Differential Effects of Modification of Membrane Cholesterol and Sphingolipids on the Conformation, Function, and Trafficking of the G Protein-coupled Cholecystokinin Receptor*

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The lipid microenvironment of receptors can influence their conformation, function, and regulation. Cholecystokinin (CCK)-stimulated signaling is abnormal in some forms of hyperlipidemia, suggesting the possibility of unique sensitivity to its lipid environment. Here we examined the influence of cholesterol and sphingolipids on CCK receptors in model Chinese hamster ovary cell systems having lipid levels modified. Cholesterol was modulated chemically or metabolically, and sphingolipids were modulated using a temperature-sensitive cell line (SPB-1). Receptor conformation was probed with a fluorescent full agonist ligand, Alexa 488-conjugated Gly-[Nle<sup>23,25,31</sup>]-CCK(26–33), shown previously to decrease in anisotropy and lifetime when occupying a receptor in the active conformation (Harikumar, K. G., Pinon, D. L., Wessels, W. S., Prendergast, F. G., and Miller, L. J. (2002) J. Biol. Chem. 277, 18552–18560). Anisotropy and lifetime of this probe were increased and prolonged with cholesterol enrichment, and decreased and shortened with depletion of cholesterol or sphingolipids. The increase in these parameters with cholesterol enrichment may reflect change in CCK receptor conformation toward its inactive, uncoupled state. Indeed, cholesterol enrichment resulted in nonproductive agonist ligand binding, with affinity of binding higher than normal and calcium signaling in response to this reduced. In cholesterol- and sphingolipid-depleted states, the receptor moved into conformations that were less than optimal. With cholesterol depletion, both ligand binding and signaling were decreased, yet internalization and trafficking were unperturbed. With sphingolipid depletion, ligand binding and signaling were normal, but internalization and trafficking were markedly inhibited. Of note, normal transferrin receptor trafficking through the same clathrin-dependent pathway was maintained under these conditions. Thus, lipid microenvironment of the CCK receptor is particularly important, with different lipids having distinct effects.

Membrane lipids are known to play major structural and functional roles in the plasma membrane, where they can have potential effects on the conformation, function, and regulation of receptors and their signaling pathways. Key candidates for study include cholesterol and sphingolipids, with both relatively enriched in the plasma membrane and further concentrated in specialized domains called rafts (2). Those microdomains are of particular interest for receptor biology, because numerous receptors and proximal signaling molecules have been localized there (2, 3). The raft regions are believed to be selective in their protein composition, resulting in specific protein-protein and protein-lipid interactions (4), yet how this is achieved and the specific effects of selected lipid components are unclear.

In this work, we have focused on the cholecystokinin (CCK)<sup>1</sup> receptor. This is a guanine nucleotide-binding protein (G protein)-coupled receptor in the rhodopsin family that plays an important role in nutrient homeostasis, mediating meal-stimulated gallbladder contraction, pancreatic exocrine secretion, and gastrointestinal motility and has even been implicated in contributing to post-cibal satiety (5). Although this receptor is known to reside within the plasma membrane, little is known regarding its specific lipid microenvironment or its structural and functional dependence on that environment. There is indirect evidence to suggest that this receptor may be uniquely sensitive to its lipid environment. The clinical association between certain hyperlipidemias with gallstones and obesity raise the intriguing possibility that these might be linked through an effect of disrupting CCK receptor function. Indeed, in prairie dogs fed a high cholesterol diet (6) and in human gallbladders with cholesterol calculi (7, 8), dysfunction of the CCK receptor has been described. The defect in these systems has been suggested to be at the level of the receptor-G protein interface (9), a physical interaction that occurs within the plasma membrane.

Membrane cholesterol has been shown to be involved in the activation or inactivation of various regulatory proteins, to influence membrane protein interactions, and to affect the function of some plasma membrane receptors (3). Included among these are the receptors for acetylcholine, oxytocin, cholecystokinin/gastrin, rhodopsin, and galanin (10–13). Membrane cholesterol depletion using methyl-β-cyclooctestrin (MβCD) has been shown to impair and to enhance the function.

