Insulation of a G Protein–coupled Receptor on the Plasmalemmal Surface of the Pancreatic Acinar Cell

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Abstract. Receptor desensitization is a key process for the protection of the cell from continuous or repeated exposure to high concentrations of an agonist. Well-established mechanisms for desensitization of guanine nucleotide-binding protein (G protein)-coupled receptors include phosphorylation, sequestration/internalization, and down-regulation. In this work, we have examined some mechanisms for desensitization of the cholecystokinin (CCK) receptor which is native to the pancreatic acinar cell, and have found the predominant mechanism to be distinct from these recognized processes. Upon fluorescent agonist occupancy of the native receptor, it becomes "insulated" from the effects of acid washing and becomes immobilized on the surface of the plasma membrane in a time- and temperature-dependent manner. This localization was assessed by ultrastructural studies using a colloidal gold conjugate of CCK, and lateral mobility of the receptor was assessed using fluorescence recovery after photobleaching. Of note, recent application of the same morphologic techniques to a CCK receptor-bearing Chinese hamster ovary cell line demonstrated prominent internalization via the clathrin-dependent endocytic pathway, as well as entry into caveolae (Roettger, B. F., R. U. Rentsch, D. Pinon, E. Holicky, E. Hadac, J. M. Larkin, and L. J. Miller. 1995. *J. Cell Biol.* 128: 1029–1041). These organelles are not observed to represent prominent compartments for the same receptor to traverse in the acinar cell, although fluorescent insulin is clearly internalized in these cells via receptor-mediated endocytosis. In this work, the rate of lateral mobility of the CCK receptor is observed to be similar in both cell types (1–3×10^{-10} cm^2/s), while the fate of the agonist-occupied receptor is quite distinct in each cell. This supports the unique nature of desensitization processes which occur in a cell-specific manner. A plasmalemmal site of insulation of this important receptor on the pancreatic acinar cell could be particularly effective to protect the cell from processes which might initiate pancreatitis, while providing for the rapid resensitization of this receptor to ensure appropriate pancreatic secretion to aid in nutrient assimilation for the organism.

GUANINE nucleotide-binding protein (G protein)[1]–coupled receptors represent the largest family of receptors recognized today. They reside within the plasmalemma in a conformation which incorporates seven transmembrane helices. Agonists approach their binding sites on such molecules from the extracellular aqueous milieu, and induce a presumed conformational change of the receptor which facilitates its association with G proteins on the cytosolic face of the plasmalemma. This ternary complex of agonist–receptor–G protein typically represents the high affinity state of the receptor and a critical step in stimulus-activity coupling. Thus, agonist access

for binding and lateral mobility in the plasma membrane are key components of the native, sensitized state of these receptors.

Desensitization is a ubiquitous process whereby continuous or repeated exposure of a cell to high concentrations of an agonist results in a reduced cellular response. This provides a critically important function of protecting the cell from over-stimulation. The mechanisms of desensitization of G protein–coupled receptors which occur after agonist occupation have been extensively studied (20). Among these are: (*a*) receptor phosphorylation which can affect arrestin-like protein binding, G protein association, and possibly even internalization processes; (*b*) receptor sequestration/internalization processes making the binding domain inaccessible to hydrophilic ligands; and (*c*) receptor down-regulation reducing the cellular complement of receptor molecules (9, 21, 39, 41). Much of the work exploring these mechanisms and the molecular determinants for their initiation has been done using recombinant wild type and mutagenized receptors expressed in a cell line. In

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1. *Abbreviations used in this paper:* CCK, cholecystokinin; CCKR, CCK receptor; DiI, 1,1' dioctadecyl 3,3,3'3' tetramethyl indocarbocyanine perchlorate; G protein, guanine nucleotide-binding protein; KRH, Krebs-Ringer-Hepes.

such cell lines, all of these processes have typically been quite apparent. It is unclear, however, whether native receptor-bearing cells use the same or different mechanisms for desensitization, and what the relative importance of each mechanism might be.

In this report, we have explored the nature of cellular handling of the cholecystokinin (CCK) receptor expressed as a native protein on the rat pancreatic acinar cell. This receptor has been the focus of a large number of studies of structure (25, 30–33, 38), function (26), and regulation (18, 27), and desensitization of CCK responsiveness in these cells has been clearly demonstrated (1). The CCK receptor is a G protein–coupled receptor in the β-adrenergic receptor family which is physiologically important in the hormonal regulation of zymogen secretion from the pancreatic acinar cell after ingestion of a meal. It is also a physiologic regulator of contraction of gallbladder and enteric smooth muscle, and affects neural activity in the enteric nervous system and distinct brain nuclei.

Consistent with established mechanisms of receptor desensitization, the CCK receptor in the acinar cell has been directly shown to be phosphorylated in response to agonist occupation (18). Morphologic demonstration of internalization of the receptor in these cells, however, has been difficult to interpret. While autoradiography of receptor-bound radioligand has localized the receptors to the expected basolateral plasmalemmal domain of the acinar cell (38), the same methodology applied to explore possible receptor internalization demonstrated only a few silver grains over lysosomal structures and the majority of grains over endoplasmic reticulum, rather than being associated with vesicular structures typical of endocytic compartments (44). This is quite different from observations we recently made in a CCK receptor–bearing CHO cell line (CHO-CCKR) using a colloidal gold–conjugated agonist ligand, in which there was prominent time- and temperature-dependent movement of the agonist-bound receptor into structures typical of the clathrin-dependent endocytic pathway, as well as caveolae (37).

This apparent difference in internalization processes in different cells led us to apply the same fluorescent localization methodology which had been used with the CHO-CCKR cells (37) to the CCK receptor native to the pancreatic acinar cell. Indeed, this demonstrated that the native cell retained essentially all agonist-occupied receptor on or near the plasma membrane. Extending these observations to the ultrastructural level failed to demonstrate receptor in distinctive plasmalemmal specializations or invaginations such as caveolae to explain this behavior.

The morphologic demonstration of agonist-bound CCK receptor remaining at the level of the plasma membrane was quite unexpected, particularly in light of previous observations that dissociation of such an agonist by an acidic medium becomes blocked over time (11, 43). This is typically interpreted as functional evidence of internalization, such as had been observed with the CHO-CCKR cells (37). Morphological analysis of the agonist-occupied CCK receptor on the pancreatic acinar cell appeared no different after warming to 37°C than when initially occupied at 4°C, however, with this change in temperature it became functionally "insulated" from the effect of the acidic medium. This was clearly distinct from the internalization ob-

served for a fluorescent insulin ligand after binding to its receptors on the same cell.

In an effort to better characterize this "insulated" state of the CCK receptor, and to attempt to better understand the mechanisms for different receptor regulation in the different cells, we also applied the technique of fluorescence recovery after photobleaching to both the acinar cell and the CHO-CCKR cell. This technique has been widely employed to study protein dynamics in the plane of the plasma membrane of living cells (2, 46). Lateral mobility of membrane proteins reflects both the fluidity of the lipid bilayer and interactions with proteins which are integral to and associated with the membrane. Like other membrane proteins, receptors move laterally within the plasma membrane, however, they are unique in that agonist binding to receptors results in changes in conformation and signalling which can potentially affect that mobility. This technique has been applied to several G protein–coupled receptors, but typically while they were being expressed on a non-native cell type (2, 4, 10, 12, 13, 16, 28, 36).

While the rates of receptor movement in the bilayer were similar in both types of cells, there were clear differences in the percentages of CCK receptors which were mobile and the impact of intracellular signalling was dramatically different in the two types of cells. Receptors on the CHO-CCKR cell line were predominantly mobile when initially occupied by agonist at low temperature, and they rapidly disappeared from the plane of the plasma membrane by processes of endocytosis upon warming (37). In contrast, the percentage of receptors on the acinar cell which were mobile when initially occupied at low temperature was slightly lower than that in the CHO-CCKR cell line, and acinar cell receptors became functionally immobilized at the level of the plasma membrane upon warming.

