicles in A431 cells bearing β-adrenergic receptors is to enter classical early endosomes, which are indistinguishable from those entered via clathrin-coated vesicles (15, 43, 44). This raises the question whether these smooth vesicles were unique organelles, or merely uncoated vesicles. Recent conjecture has been raised as to whether some of these noncoated structures might represent caveolae (28). However, if the receptor fate is to traffic through this compartment to the endosomal compartment, that fate would be significantly different from our current understanding of caveolar structures remaining attached to the plasmalemma (3).

Fluorescence studies of the endothelin receptor in endothelial cells provides strong support that indeed a G protein–coupled receptor can be localized to caveolae (9). However, since these organelles are particularly prominent in that cell type, it is not clear whether other cells handle this family of receptors similarly, and whether other receptors in this family behave similarly. We believe that caveolae also represent the identity of the minor pathway traversed by the CCK receptor in the current studies. Morphologically, this compartment has typical characteristics and the affinity-labeled receptor is clearly present in the Triton-insoluble membrane fraction. The CCK receptor sequence includes sites for fatty acid acylation, which is the type of biochemical

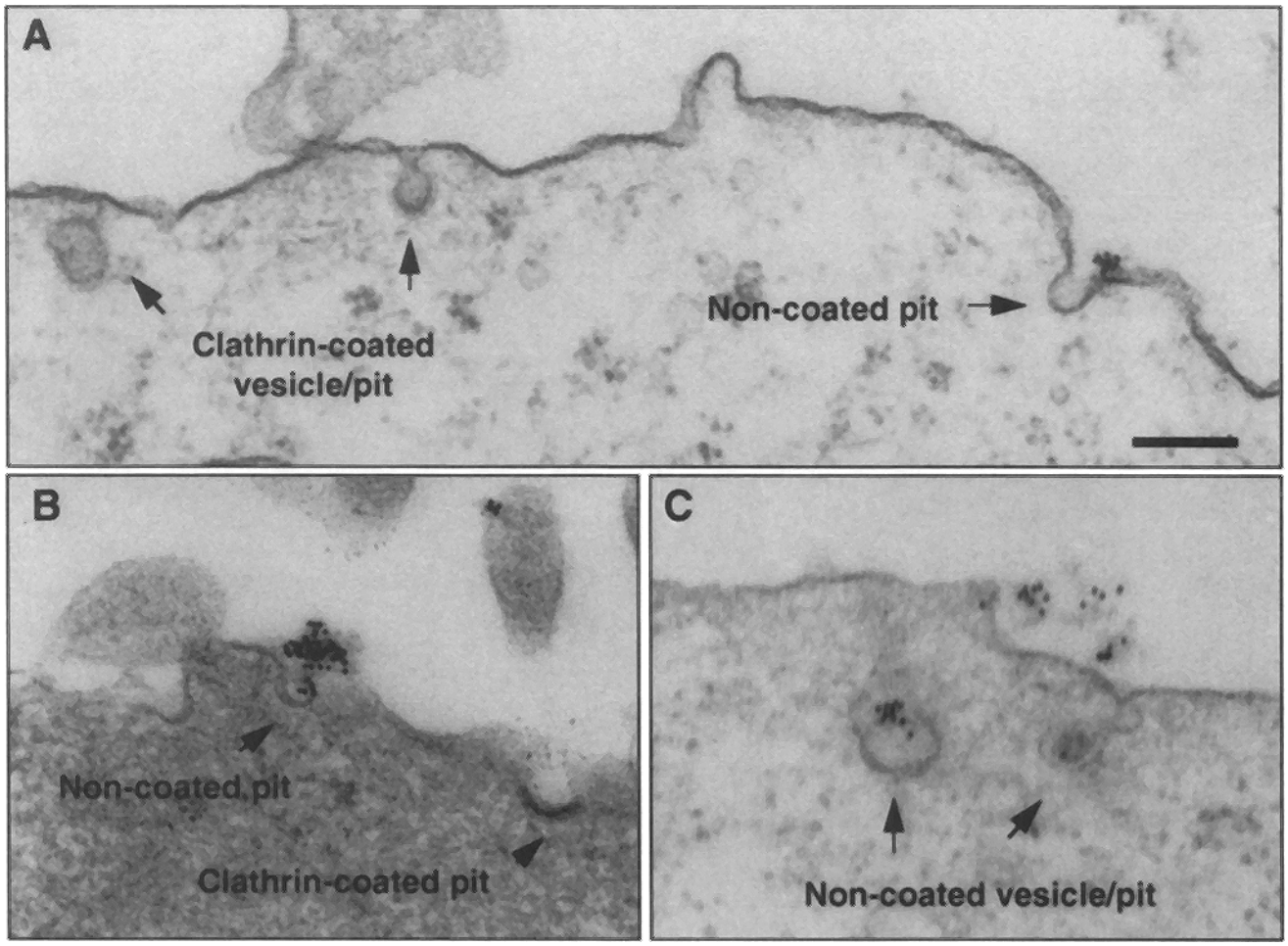

Figure 12. Localization of gold-CCK in noncoated structures in CHO-CCKR cells. (*A* and *B*) Cells were preincubated with gold-CCK for 1 h at 4°C, then warmed to 37°C before being processed for electron microscopy. (*C*) Cells were subjected to potassium depletion before being labeled with gold-CCK at 37°C. The gold observed in potassium-depleted cells was exclusively in noncoated pits and noncoated vesicles near the plasma membrane. Bar, 200 μm.