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1 The abbreviations used are: CCK, cholecystokinin; MβCD, Methyl-β-cyclooctestrin; LPDS, lipoprotein-deficient serum; CCKR, type A cholecystokinin receptor; CHO, Chinese hamster ovary; KRH, Krebs-Ringers/HEPES medium; HMEM, Hanks’ modified Eagles medium.
of different receptors (14). Cholesterol can also play a role in clathrin-mediated endocytosis, a prominent cellular mechanism for receptor desensitization (15).

In the current work, we have examined the influence of membrane cholesterol on the CCK receptor in a model cellular system that has been extensively characterized (16–18). We used two methods to deplete membrane cholesterol and one to increase it. In each experimental situation, we used fluorescence techniques to probe the environment of the ligand as bound to its receptor to gain insight into receptor conformation, as well as studying CCK receptor function and trafficking. CCK receptor conformation was sensitive to the cholesterol content of the membrane, with elevated cholesterol resulting in a change in ligand environment typical of the inactive, uncoupled state. Consistent with this, agonist binding and stimulation of intracellular calcium signaling were clearly inhibited by sphingolipid depletion and increased with cholesterol enrichment. However, agonist-induced signaling was optimal at the normal level of membrane cholesterol and was reduced with either cholesterol depletion or enrichment. Despite these effects on agonist binding and signaling, the internalization and trafficking of the CCK receptor were unperturbed with cholesterol depletion.

There is also precedent for sphingolipids to affect membrane receptor function. In sphingolipid storage diseases such as Niemann-Pick disease, endocytosis and trafficking have been shown to be abnormal (19). Also, sphingolipids like GM1 have been shown to change the conformation of the opioid receptor (20) and binding by nicotinic acetylcholine receptors, lowering their expression and trafficking to the plasma membrane (21). Metabolites of sphingolipids such as sphingosine 1-phosphate are directly involved in signaling, representing an endogenous ligand for the EDG-1 receptor (22).

In this work, we also took advantage of a unique cell line that can be depleted of sphingolipids (23) to explore the relationship of this group of lipids with the CCK receptor. This cell line represents a temperature-sensitive variant of Chinese hamster ovary (CHO-K1) cells (SPB-1 cells) that is normal when grown at the permissive temperature (33 °C), whereas it can be depleted in sphingolipids when grown in sphingolipid-deficient culture medium at nonpermissive temperature (40 °C) (23). For the current series of studies, we established an SPB-1 cell line that stably expressed the type A CCK receptor (SPB-1-CCKR cells). Agonist binding and the calcium responses to CCK were unaffected by sphingolipid depletion in these cells; however, the agonist-induced endocytosis and trafficking of this receptor were clearly inhibited by sphingolipid depletion and were restored to normal upon sphingolipid replenishment. This work establishes distinct roles for different lipids in the plasma membrane in affecting G protein-coupled receptor function and regulation. It provides strong rationale for further detailed analysis of the natural microenvironment of the CCK receptor as it resides in normal healthy cells and in various pathologic states.

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic CCK-8 (the sulfated carboxyl-terminal octapeptide of cholecystokinin) was purchased from Peninsula Laboratories (Belmont, CA). Fura 2-AM, Alexa 488-N-hydroxysuccinimide ester, Alexa 594-transferrin, and the Amplex cholesterol assay kit were from Molecular Probes (Eugene, OR). Cells were plated at a density of 50,000–100,000 cells/well 3 days before the radioligand binding assays. The cells were incubated with 5 pm radioligand (125I-1-Tyr-Gly-[Nle28,31]-CCK-26–33) or 125I-1[Tyr10]-secretin-27) in the absence and presence of increasing concentrations of unlabeled hormone in 0.5 ml of KRH medium at room temperature for 60 min. Non specific binding was defined as radioactivity bound in the presence of 1 μM competing unlabeled hormone. After termination of the binding reaction by
washing the cells twice with ice-cold medium, cells were lysed with 0.5 M NaOH, and the radioactivity in the lysate was quantified by a gamma counter. Data were analyzed using the LIGAND program of Munson and Rodbard (29), and were graphed using the nonlinear least squares curve fitting routine in the Prism suite of programs by GraphPad (San Diego, CA).