Based on these observations, we believe that we have identified a previously unrecognized mechanism for receptor desensitization. This state of the receptor is particularly prominent in a distinct native receptor–bearing cell, while it represents at most a transient state in a CHO cell line which expresses the identical receptor protein. We postulate that this "insulated" state of the receptor represents a conformational change in this molecule, as well as a physical interaction between it and the cytoskeleton or a cytoskeleton-associated protein. While these differences could be explained by cellular differences in plasmalemmal structure and microdomains, in signalling events, or in the cellular complement of proteins which might mediate receptor adherence to cytoskeletal components, recognition that this new mechanism for desensitization exists should permit focused evaluation of the molecular details which are responsible for it. Awareness of this will be key in understanding regulation of receptor function in normal epithelia.

Materials and Methods

Reagents

1,1′ dioctadecyl 3,3,3′3′ tetramethyl indocarbocyanine perchlorate (DiI) were purchased from Molecular Probes (Eugene, OR), and rhodamine-labeled concanavalin A was from Sigma Chemical Co. (St. Louis, MO). Hams F-12 medium, essential amino acids, and non-essential amino acids were purchased from GIBCO BRL (Gaithersburg, MD). EM grade paraformaldehyde was purchased from Electron Microscopy Sciences (Ft. Washington, PA). All chemicals were reagent grade.

Receptor and Membrane Probes

The CCK analogue, Gly-([Nle^{28,31}]CCK 26-33), and its rhodamine and colloidal gold–conjugates and radiolabeled forms were synthesized in our laboratory as we previously described (33, 34, 37). The fluorescent CCK analogue (Rho-CCK) has been demonstrated to be a fully efficacious agonist with slightly reduced potency compared with native CCK-8, but with high receptor binding affinity (37). The electron-dense conjugate (gold-CCK) was prepared by adsorbing the CCK analogue to 10 nm colloidal gold as we previously described (37). Radiolabeled forms of this CCK analogue were prepared by synthesizing forms having D-Tyr on their amino terminus, with one form also incorporating a pNO$_2$-Phe residue in position 33 for photoaffinity labeling. For both of these reagents, oxidative radioiodination was performed and products were purified on HPLC as we have described (33, 34).

Rhodamine-conjugated synthetic human insulin was prepared in manner analogous to that described for the CCK analogue (37). This was also purified by reversed phase HPLC.

To prepare a fluorescent marker to label the lipid component of the plasma membrane, 0.125 mg of the fluorescent lipid analogue, DiI, was dissolved in 1 ml of ethanol. This solution was sonicated 10 min, centrifuged 5 min at 14,000 rpm (15,800 g) and dispersed (1:100) in 37°C PBS (1.5 mM NaH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$, 0.145 M NaCl, 0.1 mM MgCl$_2$, 0.08 mM CaCl$_2$, pH 7.4).

Cell and Tissue Preparations

Dispersed rat pancreatic acini and enriched plasma membranes were prepared from male Sprague-Dawley rats weighing 125–150 g, as we have described (7, 23). All procedures involving animals were approved by the Mayo Clinic Animal Care and Use Committee.

CHO cells expressing the rat pancreatic CCK receptor (CHO-CCKR) were established and validated previously (37). Receptors expressed on this cell line are appropriately coupled to signal transduction machinery as demonstrated by a prompt increase in intracellular calcium following agonist occupation. CHO-CCKR cells were grown in Hams F-12 medium in a 37°C humidified incubator containing 5% CO$_2$, and plated on coverslips 2–3 d before microscopic observation.

Fluorescent Labeling of CCK Receptors and Lipid Components of CHO-CCKR Cells

CHO-CCKR cells grown on coverslips were washed three times with iced PBS and incubated for 1 h at 4°C with 50 nM Rho-CCK in the absence or presence of competition with 5 μM non-fluorescent CCK-8. For receptor distribution studies, subsequent incubation was performed at 4° or 37°C for the indicated times. Afterwards, the coverslips 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 coverslips were then washed and mounted on glass slides. For photobleaching experiments, cells were plated on No. 1 22-mm-square glass coverslips (Baxter, McGaw Park, IL) in individual 35-mm polystyrene dishes (Becton Dickinson, Lincoln Park, NJ). Following labeling, coverslips were placed cell side down over 20 μl of 4°C PBS on a chilled glass slide, blotted, and sealed. Fluorescence recovery studies were performed as described below, immediately following preparation of these samples.

To label the lipid portion of the plasma membrane, CHO-CCKR cells grown on coverslips were washed three times with PBS at 37°C, and incubated with the DiI solution for 5 min in a shaking water bath at 37°C. The coverslips were then washed with 37°C PBS and placed cell side down over 20 μl of PBS on glass slides, blotted, and sealed. Fluorescence recovery studies were conducted immediately following preparation of these samples. The DiI appeared to be homogeneously distributed throughout the plasma membrane over the time period of the optical experiments.

Fluorescent Labeling of CCK Receptors on Rat Pancreatic Acini

Freshly prepared dispersed rat pancreatic acini were collected by centrifu-

gation at 300 rpm for 3 min and then washed and resuspended in iced Krebs-Ringer-Hepes medium (KRH) (25 mM Hepes, pH 7.4, 1 mM KH$_2$PO$_4$, 104 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1.2 mM MgSO$_4$, 2.5 mM D-glucose, essential and nonessential amino acids, 2 mM glutamine, 0.2% [wt/vol] bovine serum albumin and 0.01% [wt/vol] soybean trypsin inhibitor). CCK receptors were then labeled at 4°C for 20 min with 50 nM Rho-CCK in the absence or presence of competition with 5 μM non-fluorescent CCK-8. For receptor distribution studies, cells were either labeled at 4°C as described above, or labeled with Rho-CCK at 37°C for 5 to 30 min. For photobleaching studies, a small drop of the fluorescently labeled cell suspension was placed on a chilled glass slide and covered with a No. 0 22-mm-square glass coverslip (Baxter, McGaw Park, IL). Cells were observed immediately following sample preparation.

Acidic Medium Dissociation Studies

Freshly prepared dispersed acini were fluorescently labeled with 50 nM Rho-CCK for 15 or 30 min at 37°C before being washed with iced KRH. The cell samples were further washed with either iced glycine buffer (50 mM glycine, 150 mM NaCl, pH 3.0) or an acetic acid buffer (DME medium adjusted to pH 3.5 with glacial acetic acid, 2% BSA) for 0.5 min, and then resuspended in iced KRH. Control cells were fluorescently labeled with 50 nM Rho-CCK for 20 min at 4°C, before being washed with iced KRH. The cells were then washed with the acidic medium as described above. Both of the acidic media gave similar results.

Analogous acidic medium dissociation studies were performed with the CCK analogue radioligand in place of the fluorescent ligand, for ease of quantitation. For this, a time-course out to 60 min was performed, following the percentage of saturable binding (determined in the presence of 0.1 μM CCK-8) which was removed by the acid wash conditions.

Surface Digestion of CCK Receptor

CCK receptors on pancreatic acini and CHO-CCKR cells were affinity labeled after incubation with ^{125}I-D-Tyr-Gly-([Nle^{28,31},pNO$_2$-Phe^{33}]CCK-26-33) at 4°C for 60 min or at 37°C for 30 min, using photolysis conditions previously established (33). Cells were then washed and treated on ice with either papain (500 μg/ml) in KRH containing 0.1 mM EDTA, 10 mM DTT, and 60 μM β-mercaptoethanol which had been preactivated at 37°C for 10 min, or the same mixture which had been inactivated with 5 mM iodoacetamide. Cells were then washed extensively with KRH containing a protease inhibitor cocktail and 5 mM iodoacetamide, rapidly fractionated to yield a particulate fraction (18), solubilized with SDS sample buffer, and separated by electrophoresis on a 10% SDS–polyacrylamide gel using the conditions of Laemmli (19). The labeled receptor bands were then visualized and quantified by exposure to a phosphorimager (Molecular Dynamics).