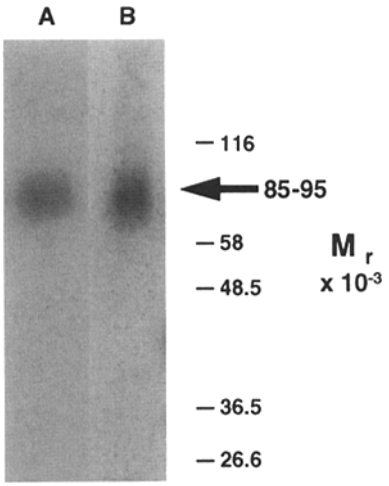

Figure 13. Detection of CCK receptors in the Triton-insoluble complexes to CHO-CCKR cells. Shown is an autoradiograph of a 10% SDS-polyacrylamide gel used to resolve affinity-labeled CCK receptor present in enriched plasma membranes (lane *A*) and Triton-insoluble complexes (lane *B*). Both clearly demonstrated the typical M_r = 85,000–95,000 band known to represent the CCK receptor. Exposures of lanes were normalized to provide similar signal intensities.

determinant that has been associated with caveolar localization (4). Vicinal Cys residues in the carboxyl-terminal tail of the CCK receptor have been demonstrated to be palmitoylated in some G protein–coupled receptors (40), with this process possibly regulated in response to agonist occupation (29, 37).

Of note, cellular manipulations that are known to interfere with clathrin-dependent endocytosis do not interfere with agonist-occupied receptor entry into the noncoated plasmalemmal pits and vesicles. The persistent proximity of these structures to the plasmalamma may suggest that they function as a reservoir for rapid resensitization, while the classical endosomes could be available for similar processes that occur more slowly. Furthermore, the classical endocytic pathway is the likely predominant route for receptor deg-

radation in lysosomes. Both types of compartments were directly observed in untreated cells at the ultrastructural level using a novel colloidal gold conjugate of a CCK analogue, while the smooth structures were the exclusive site of gold localization after treatment of the cells with hypotonic potassium-depleted media. With prolonged incubation, these structures continued to be observed adjacent to the plasmalemma, and not deeper within the cell.

Dual pathways of receptor internalization may provide cellular mechanisms to assure rapid desensitization and carefully regulated resensitization. Desensitization of the pancreatic acinar cell CCK receptor may be critical to protect the cell from potentially damaging overstimulation. Indeed, hyperstimulation with CCK is a common animal model for acute pancreatitis, and that pathologic process can be associated with life-threatening complications (47). In addition, timely resensitization may be just as critical for the organism since adequate pancreatic exocrine secretory responses to nutrient ingestion are necessary for digestion and nutrient assimilation. The caveolae-like compartment closely associated with the plasmalemma may serve this function, while the more predominant recycling endosomal compartment may provide a similar, but more leisurely, response.

The time course of previously observed CCK receptor phosphorylation events is consistent with roles for the plasmalemma, as well as for both clathrin-dependent and -independent vesicular compartments. The rapid onset of agonist-stimulated receptor phosphorylation suggests that this covalent modification occurs while the receptor resides at the plasmalemma (20). Since protein kinase C is the predominant kinase to phosphorylate this receptor after both homologous and heterologous agonist stimulation, and since this enzyme translocates to the plasmalemma, this is a reasonable hypothesis (13, 20, 58). Another kinase postulated to act on the CCK receptor is a member of the G protein–coupled receptor kinase family, known to act exclusively on the activated form of the receptor (13). This kinase also would be expected to reside at the level of the plasmalemma, where the ternary complex is formed with the relevant G protein (11).

The most prominent time of receptor dephosphorylation is between 2 and 15 min after agonist stimulation (31), suggesting that this process occurs predominantly in intracellular compartments. During this time period, the principal location of the agonist-occupied receptor is within the endosomal compartments. However, by 30 min after stimulation, the receptor returns to its basal state of phosphorylation, suggesting that receptor molecules in each of the intracellular compartments are being fully dephosphorylated. Phosphorylated residues of the receptor reside in its endodomain, and they would be accessible to cytosolic phosphatases as the receptor traffics through intracellular compartments. It is also possible that a regulated form of this enzyme could be targeted to a particularly critical compartment, such as a smooth vesicle compartment near the plasmalemma. Now that the existence of such a compartment has been recognized, it will be important to explore this question directly.

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