Biological Activity—Biological activity was assessed by measurement of intracellular calcium stimulation in response to CCK. This utilized a well established assay of intracellular calcium in Fura-2-loaded CHO-CCKR cells (17). In this assay, ~2 million receptor-bearing cells were loaded with 5 µM Fura-2 AM in Ham’s F-12 medium for 30 min at 37 °C. Cells were then washed and stimulated with varied concentrations of CCK at 37 °C, and fluorescence was quantified in a PerkinElmer Life Sciences LS50B luminescence spectrophotometer. Emission was determined at 520 nm after excitation at 340 and 380 nm, and calcium concentration was calculated from the ratio of the two intensities (29). This method of cellular calcium spectrofluorimetry was utilized to determine the agonist concentration dependence of this biological response. As an additional control, the biological activity of secretin in cholesterol-depleted CHO-secR cells was monitored by measuring cAMP after stimulation with secretin, as we have described previously (30).

Fluorescence Spectroscopy—Steady-state fluorescence spectra were recorded on a SPEX Fluorolog fluorometer (SPEX Industries, Edison, NJ) at 20 °C using a 1-mL quartz cuvette. Fluorescence measurements were performed in cells at ambient temperature. Buffer was degassed by bubbling nitrogen to prevent quenching of fluorescence by soluble oxygen. Under the conditions employed, fluorescence was stable to photobleaching. Unlabeled cells were used to correct background fluorescence and light scatter. Cells were disdissolved by using cell dissociation buffer (Invitrogen) pelleted, and resuspended in Fura-2AM permeabilization buffer. Cells were then labeled with 50 nM Alexa 488-CCK in KRH buffer, pH 7.4, for 2 h at 4 °C. The cell suspension was then centrifuged and washed repeatedly with ice-cold buffer to remove unbound ligand and then resuspended in ice-cold buffer for fluorescence measurements.

Fluorescence Anisotropy and Lifetime Measurements—Anisotropy measurements were recorded using an Edinburgh spectrofluorophotometer equipped with polarizers and a thermosmotically regulated cuvette. Measurements were performed with constant optimal wavelengths for excitation and emission of the fluorophore. Emission intensities were measured with excitation side polarizer in the vertical position (V) and emission side polarizer in the horizontal (H) and vertical (V) positions. Excitation wavelengths were maintained at 482 nm. The measurements were performed at both 4 °C and 30 °C. Anisotropy was calculated according to the equation, A = I_{HH} - G(I_{VV} + 2I_{HV})/2, where I_{HH} is the intensity measured with both the excitation side and emission side polarizers in the vertical positions, and I_{HV} is the intensity measured with the excitation side polarizer in the vertical position and the emission side polarizer in the horizontal position. The value of G was calculated by the equation G = I_{HV}/I_{HH}. 

The fluorescence lifetime measurements for the Alexa 488-Gly-[Nle]AlCC (26–33) (Alexa 488-CCK) bound to receptor was measured as described previously using time-resolved fluorescence spectroscopy (1). Briefly, cells labeled with Alexa 488-CCK were excited using a pulse-picked, frequency-doubled titanium-sapphire picosecond laser source (Coherent Mira 900, Palo Alto, CA). Fluorescence emission was collected through interference filters with 6.8-nm bandwidth. Measurements were taken at 4 °C. The excitation wavelength was tunable with a pulse width of ~2 picoseconds full-width at half-maximum. Data were collected in 1080 channels, with a width of 10.05 picoseconds/channel. Fluorescence intensity decay analysis was performed using the GLOBALS Unlimited program package (31). Models of a single exponential and two discrete exponential lifetime components were utilized. The quality of fit was judged by the value of χ² statistics. Analysis of these measurements has been fully validated previously (1).

CCK Receptor Internalization, Trafficking, and Recycling—Agonist-stimulated CCK receptor internalization, trafficking, and recycling were assayed morphologically using a fluorescent ligand that has been fully validated and characterized previously (16). The fluorescent ligand, Alexa 488-CCK, is a full agonist and binds to the CCK receptor with high affinity. In short, for internalization studies, cells grown on coverslips were incubated with 50 nM of Alexa 488-CCK for 1.5 h at 4 °C to saturate surface receptors. The cells were then warmed to 37 °C for various times before washing and fixing with 2% paraformaldehyde. The cells were examined using a Zeiss 510 confocal microscope using ×63 1.4 numerical aperture oil immersion lens.