Microscopy

Acinar cells were examined with a Zeiss Axiophot microscope (Oberkochen, Germany) equipped for epifluorescence. A 75 W xenon lamp was used with a 20 nm bandpass excitation filter centered at 546 and a 590 nm emission filter. CHO-CCKR cells were examined with a Nikon Microphot FXA microscope (Frank Fryer, CE, Huntley, IL) equipped for epifluorescence. Samples were illuminated with a 100 W mercury lamp using a 20 nm bandpass excitation filter centered at 546 and a 590 nm emission filter. Photographs were taken with a 35 mm camera using Hypertech film (Microfluor, Stony Brook, NY).

Confocal Microscopy

Acini labeled with 100 nM Rho-CCK or Rho-insulin were examined with a Zeiss LSM-410 microscope equipped with a helium neon laser and a 40× objective (1.3 NA). Samples were excited at 543 nm and detected using a 590-nm-long pass emission filter. The pinhole was aligned and set with a Z resolution nominally 1 μm.

Electron Microscopy

Dispersed pancreatic acini were incubated with gold-CCK at 6° or 37°C for 30 min, and washed in iced KRH. Selected samples were then acid washed as described above. Control studies included incubations with BSA adsorbed to the same size colloidal gold and competition for the gold-CCK conjugate with 1 μM CCK-8. The cells were fixed and prepared for electron microscopy as we previously described (37).

Fluorescence Photobleaching Recovery Measurements

Fluorescence recovery after photobleaching measurements were carried out either using a cooled ($10° \pm 1°C$) or room temperature ($24° \pm 1°C$) stage following the procedure described by Jans et al. (13). Briefly, fluorescence was excited by the 514-nm line of an Innova 90 argon laser (Coherent, Palo Alto, CA). Laser illumination intensity was controlled using the first order diffraction from an acousto-optic modulator (IntraAction Corp, Bellwood, IL). The beam then passed through a spatial filter and then into a Zeiss Axioplan microscope equipped for epifluorescence. A $100\times$ (1.3 NA) oil objective was used to focus the beam onto the sample. The circular area illuminated on the sample was approximately 1 μm in diameter, and the fluorescence emitted from the sample was detected continuously using a single photon counting module (EG&G Opto-electronics, Vaudreuil, Quebec) interfaced to a 486 computer. The diffusion coefficient, D, was determined from the fluorescence recovery half-time, $t_{1/2}$, the size of the illuminated area, and a correction for bleach depth, assuming a gaussian intensity distribution of the beam as described by Axelrod et al. (3). The percentage of mobile receptors was defined by 100 $(F_{rec}-F_B)/(F_o-F_B)$ where F_{rec} is the final recovered fluorescence intensity attained after bleaching, F_B is the measured fluorescence immediately following the bleach, and F_o is the fluorescence intensity level prior to the bleach.

To measure fluorescence in the plane of the plasma membrane, the microscope was first focused visually at the plasma membrane using transmitted light, then fluorescence intensity was monitored to check that fluorescence was maximal at this position, or with only slight adjustment of focus. The observation that little or no adjustment was necessary agrees with the confocal microscopy result that Rho-CCK was localized to the cell surface. As a control, this focusing technique was also used and validated on pancreatic acini using 0.1 μM rhodamine–concanavalin A which brightly labeled the plasma membrane on this cell.

Statistical Analysis

The number of replicate experiments is noted for each figure or presentation of data. Values represent means $\pm$ SEM of experimental replicates. Significant differences were determined by the Mann-Whitney non-parametric test of unpaired values, with $P < 0.05$ considered to be significant.

Results

Fluorescent Labeling of Plasma Membrane CCK Receptors

To establish the usefulness of the fluorescent CCK agonist ligand, Rho-CCK, to label CCK receptors on the pancreatic acinar cell, this reagent was applied to the acinar cells and compared with its application to the CCK receptor-bearing CHO cell line (as we previously reported; see reference 37). Indeed, like the CHO-CCKR cells, the pancreatic acinar cells were specifically labeled with the Rho-CCK on the plasma membrane. While the CHO-CCKR cells were labeled diffusely over all plasma membrane domains (Fig. 1 c), the acinar cells were labeled only on the basal and lateral plasma membrane (Figs. 1 a and 3). The intensity of fluorescent labeling of the acinar cells was substantially less than that of the CHO-CCKR cells, representing only 8% of that observed with the cells overexpressing the recombinant CCK receptor (125,000 sites per cell) (37). The pattern of cell labeling was identical using fluorescent ligand concentrations ranging from 1–100 nM. In both types of cells, the fluorescent signal was eliminated in the presence of excess non-fluorescent CCK-8 (Fig. 1, b and d). Non-receptor–bearing CHO cells demonstrated no fluorescent labeling with Rho-CCK (data not shown).
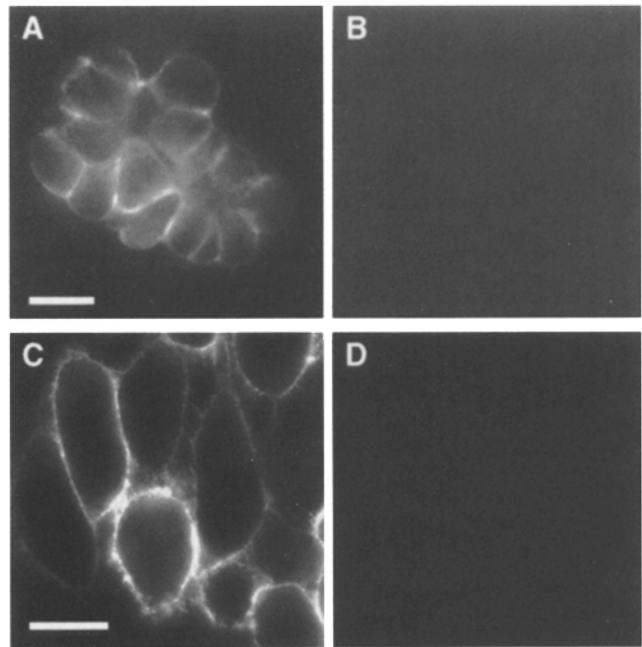

Figure 1. Saturability of labeling CCK receptors on pancreatic acini and receptor-bearing CHO-CCKR cells. Rat pancreatic acini labeled with 50 nM Rho-CCK alone (*a*), or in the presence of 5 μM CCK-8 (*b*). CHO-CCKR cells labeled with 50 nM Rho-CCK alone (*c*), or in the presence of 5 μM CCK-8 (*d*). Images are representative of five separate experiments. Bar, 20 μm.

Cellular Handling of Agonist-occupied CCK Receptors at 37°C

The distribution of agonist-occupied CCK receptors on acini following warming to 37°C was examined using epifluorescence microscopy. Upon warming, the distribution of the fluorescent Rho-CCK signal on the native acinar cell did not change (Fig. 2, *a–c*), unlike its behavior on the CHO cell line in which this signal was observed to traverse internalization pathways deep within the cell in a clear and prominent manner (Fig. 2, *d–f*) (37). The internalization behavior of Rho-CCK in another CHO cell line established to express one order of magnitude fewer recombinant CCK receptors was identical to that observed with the CHO-CCKR cell line (except for the signal being much less prominent) (data not shown).

These observations of fluorescent ligand handling were further confirmed utilizing confocal microscopy of living acinar cells, with optical sectioning through the entire cell (Fig. 3). Under the conditions used, all specific fluorescence in these cells was associated with the plasma membrane. Occasional small punctate areas of fluorescence were observed within the cell, however, these largely represented autofluorescence as determined by their emission spectra and their presence in cells never incubated with Rho-CCK. Three dimensional images were constructed from these sections, confirming the basolateral plasma-lemmal location of the predominant fluorescent signal.

Fluorescent insulin provided a positive control to show that these acinar cells were indeed capable of more prominent internalization of a receptor ligand. Fig. 4 demon-
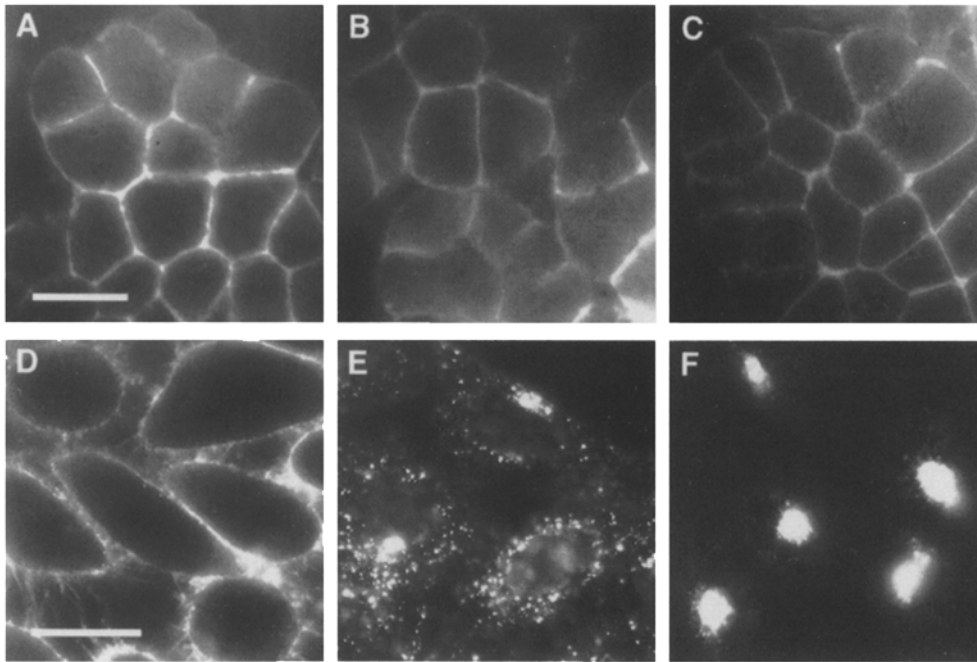
Figure 2. Comparison of cellular handling of CCK receptors by pancreatic acinar cells and CHO-CCKR cells. Dispersed rat pancreatic acini were labeled with Rho-CCK at 4°C for 20 min (*a*), and at 37°C for 5 min (*b*) and 30 min (*c*). CHO-CCKR cells were labeled with Rho-CCK at 4°C for 1 h (*d*), then warmed to 37°C for 5 min (*e*), and 30 min (*f*). Images are representative of seven separate experiments. Bar, 20 μm.

strates representative confocal images of acini which had been incubated with Rho-insulin for 60 min at 4°C, or at 37°C for 30 min in the absence or presence of 100-fold excess non-fluorescent insulin. At the lower temperature, all rhodamine fluorescence remained at the level of the plasma membrane, whereas at physiologic temperature clear and prominent labeling was observed over punctate structures within the cells. Careful analysis of the emission spectrum of this fluorescence demonstrated it to represent rhodamine, and it fully disappeared in analogous studies performed in the presence of competing non-fluorescent insulin. These structures have been previously characterized ultrastructurally to represent endosomes (5).

Acidic Medium Dissociation Studies

To determine the accessibility of the agonist–receptor complex to dissociation, studies incorporating acid washing were used. This manipulation is a well established way to remove surface-accessible ligands whose binding is sensitive to low pH. Two distinct acidic media (representing acidification with glycine and acetic acid) were used without any difference in the results. The Rho-CCK fluorescent ligand was completely dissociated after acid washing acinar cell receptors which had been occupied at 4°C (Fig. 5, *a* and *b*), however after acini had been warmed to 37°C labeling at the level of the plasma membrane became resistant to this treatment (Fig. 5, *c* and *d*).
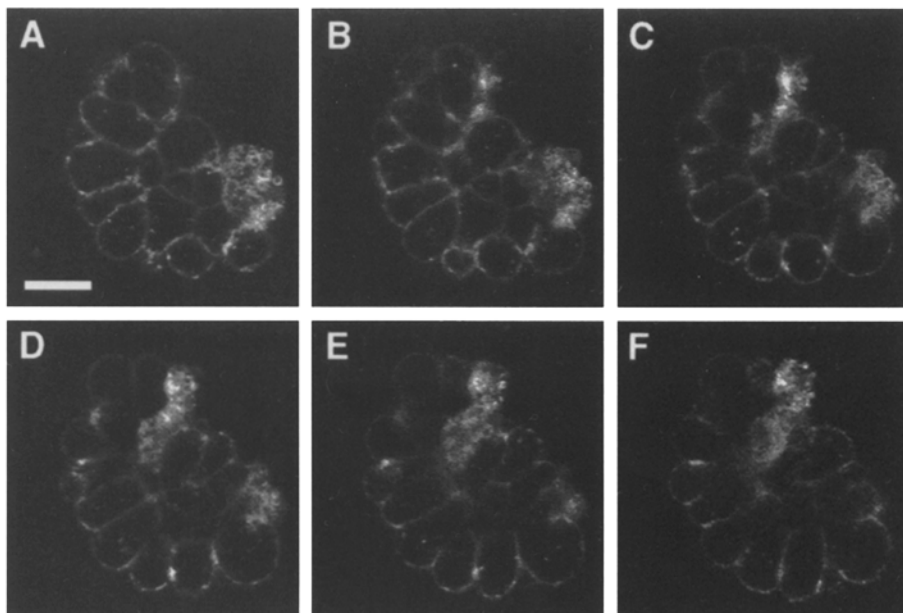

Figure 3. Confocal microscopy of labeled acini. Shown are serial confocal optical sections of Rho-CCK binding to receptors on the plasma membrane of pancreatic acini which had been incubated with fluorophore for 30 min at 37°C. Sections were taken 16 (*a*), 18 (*b*), 20 (*c*), 22 (*d*), 24 (*e*), and 26 μm (*f*) above the base of the acinar cells. Three dimensional reconstruction of a series of such images clearly shows diffuse basolateral plasmalemmal labeling without labeling over apical plasmalemmal domains (best seen in *e* and *f*). Images are representative of six separate experiments. Bar, 20 μm.
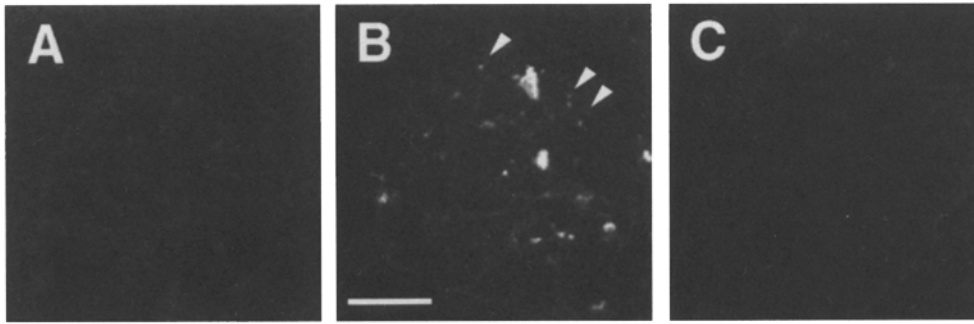
Figure 4. Internalization of receptor-bound insulin. Shown are representative confocal optical sections through pancreatic acini which had been incubated with Rho-insulin at 4°C for 60 min (*a*), or at 37°C for 30 min in the absence (*b*) or presence of 100-fold excess non-fluorescent insulin (*c*). Images reflect identical exposures, and are representative of five separate experiments. There was a clear temperature-dependent difference in cellular handling of this fluorescent ligand. At the lower temperature, saturable binding was quite dim and it remained at the level of the cell surface (all intracellular fluorescence observed was found to represent autofluorescence, not correlating with the spectrum of rhodamine), whereas at 37°C there was clear and prominent labeling of punctate structures within the cell (with spectral characteristics of rhodamine). These structures were clearly seen on multiple sequential sections, and three-dimensional reconstructions clearly demonstrate their intracellular localization. In the presence of saturating concentrations of non-fluorescent insulin, labeling of these structures was completely eliminated although a small amount of surface staining remained. Bar, 15 μm.

Studies were also performed under analogous conditions with a radioiodinated CCK analogue to allow sensitive quantitation of the degree of acid lability of ligand binding (Fig. 5 *e*). When cells were kept at 4°C, all saturably bound ligand was eluted with the acidic wash. After exposure to 37°C for 5 min, 51 ± 7% of saturably bound ligand became resistent to acidic elution, and after 30 min of such treatment this value reached a plateau at approximately 80% of ligand which was saturably bound to cells being resistent to acidic elution.