For studying recycling of internalized receptors back to the cell surface, cells were incubated with 50 nM CCK for 1 h at 4 °C (saturation of surface receptors with nonfluorescent ligand). Cycloheximide (150 µg/ml) was added during the final 15 min of the incubation, and it was maintained for subsequent incubations. Cells were washed with 37 °C PBS buffer and incubated for times ranging from 0 to 90 min. At appropriate time periods, cells were rinsed with ice-cold PBS buffer, before labeling surface receptors with 50 nM Alexa 488-CCK at 4 °C for 1 h. This assay has been validated previously (16).

SPB-1-CCKR cells grown on glass coverslips were treated with inhibitors of endocytosis as follows. (a) Cells were incubated with 6 µg/ml of chlorpromazine, a known inhibitor of clathrin-dependent endocytosis, in Hanks’ modified Eagles’ medium (HMEM) for 30 min at 37 °C. The cells were then labeled with 50 nM fluorescent CCK at 4 °C for 90 min, washed, and incubated for 20 min at 37 °C in the presence of chlorpromazine. (b) Cells were incubated with 25 µg/ml nystatin, a known inhibitor of internalization via caveolae, in serum-free HMEM for 30 min at 37 °C, washed, with HMEM containing 25 µg/ml nystatin, and then labeled with 50 nM fluorescent CCK at 4 °C for 90 min, washed, and incubated for 20 min at 37 °C in the presence of nystatin. For each inhibitor treatment, cell viability was greater than 90% (32).

Co-localization of CCK Receptor with the Transferrin Receptor—SPB-1-CCKR cells were washed with HMEM and incubated with Alexa 488-CCK and 30 µg/ml of Alexa Fluor 594-labeled transferrin at 4 °C for 90 min, washed, and incubated at 37 °C for 90 min. Samples were then assayed morphologically using a fluorescent ligand that has been fully validated previously (16). Bovine serum albumin complex of lactosyl cerebroside or sphingomyelin was made as described previously (33, 34). This was added to cells in culture to make a final concentration of 5 µM and incubated overnight. Fluorescence microscopy was performed with an Olympus IX70 fluorescence microscope, as described (34). Quantitative image analysis was performed using Metamorph software (Universal Imaging, Media, PA).

Analysis—Data were analyzed using Student’s t test for unpaired values. Significant differences were considered to be at the p < 0.05 level.

RESULTS

Manipulation of Cellular Lipid Levels—Each method for manipulation of cellular cholesterol content (depletion or augmentation) achieved its stated goal. The chemical method of cholesterol depletion was more effective (~40% depletion) than the metabolic method (~32% depletion). Cellular cholesterol was analyzed for each of these manipulations (in µg/mg protein, means ± S.E. of data from four independent experiments: control, 42.3 ± 6.1; MjCD chelator, 24.1 ± 4.5, p < 0.01; LPDS plus hydroxymethylglutaril-CoA reductase inhibitor, 28.6 ± 5.4, p < 0.01). Plasma membrane cholesterol levels were also examined qualitatively by fluorescence microscopy using the polyene antibiotic, filipin. Fig. 1 shows that filipin staining correlated with the expected and assayed levels of cholesterol for each experimental condition employed.

The SPB-1-CCKR cells were utilized for modification of sphingolipid levels. When grown at the permissive temperature (33 °C), these cells had normal composition and behaved like normal CHO cells, whereas growth at the nonpermissive temperature (40 °C) resulted in a significant reduction in levels of endogenous sphingolipid. Hanada et al. (23) demonstrated previously that growing these cells in sphingolipid-deficient medium for 48 h leads to approximately an 80% decrease in sphingolipid content relative to that in wild type cells. Parental CHO-CCKR cells were also grown at 40 °C and studied as a control to demonstrate that there were no other relevant non-specific metabolic effects of this treatment. Of note, CHO cells grown at this nonpermissive temperature have been shown previously (23) to have no adverse effects on the synthesis of membrane lipids or in any change in cell viability.

Characterization of CCK Receptor Function in Different Cellular Lipid Environments—Cholesterol depletion either with MjCD or by growing the CHO-CCKR cells in LPDS medium supplemented with metabolic inhibitors resulted in a shift to lower affinity binding of CCK. This reached statistical significance (p < 0.01) for both the MjCD- and LPDS-treated cells (Fig. 2 and Table I). This correlated with the levels of cholesterol depletion achieved by these two treatments. In contrast,
the binding affinity of CCK was increased with augmentation of cholesterol levels in the cells (Fig. 2 and Table I). There was no significant change in the density of binding sites on the cells undergoing these treatments. Control studies with the CHO-SecR cells revealed no effect of cholesterol depletion using MβCD on secretin receptor binding (Fig. 2).

The SPB-1-CCKR cells bound CCK with normal affinity both when grown at the permissive and nonpermissive temperatures. Sphingolipid depletion of these cells caused no significant change in the CCK binding affinity (Fig. 2), although there was a significant decrease in the apparent density of binding sites (Table I). This is likely explained by the influence of this treatment on CCK receptor trafficking (shown below).

Signaling originating at the CCK receptor was assessed by the ability of CCK to stimulate an increase in intracellular calcium. The magnitude of intracellular calcium responses in the CHO-CCKR cells was decreased for each of the conditions of cholesterol modulation (peak intracellular calcium responses, nm: control, 168 ± 21; MβCD, 97 ± 11; LPDS, 98 ± 32; cholesterol enrichment, 58 ± 12). Consistent with the apparent decrease in receptor binding affinity in cholesterol-depleted cells, there was an approximate 8-fold decrease in the potency of CCK to stimulate an intracellular calcium response (Fig. 2). This reached statistical significance for both methods of cholesterol depletion (p < 0.01). Repletion of cholesterol after the MβCD treatment reversed this effect. Of note, a further increase of cholesterol levels beyond normal also resulted in a decrease in potency of CCK to stimulate a calcium response of magnitude similar to that observed in the cholesterol-depleted cells. As an additional control, cholesterol depletion of the CHO-SecR cells using MβCD had no effect on secretin-stimulated cAMP (Fig. 2). Consistent with the binding studies, CCK stimulated a normal intracellular calcium response in the SPB-1-CCKR cells, when grown at permissive and nonpermissive temperatures (Fig. 2 and Table I).

Fluorescence Analysis of the CCK Receptor in Different Cellular Lipid Environments—To explore the conformation of the CCK receptor in lipid-modulated cells, the fluorescence properties of a receptor-bound fluorescent agonist probe, Alexa 488-Gly-[Nle\textsuperscript{28,31}]CCK-(26–33) (Alexa 488-CCK), were monitored. This fluorescent ligand has been characterized previously as a potent and fully efficacious agonist that binds specifically and saturably to the CCK receptor (1). We measured the fluorescence anisotropy and lifetime of this fluorescent probe when bound to receptors in each of the CCK receptor-bearing cells (Fig. 3, Table II).

Membrane cholesterol is a major component of the plasma membrane that is known to affect physical parameters such as fluidity (35). Cholesterol depletion of the CHO-CCKR cells decreased the anisotropy of this probe, whereas excess cholesterol increased its anisotropy (Fig. 3). Similarly, measurements demonstrated that the mean fluorescence lifetime of the probe was shorter than normal in cholesterol-depleted cells, whereas cholesterol augmentation resulted in a longer lifetime (Fig. 3). The increase in fluorescence anisotropy and lifetime observed in cholesterol enrichment may reflect the change in CCK receptor conformation toward its inactive, uncoupled state, as demonstrated previously (1).

The fluorescence properties of CCK receptor-bound Alexa 488-CCK were also analyzed in the SPB-1-CCKR cells. Fluorescence anisotropy and lifetime of this receptor-bound probe were significantly decreased when the cells were grown at the nonpermissive temperature (40 °C) (Fig. 3).

Internalization of the CCK Receptor in Cells Having Different Lipid Compositions—Clathrin-dependent internalization has been shown to be the major cellular endocytic pathway for many G protein-coupled receptors, including the CCK receptor (16). Cholesterol is believed to play a key role in the endocytosis of some membrane proteins (15); however, its contribution to G protein-coupled receptor vesicular trafficking has not yet been established. Internalization and trafficking were investigated morphologically by using confocal microscopy, following Alexa 488-CCK after binding to receptors on the cell surface and allowing internalization to take place for various times at 37 °C. Results showed that CCK receptors internalized and trafficked normally after agonist occupation and that this was unaffected by cholesterol depletion (10 mM MβCD, 30 min, 37 °C) or cholesterol enhancement relative to control cells (Fig. 4).