Acidic media was also used to dissociate >90% of radioligand which was saturably bound to CCK receptors in acinar cell membranes at 4°C. Of particular interest, bringing the receptor-bearing membranes to 37°C failed to protect this binding from the ability of acidic media to dissociate it. Thus, the insulation phenomenon cannot be reproduced by conformational changes induced by binding to the receptor in an isolated plasma membrane preparation, but requires additional factors provided by the intact cell.

Ultrastructural Characterization of Receptor Localization on the Plasmalemma

To establish whether the agonist–receptor complex was actually at the level of the plasmalemma, or whether it was present within membrane invaginations such as a caveolae or within some other distinctive vesicular structure associated with the membrane, electron microscopic studies were performed. For these, we utilized as ligand a CCK analogue adsorbed to the surface of 10 nm colloidal gold which we recently used to characterize the vesicular compartments of internalization of the CCK receptor in the CHO cell line (37). After binding at 37°C for 30 min, cells were acid washed, and fixed in preparation for electron microscopy. The colloidal gold probe was observed predominantly at the basolateral surface of the plasma membrane of the acinar cell, but not within caveolae or distinct vesicles (Fig. 6 *a*). While coated pits and structures resembling caveolae were present on the basolateral surfaces of these cells (Fig. 6, *b* and *c*), as previously reported (24), their density on the healthy acinar cell is quite low. There was an average of 23.3 gold particles observed per 0.1-

μm-thick section per cell, with 99.5% at the level of the plasma membrane and only 0.5% over internal structures (*n* = 422). The domains of the plasmalemma which were labeled with this reagent often appeared to have a prominent glycocalix, but had no other specific distinguishing morphological characteristics which we could identify (Fig. 6, *d–g*). Experiments performed with albumin-coated colloidal gold and peptide-competition controls for the CCK-coated gold reagent were appropriately negative.

Surface Digestion of CCK Receptor

To further confirm the surface localization of the CCK receptor on the acinar cell after binding agonist ligand and warming the cells which was suggested by the ultrastructural studies, papain digestion of affinity-labeled receptor protein accessible to the outside of the cell was performed as described by Jesaitis et al. (14). Indeed, 85–90% of affinity-labeled CCK receptors on the acinar cells were digested by this treatment (Fig. 7). This was similar to the CCK receptors on the surface of the CHO-CCKR cells at 4°C, whereas, as expected, a substantial percentage of those receptors were protected from proteolysis after short exposure of the CHO-CCKR cells to 37°C (Fig. 7).

Lateral Mobility Characteristics of CCK Receptors on the CHO-CCKR Cell Line

To investigate the lateral dynamics of CCK receptors on CHO-CCKR cells, we conducted fluorescence recovery after photobleaching experiments. To minimize effects of receptor clustering and redistribution following agonist occupation of receptors, all readings were taken immediately following sample preparation, while fluorescence appeared to be homogeneously distributed over the plasma membrane. Control studies were performed to ensure that ligand-receptor binding and dissociation kinetics did not contribute to the fluorescence recovery curves. For these, receptors were first fixed in position on the plasma membrane with 4% paraformaldehyde and washed prior to labeling with Rho-CCK. This measured diffusion coefficient on these specimens was 1×10^{-12} cm²/s, two orders of
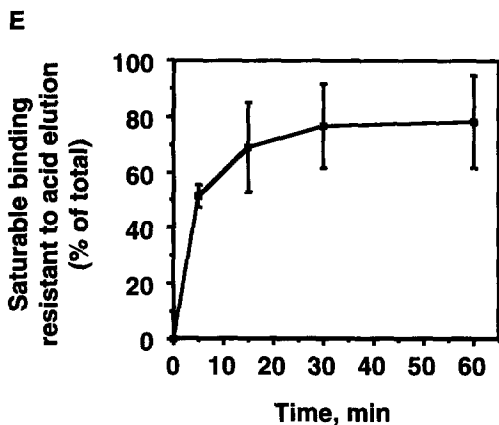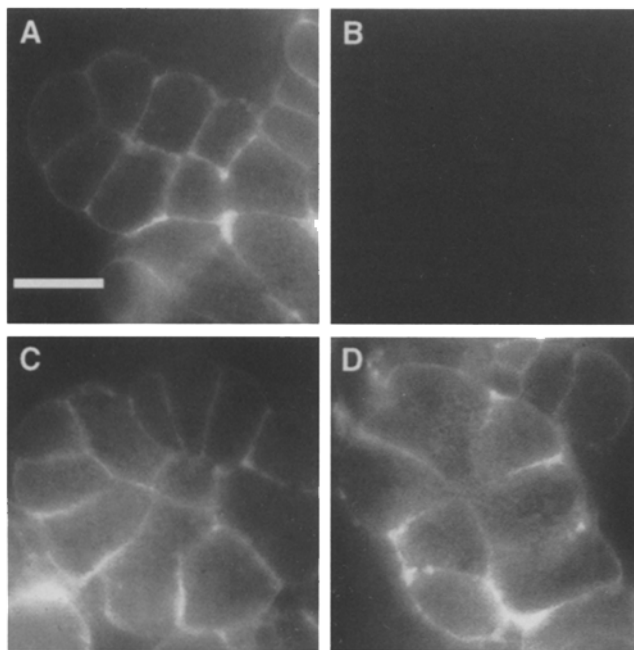
Figure 5. Insulation of CCK receptors in acid-resistant compartments on acinar cell surface. Dispersed pancreatic acini were labeled with Rho-CCK at 4°C (a) and at 37°C (c). Acid-washed acini after labeling at 4°C (b), and at 37°C for 30 min (d). Images are representative of 9 separate experiments. Also shown is a time-course of quantitation of the resistance of the saturably-bound CCK ligand to elution by acidic media (e). Values represent means ± SEM for three separate experiments performed in duplicate. Bar, 20 μm.

magnitude less than values of D determined for receptors on cells that had not been fixed.

As shown in Table I, receptors on the CHO-CCKR cells were predominantly mobile in the plane of the membrane at 10°C. The average percentage of mobile receptor was 88 ± 2%, and was significantly different from 100%, indicating that a small but measurable population of receptors were immobile on the cell surface. The measured lateral diffusion coefficient of the mobile receptors was $1.4 \pm 0.2 \times 10^{-10}$ cm²/s.

The lateral diffusion coefficient for these receptors measured at room temperature (D = $3.1 \pm 0.4 \times 10^{-10}$ cm²/s) increased more than 2-fold from the value determined at

10°C (D = $1.4 \pm 0.2 \times 10^{-10}$ cm²/s) (P = 0.0003) (Fig. 8, a and b). This paralleled the change observed in lipid mobility in the cell membrane, increasing from $0.8 \pm 0.04 \times 10^{-9}$ cm²/s at 10°C to $2.7 \pm 0.5 \times 10^{-9}$ cm²/s at 24°C (P = 0.006) (n = 4 experiments performed in quadruplicate) (Fig. 8, c and d). Despite the change observed in the rate of receptor diffusion, the percentage of mobile receptors (R = 92 ± 4%) was not statistically different from that determined at 10°C for cells labeled under the same conditions.

Studies of agonist-occupied receptor mobility on the plasma membrane of CHO-CCKR cells were not feasible at physiological temperature. Warming the CHO-CCKR cells to 37°C results in the prompt internalization of fluorescent agonist-occupied receptors, eliminating the surface fluorescence necessary for photobleaching studies (37).

Lateral Mobility Characteristics of CCK Receptors on Rat Pancreatic Acini

Fluorescence recovery after photobleaching studies were also conducted to determine the lateral mobility parameters of CCK-A receptors in the native cellular environment. CCK receptors are expressed more sparsely on rat pancreatic acini than on the recombinant CHO cell line (37), resulting in a reduced fluorescent labeling intensity. While the fluorescent label on the basal and lateral plasma membrane of the acinar cells appeared to be uniform when examined on a standard fluorescence microscope, optical sections examined by confocal microscopy demonstrated domains of varied intensities. This was supported by variations in the measured intensities of fluorescence within small areas (d = 1 μm) at discrete locations on the membrane.

At 10°C, CCK receptors on native pancreatic acini moved at the same rate ($1.7 \pm 0.4 \times 10^{-10}$ cm²/s) as those on CHO-CCKR cells ($1.4 \pm 0.2 \times 10^{-10}$ cm²/s) under these conditions (Table I). However, the percentage of these receptors which were mobile was significantly lower in the native cells (17 ± 5%). As temperature was increased to 24°C, the lateral diffusion coefficient D, of native acinar cell receptors increased from $1.7 \pm 0.4 \times 10^{-10}$ cm²/s to 2.2 ± 0.4 × 10⁻¹⁰ cm²/s, similar to those on the CHO-CCKR cells. Under these conditions, the percentage of mobile receptors increased significantly to 65 ± 9%. All measurements of mobile receptor percentages were statistically different from 100%, indicating that a component of the receptor population was either immobilized or moving too slowly to be detected under these experimental conditions.

Unlike the CHO-CCKR cells, when acinar cells were warmed to 37°C a substantial fluorescent signal persisted at the level of the plasma membrane. This provided an opportunity to examine the effect of agonist-initiated intracellular signalling cascades which are known to be temperature-dependent. For purposes of comparison, receptor mobility was studied with the microscope stage at room temperature (24°C), taking measurements rapidly after cells were transferred to the stage from their respective treatments. An additional treatment was added in this series of studies to determine if the ability to dissociate bound ligand with acidic medium could be correlated with receptor mobility status.

Although acini incubated with Rho-CCK at 37°C demonstrated diffuse fluorescent labeling over the basal and
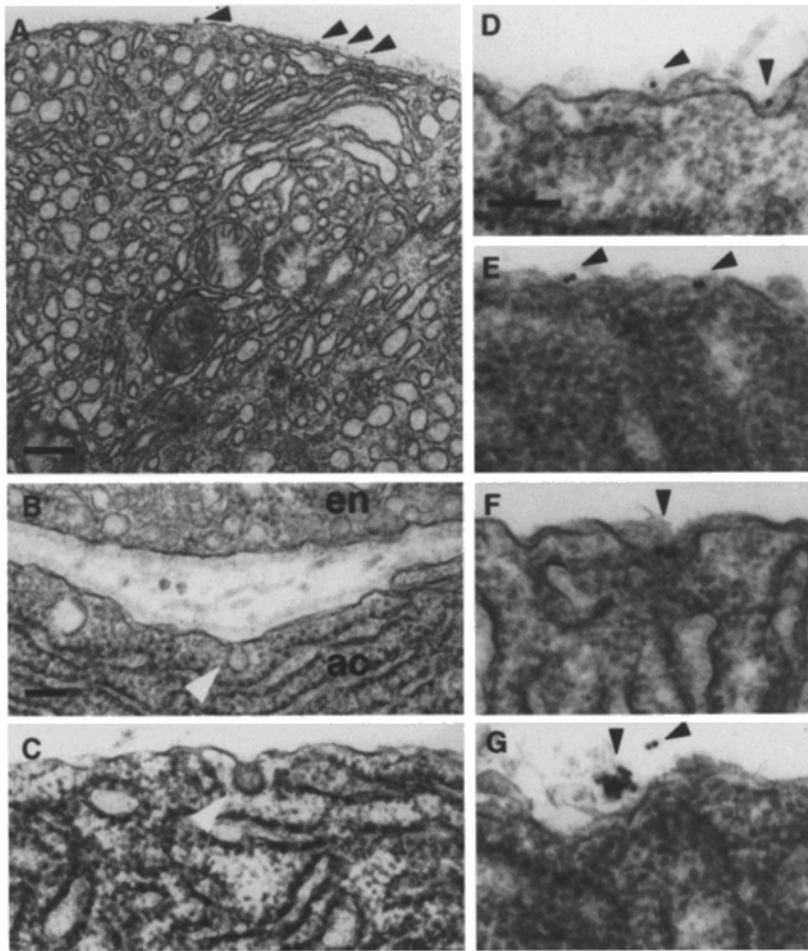
Figure 6. Ultrastructural localization of insulation compartment on pancreatic acini. Dispersed pancreatic acini labeled with gold-CCK at 37°C for 30 min, demonstrating predominant labeling on the cell surface (only 0.5% of gold was intracellular) (*a*). Bar 500 nm. Shown with white arrows is a caveolus on an acinar cell (*ac*) directly opposite a caveolae-rich endothelial cell (*en*) (*b*), and a coated pit on the basolateral surface of an acinar cell (*c*). These membrane specializations are quite rare on these cells, and were never observed to contain gold-CCK. Higher magnification of gold-CCK localization (*black arrows*) on acinar cells after 30 min at 37°C and acid washing (*d–g*). Images are representative of six separate experiments. Bars: 100 nm.

lateral plasma membrane (Fig. 9 *c*) which was morphologically indistinguishable from the same cells labeled at 4°C (Fig. 9 *a*), the acid lability of these signals was quite different. Acidic glycine washes eliminated the labeling performed at 4°C but not that after warming. Consistent with this difference, typical fluorescence recovery curves for CCK receptors labeled at 4°C showed rapid recovery with a high percentage of mobile receptors (65 ± 9%, *n* = 8) (Fig. 9 *b*), while that of the warmed cells was flat (4 ± 3%, *n* = 15) indicating that the receptors had become immobile (Fig. 9 *d*). The percentage of non-acid labile ligand–receptor complexes that were mobile was not statistically different from zero (Fig. 9 *e*). Thus, although the cellular receptors were visually similar at the two temperatures, receptor lateral dynamics were strikingly different. Non-insulated receptors were predominantly mobile, while receptors in the insulated state had become immobilized.

Discussion

In this report, we have explored the cellular handling of the CCK receptor expressed as a native protein on the rat pancreatic acinar cell, and have demonstrated a clear difference from our recently reported experience with the recombinant receptor protein stably expressed on a CHO cell line (37). Since functional desensitization has been clearly demonstrated to occur for both cells (1, 18), the molecular mechanisms involved must be quite distinct.

The receptor-bearing CHO cell line demonstrates all of the previously described and expected mechanisms, with internalization deep within the cell most prominent (37). In the native cell, however, we find clear evidence for a predominant process which occurs at physiologic temperature and which has not been previously recognized, which we now categorize as "insulation." This occurs after agonist occupation, but before frank movement of the receptor from the plasmalemma to a distinct vesicular compartment (such as occur in the process[es] of sequestration/internalization). We postulate that this process requires a conformational change of the receptor, as well as a physical interaction between the receptor and the cytoskeleton or a cytoskeleton-associated protein. Consistent with this, it does not occur in receptor-bearing plasmalemmal preparations, but only in intact cells. Of particular interest is the prominence of this state of the receptor in a distinct native receptor-bearing epithelial cell, while it represents at most a transient state in a CHO cell line which expresses the identical receptor protein. A similar process may occur for the formyl peptide receptor on the neutrophil when incubated with agonist at 15°C (14, 15), however warming that cell to 37°C results in prominent receptor internalization.