Recent studies have also shown that sphingolipids may be endocytosed through clathrin-dependent and -independent mechanisms (34). Therefore, it was of substantial interest to explore CCK receptor endocytosis and trafficking under conditions of manipulation of sphingolipids. Indeed, under control conditions (grown at the permissive temperature), CCK receptors on SPB-1-CCKR cells were promptly internalized and trafficked normally in response to agonist occupation, following the same time course as observed in the CHO-CCKR cells (Fig. 4). Of note, these processes were quite sensitive to sphingolipid depletion, with the process of endocytosis and trafficking disrupted when the cells were grown at the nonpermissive temperature (40 °C) (Fig. 4).

As expected, the pattern of endocytosis was markedly affected by interference with clathrin in the SPB-1-CCKR cells. Chlorpromazine treatment largely eliminated CCK-induced internalization at the permissive temperature, whereas nystatin that acts via inhibition of internalization through caveolae had no effect (Fig. 5).

Studies were also performed using fluorescent transferrin to track the transferrin receptor, another receptor known to be endocytosed via a clathrin-dependent pathway (36) (Fig. 6). Both CCK receptors and transferrin receptors were extensively co-localized after each of the cholesterol manipulations in the CHO-CCKR cells. The percentages of endosomes staining for both CCK and transferrin after 30 min of agonist treatment...
FIG. 2. CCK binding and biological activity in lipid-modulated cells. Shown are competition-binding curves (left) for binding of a CCK-like radioligand to cholesterol (Chol)-modulated CHO-CCKR cells (top) and sphingolipid-modulated SPB-1-CCKR cells (bottom), and for binding of a secretin-like radioligand to cholesterol-modulated CHO-SecR cells (middle). Values reflect saturable binding as a percentage of that occurring in the absence of competitor. Points represent means ± S.E. of data from 3 to 5 independent experiments performed in duplicate. Shown also are curves representing the concentration dependence for CCK to stimulate intracellular calcium responses (right) in cholesterol-modulated CHO-CCKR cells (top) and sphingolipid-modulated SPB-1-CCKR cells (bottom), and for secretin to stimulate cAMP in cholesterol-modulated CHO-SecR cells (middle). Values were normalized to maximal calcium responses under each condition. Points represent means ± S.E. of data from 3 to 5 independent experiments performed in duplicate.

**TABLE I**

<table>
<thead>
<tr>
<th>Condition</th>
<th>$K_i$ (nM)</th>
<th>No. sites/cell $\times 10^5$</th>
<th>Stimulation of intracellular Ca$^{2+}$ $EC_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.4 ± 0.5</td>
<td>0.85 ± 0.1</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>MJICD</td>
<td>8.7 ± 0.3$^a$</td>
<td>0.71 ± 0.14</td>
<td>0.37 ± 0.01$^a$</td>
</tr>
<tr>
<td>LPDS</td>
<td>5.9 ± 1.2$^a$</td>
<td>0.53 ± 0.11</td>
<td>0.21 ± 0.04$^a$</td>
</tr>
<tr>
<td>Excess cholesterol</td>
<td>1.2 ± 0.4$^a$</td>
<td>0.70 ± 0.09</td>
<td>0.21 ± 0.01$^a$</td>
</tr>
<tr>
<td>MJICD + cholesterol</td>
<td>ND$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPB-1-CCKR (33 °C)</td>
<td>2.7 ± 0.5</td>
<td>2.02 ± 0.38</td>
<td>0.08 ± 0.02$^a$</td>
</tr>
<tr>
<td>SPB-1-CCKR (40 °C)</td>
<td>1.6 ± 0.4</td>
<td>0.44 ± 0.14$^a$</td>
<td>0.12 ± 0.03</td>
</tr>
</tbody>
</table>

$^a$ p < 0.01 versus control, representing normal CHO cells in the cholesterol studies and SPB-1-CCKR cells at 33 °C for the sphingolipid studies.

$^b$ ND indicates not determined.
were as follows: control without lipid modification, 86 ± 5%; MβCD treatment, 93 ± 2%; LPDS treatment, 96 ± 1%, and excess cholesterol, 89 ± 2% (not significantly different from each other, \( p > 0.05 \)). This further supports our conclusion that cholesterol modulation did not affect CCK receptor internalization or targeting.

We also monitored the internalization of the transferrin receptor in the SPB-1-CCKR cells (Fig. 6). Both the CCK receptor and the transferrin receptor were endocytosed and trafficked normally, being co-localized in recycling endosomes in these cells under the control conditions. However, under conditions of sphingolipid depletion, the internalization and trafficking of the CCK receptor was clearly disrupted, whereas the transferrin receptor continued to be internalized and to traffic normally (Fig. 6). The abnormality of CCK receptor internalization and trafficking was reversed by supplementation of these cells with sphingolipid analogues such as lactosyl cerebroside or sphingomyelin. Under these conditions, co-localization of the CCK and transferrin receptors was restored (Fig. 6). The percentages of co-localization observed between CCK and transferrin receptors were 71% with lactosyl cerebroside and 72% with sphingomyelin.

Because our studies demonstrated that CCK receptor internalization and trafficking were perturbed by sphingolipid depletion and that supplementation of sphingolipids corrected this defect, we also studied the effect of sphingolipid depletion on receptor recycling (Fig. 7). The results showed that ~90% of internalized CCK receptors were able to recycle to the cell surface of SPB-1-CCKR cells grown at the nonpermissive temperature in the presence of sphingomyelin, whereas this was

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**TABLE II**

Fluorescence lifetime distribution of CCK receptor-bound Alexa488-CCK in lipid-modified CHO cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>( \tau_1 ) (ns)</th>
<th>( \tau_2 ) (ns)</th>
<th>( \chi^2 )</th>
<th>Average lifetime (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>4.01 ± 0.13</td>
<td>0.79 ± 0.04</td>
<td>ns</td>
<td>1.08 ± 0.03</td>
</tr>
<tr>
<td>MβCD</td>
<td>4.04 ± 0.09</td>
<td>0.71 ± 0.02</td>
<td>ns</td>
<td>1.10 ± 0.01</td>
</tr>
<tr>
<td>LPDS</td>
<td>3.97 ± 0.04</td>
<td>0.77 ± 0.005</td>
<td>ns</td>
<td>1.05 ± 0.03</td>
</tr>
<tr>
<td>Excess cholesterol</td>
<td>4.05 ± 0.05</td>
<td>0.81 ± 0.005</td>
<td>ns</td>
<td>1.07 ± 0.03</td>
</tr>
<tr>
<td>SPB-1-CCKR (33 °C)</td>
<td>3.90 ± 0.05</td>
<td>0.84 ± 0.02</td>
<td>ns</td>
<td>1.11 ± 0.08</td>
</tr>
<tr>
<td>SPB-1-CCKR (40 °C)</td>
<td>3.76 ± 0.01</td>
<td>0.73 ± 0.01</td>
<td>ns</td>
<td>1.00 ± 0.04</td>
</tr>
</tbody>
</table>

\( ^a p < 0.01 \) versus control, representing normal CHO cells in the cholesterol studies and SPB-1-CCKR cells at 33 °C for the sphingolipid studies.
also quite impaired when these cells were deprived of sphingo-
lipids. Once again, this impairment was corrected by supple-
menting the cells with sphingomyelin (Fig. 7).

**DISCUSSION**

Until recently, G protein-coupled receptors were believed to
function as single molecules, only interacting with their het-
erotrimeric G protein proximal effectors after agonist occupa-
tion. The lipid environment of these receptors and the recep-
tor-G protein interface were considered to be relatively
unimportant, with the vast majority of receptors working simi-
larly in their natural cellular environments and when ex-
pressed in any of a long series of tissue culture cell lines.
Indeed, this has been the basis of expression cloning that was
the way many of these receptors were originally identified, and
it is how the pharmacological characteristics of many of these
receptors have been investigated (37, 38).

More recently, it has become clear that G protein-coupled
receptors can interact with a number of other membrane pro-
teins in addition to the heterotrimeric G proteins, and that the
lipid environment can affect these molecular interactions (39–
41). G protein-coupled receptors can interact with each other in
the plasma membrane to form homodimers and can interact
with some other structurally related members of this super-
family to form heterodimers (42). They can also interact with a
number of other membrane proteins, such as receptor-associ-
ated membrane proteins that have the potential to modify
and/or regulate their function (40). It has been proposed that
the lipid environment is critical for these molecular interac-
tions in the plasma membrane to occur (43). One of the most
interesting of such environments is the lipid rafts, known to be
enriched in cholesterol and sphingolipids relative to the rest of
the plasma membrane (2). Indeed, receptor environment may
be one of the critical determinants of distinct receptor function
and regulation in different cell types (6, 9).