We used a fluorescently labeled analogue of CCK for morphological localization of the agonist-bound receptor. This analogue, rhodamine-Gly-([Nle^{28,31}]CCK-26-33) (Rho-CCK), was synthesized, purified, and characterized as a fully biologically active, high affinity and specific ligand of
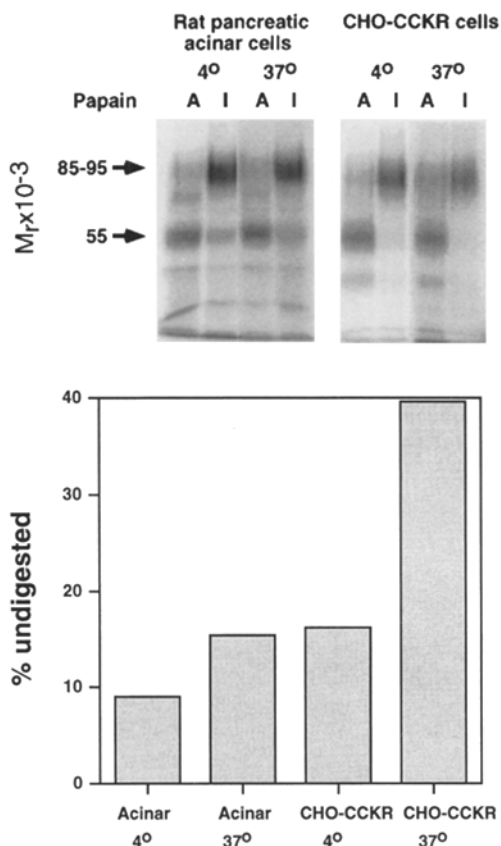
Figure 7. Surface digestion of affinity-labeled CCK receptors. Shown is an autoradiograph and densitometric quantitation of SDS–polyacrylamide gels used to separate particulate proteins from cells which had been affinity labeled after incubation at the temperatures noted, and which were then exposed to active (A) or inactive (I) papain prior to fractionation. This is representative of three separate experiments. The intact receptor migrates at $M_r = 85,000$–$95,000$ (30).
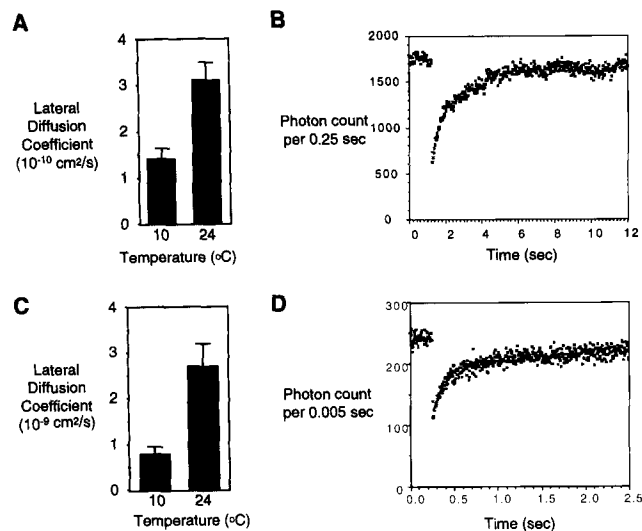

Figure 8. Temperature dependence of lateral diffusion coefficients in CHO-CCKR cells. Lateral diffusion coefficients of CCK receptors on cells at 10 and 24°C (*a*). Representative photobleaching recovery curve of CCK receptors at 10°C (*b*). Lateral diffusion coefficients of the lipid analogue, DiI, on cells at 10° and 24°C (*c*). Representative photobleaching recovery curve for DiI at 10°C (*d*).

the CCK receptor (37). Like in the receptor-bearing CHO cell (37), when binding was performed at 4°C, this reagent clearly labeled receptors residing on the plasmalemmal surface of the pancreatic acinar cell. Upon warming to 37°C, the distribution of the fluorescent signal did not change on the native acinar cell, unlike its behavior on the CHO cell line in which this signal was observed to traverse internalization pathways deep within the cell in a clear and prominent manner (37). This was further confirmed utilizing confocal microscopy of living cells, with optical sectioning through the entire cell. Of note, the acinar cell is

fully capable of receptor-mediated endocytosis as demonstrated by its handling of a fluorescent insulin ligand in which this enters an intracellular vesicular compartment previously characterized ultrastructurally as endosomes (5).

These results were quite unexpected, given the previous demonstration that upon incubation at warm temperatures, CCK radioligand binding to the intact pancreatic acinar cell becomes progressively more resistant to dissociation by acidic medium (11, 43). The incubation times and temperature for the current morphologic observations were fully consistent with those in which dissociation of the ligand has been reported to become acid resistant. Acid washing is a well established manipulation to remove surface-accessible ligands whose binding is sensitive to low pH. It has been utilized to demonstrate the time- and temperature-dependent sequestration of receptors (43). Such a manipulation has been widely applied and interpreted as consistent with agonist-stimulated receptor internalization. Indeed, consistent with that interpretation, our recent work with the CCK receptor–bearing CHO cell line demonstrated clear and prominent internalization into vesicular structures deep within the cell, with morphological characteristics of classical endosomes (37). The current data, however, suggest that this is explained by the distinct mechanism of receptor insulation on the surface of the native acinar cell.

The acid-lability of the binding of CCK radioligands to the CCK receptor can be clearly demonstrated with an enriched plasmalemmal preparation from acinar cells (11, 43). In that preparation, acidic media dissociated radioligand which was saturably bound at 4°C. Of further interest, bringing those receptor-bearing membranes to 37°C failed to "insulate" this binding from the effect of acid to dissociate bound ligand. Performing the same experiment in intact acini resulted in a time- and temperature-depen-

Table I. Lateral Mobility of CCK-A Receptors

Cell type	Temperature	n	D	R
	°C		$\times 10^{2-10} cm^2/s$	%
CHO-CCKR	10	26	1.4 ± 0.2	88 ± 2
CHO-CCKR	24	14	3.1 ± 0.4	92 ± 4
Acinar cells	10	24	1.7 ± 0.4	17 ± 5
Acinar cells	24	8	2.2 ± 0.4	65 ± 9

Measurements of lateral mobility of the CCK receptor were conducted using fluorescence recovery after photobleaching. n is the number of observations, D is the lateral diffusion coefficient, and R is the percentage of receptors that were mobile. Mean values for D and R are given ± standard error of the mean.
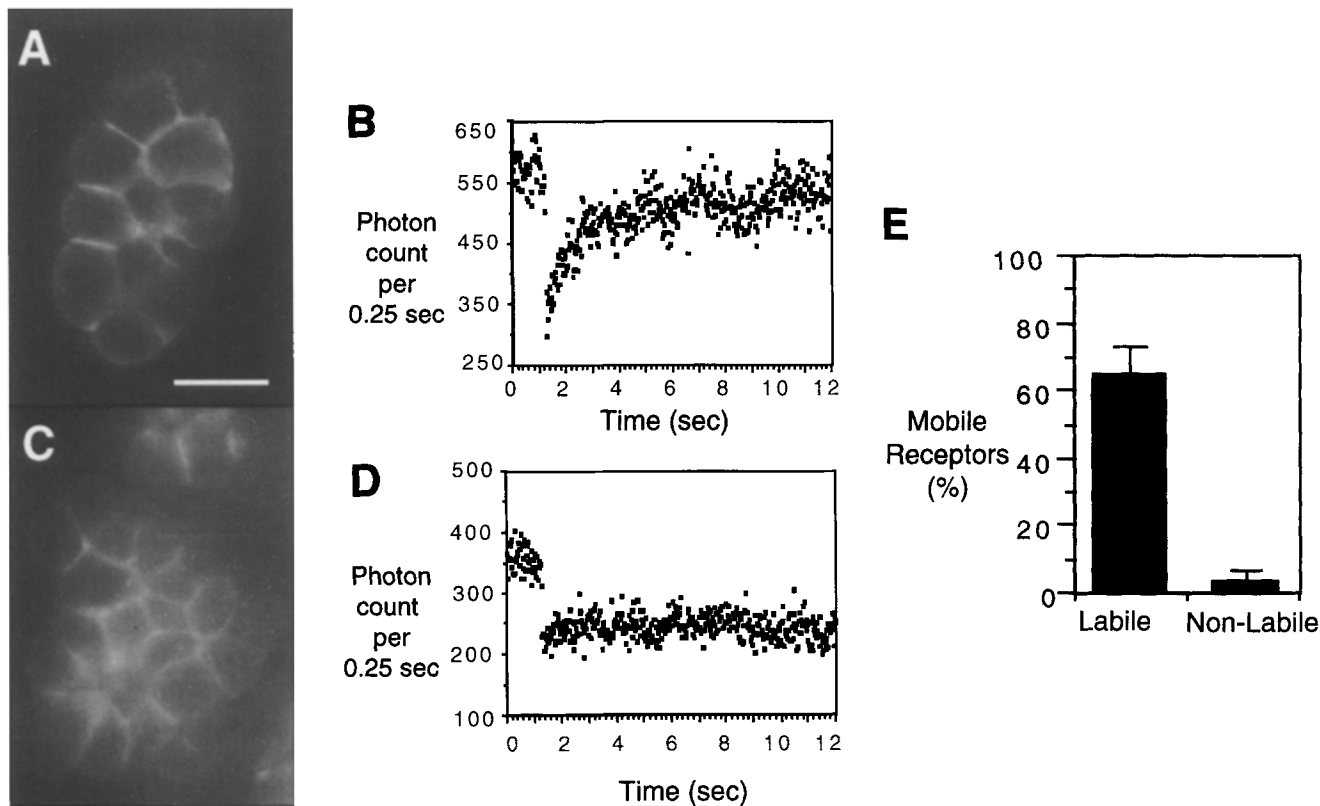
Figure 9. Immobilization of CCK receptors in insulation compartment. Acini labeled with 50 nM Rho-CCK at 4°C for 20 min (*a*), and corresponding typical photobleaching recovery curve (*b*). Acini labeled with 50 nM Rho-CCK at 37°C for 15 min followed by acid washing (*c*), and corresponding typical photobleaching recovery curve (*d*). Comparison of percentages of mobile receptors on acini labeled at 4°C and those labeled at 37°C followed by an acid wash (*e*). Bar, 20 μm.

dent "insulation" phenomenon. This may be analogous to the "sequestration" phenomenon reported for nutrient receptors, but since that term has already been used differently with G protein–coupled receptors (representing a functional definition which is compatible with internalization deep within the cell, rather than a morphological definition) (45), we felt it to be important to introduce this new terminology. The traditional definition of G protein–coupled receptor "sequestration" represents the movement of the receptor into a cellular compartment which is accessible to hydrophobic, but not hydrophilic ligands (45). Cellular fractionation studies have even correlated this with receptor localization in a vesicular compartment which may be distinct from the plasmalemma (40, 41). This further emphasizes the need for a distinct terminology for the "insulation" which occurs at the level of the plasmalemma.

To further confirm that the agonist–receptor complex in the "insulation compartment" continued to reside at the level of the plasmalemma, rather than being present within a membrane invagination such as a caveolus or within a distinct vesicular structure associated with the membrane, electron microscopic studies were performed. For these, we utilized as ligand a CCK analogue adsorbed to the surface of 10 nm colloidal gold which we recently utilized to characterize the vesicular compartments of internalization of the CCK receptor in the CHO cell line (37). The colloidal gold was observed at the surface of the basal and lateral plasmalemma of the acinar cell, but not within caveo-

lae or distinct vesicles. Domains of the plasmalemma which were labeled with the CCK analogue-coated colloidal gold appeared to have a prominent glycocalix, but had no other specific distinguishing morphological characteristics which we could identify. In addition to the ultrastructural evidence of surface localization, the CCK receptor in this compartment was also demonstrated to be fully accessible to proteolytic cleavage with a cell-impermeant protease.

A particularly interesting correlation with the agonist-receptor complex within this "insulation compartment" was our observation of a clear change in lateral mobility of the receptor within the plane of the plasmalemma. Application of the technique of fluorescence recovery after photobleaching to the CCK receptor expressed on the pancreatic acinar cells demonstrated that the majority of receptors were in the mobile state when initially occupied, and all receptors in the "insulation compartment" of the plasmalemma became fully immobile. Immobility of a G protein–coupled receptor has been implicated in decreased effector responses (13), as might be expected from a system in which the association of two molecules must occur within a fluid lipid bilayer. A similar phenomenon has been suggested for the formyl peptide receptor, based on fractionation studies performed after incubation at 15°C suggesting that the receptor can move into a plasmalemmal subdomain which is devoid of G proteins (14, 15, 17).

In contrast to the acinar cell data, almost all of the CCK receptors expressed on the surface of the CHO cell were

mobile when initially occupied, and remained in that state through the time points at which they were internalized via the classical endocytic pathway (37). Of note, mobile receptors on both types of cells had similar diffusion coefficients, indicating that their rates of movement within the bilayer were not different, and therefore intrinsic differences in the lipid fluidity of the cells is not a likely cause of the prominence of the "insulation" phenomenon in the acinar cell. The absolute values for the receptor diffusion coefficients in this study were well within the range of values reported for other G protein-coupled receptors in a variety of cell types (1×10^{-10} cm^2/s to 5×10^{-10} cm^2/s) (4, 10, 12, 16, 28, 36).

Similarly, differences in the primary structure of the receptor molecule in the CHO cell line and the native acinar cell can be ruled out as a cause of the different processes of internalization and desensitization observed. Also, the recombinant receptor in the CHO cell is known to undergo a conformational change which initiates an appropriate stimulus-activity cascade as demonstrated by the intracellular calcium response stimulated by CCK in these cells (37). The activation of cellular processes has been previously correlated with changes in receptor lateral mobility (16, 28, 36).

This focuses attention on possible differences in more distal signalling events or in receptor-associated proteins as potential explanations for the differences in behavior of the agonist-occupied receptors in the two types of cells. Indeed, even though receptors on both the native acinar cell and on the receptor-bearing CHO cell are phosphorylated in response to agonist occupation, we have preliminary evidence for differences in the sites of receptor phosphorylation in the two types of cells (29). This supports differences in signalling processes between the cells. The differences we observed in the photobleaching studies are particularly supportive of a difference in the complement of or in the regulation of receptor-associated proteins which are intrinsic to or bind to the cytoskeleton. Potential candidates for such an activity include dynamin-2 and members of the rab family (35, 42). The apparent immobilization of the "insulated" state of the agonist-occupied receptor within the highly-mobile lipid phase of the plasmalemma suggests that there is a direct or indirect association between the receptor and the cytoskeleton. Given the small number of receptors present on the native cell relative to the CHO cell line, it is possible that potential adapter proteins which could keep the agonist-bound receptor immobilized on the surface of a living cell could be stoichiometrically overwhelmed in the model system in which receptor is overexpressed. This is somewhat unlikely, based on our observation that a distinct CHO cell line established to express a similar number of receptors to that expressed on the acinar cell internalized the CCK ligand identically to the receptor-overexpressing cell line. It is also possible that the molecular association postulated to exist is regulated by a process unique to the native acinar cell.

One would expect a functional reason for the prominence of this potential cellular mechanism for receptor desensitization in the acinar cell. This may have a relationship to a need for rapid resensitization in this cell. Clearly, desensitization is necessary in this cell since hyperstimulation of the acinar cell may be associated with intracellular

activation of zymogens with the possibility of a life-threatening complication of pancreatitis (8), but timely resensitization is also critical since nutrient-regulated pancreatic enzyme secretion is necessary for nutrient assimilation (6). In recent work, we demonstrated that CCK receptor phosphorylation in response to agonist stimulation of the pancreatic acinar cell occurred rapidly and quite transiently (18). It is even possible that this rapidly reversible biochemical event has mechanisms of regulation which might include localization of key enzymes within the specialized membrane microdomains of receptor insulation. Indeed, we recently reported that CCK stimulation of the pancreatic acinar cell is associated with the activation of a protein phosphatase type 2a-like activity specifically directed toward the CCK receptor (22). The possibility of this enzyme or a factor which regulates it residing in this cellular compartment needs to be tested. This and other interesting possibilities can now be pursued with the new recognition of this phenomenon potentially important to the protection of native cells.

Thus, this work expands our understanding of the cell's repertoire of receptor desensitization mechanisms. Immobilization of the receptor on the cell surface in a conformation which insulates it from the effect of acidic medium to dissociate bound ligand represents a newly recognized mechanism for desensitization. Further, this provides clear evidence that the same receptor can be regulated quite differently in different cells. In particular, the native cellular environment may provide an organization and display of interacting molecules which is quite different from transfected cells which are commonly used to evaluate the impact of receptor mutations on receptor regulation. It will be important to better understand these processes, and to incorporate these concepts into future experimental designs.

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