The CCK receptor may be unusually sensitive to its lipid
environment. This could explain the reduced responsiveness to
CCK in some experimental models, such as the prairie dog fed
a high cholesterol diet (6). This could even play a role in the
clinical setting of the high incidence of cholesterol gallstones in
patients with hyperlipidemia and with obesity (9, 44). Previous
investigations have demonstrated that patients with choles-

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**FIG. 4. Cellular trafficking of the CCK receptor.** Shown are the represent-
ative fluorescence images of Alexa 488-
CCK internalization over time for CHO-
CCKR cells under control conditions and
with modification of cholesterol content
(A) and SPB-1 cells under control and
sphingolipid-modified conditions (B).
Cells internalized the ligand normally un-
der each of the cholesterol-modified con-
ditions, whereas sphingolipid depletion
markedly inhibited internalization. Im-
ages are representative of 4 independent
experiments. Bar, 20 μm.

**FIG. 5. Clathrin-dependent internalization of the CCK recep-
tor.** Shown are the effects of chlorpromazine and nystatin on CCK
receptor internalization in SPB-1-CCKR cells. After occupying receptor
with fluorescent CCK (50 nM Alexa 488-CCK) at 4 °C, the cells were
washed with iced buffer and incubated for 15 min at 37 °C. Untreated
cells were used as a control.
terol gallstones have a normal CCK receptor gene and normal splicing of the mRNA for this receptor (8), although a rare patient with what appears to represent a familial syndrome of early onset gallstones and morbid obesity was shown to have abnormal splicing of a normal CCK receptor gene (45). Similarly, the G protein effector has been found to be normal in patients with cholesterol gallstone disease (46). However, the phosphatidylinositol response to CCK has been found to be abnormal in this clinical setting, suggesting a possible problem at the level of the receptor-G protein interface (9, 46). It is noteworthy that gallbladder muscularis membrane cholesterol has been demonstrated to be elevated in patients with cholesterol gallstones relative to those with pigment gallstones (7).

In the current work, we have performed an extensive analysis of the CCK receptor and its function in lipid-modified environments. Of particular interest were the fluorescence studies that revealed a change in CCK receptor conformation from the control state, with a clear change in the environment and mobility of the fluorescent indicator. The cholesterol-enriched condition was associated with the receptor in the inactive, uncoupled conformation. Cholesterol is known to induce changes in phospholipid packing in lipid bilayers, and it could thereby alter CCK receptor conformation. Cholesterol could also modify the interaction of this receptor with its effector or regulatory proteins. An analogous effect was reported previously for rhodopsin, where depletion or enrichment of cholesterol perturbed rod disk membranes and inhibited its activation (13).

Our results showed that cholesterol levels in the plasma membrane affect CCK receptor binding and signaling. Cholesterol depletion significantly decreased the affinity of agonist binding to this receptor. The CCK receptor is normally coupled with Gq and elicits a phospholipase C response, resulting in an increase in intracellular calcium. Changes in cholesterol levels, including both depletion and enrichment from normal levels, interfered with the ability of the CCK receptor to stimulate intracellular calcium.

Many receptors, including the G protein-coupled CCK receptor (16), are internalized via clathrin-dependent endocytosis. Cholesterol is believed to contribute to the formation of clathrin-coated vesicles (15), and acute cholesterol depletion has been reported to decrease budding of clathrin-coated pits and to reduce the rate of endocytosis (47). This treatment may also change the size and shape of cells, as a result of the effect of cholesterol on lipid dynamics, membrane curvature, thickness, and tension (47). However, under the conditions of cholesterol modulation used in the present study, there was no effect

**FIG. 6.** Co-localization of CCK receptors with transferrin receptors. Shown are representative images of CCK receptor co-localization with transferrin receptor in cholesterol (Chol)- and sphingolipid-modulated CHO-CCKR cells. Cells were incubated with 50 nM Alexa 488-CCK and Texas Red-transferrin for 1.5 h at 4 °C. After washing the cells with iced buffer, they were incubated for 15 min at 37 °C and fixed using paraformaldehyde. Bar, 20 μm. LacCer, lactosyl cerebroside; SM, sphingomyelin.

**FIG. 7.** Sphingolipid feeding restores the recycling of CCK receptors in SPB-1-CCKR cells. Shown are representative images of the CCK receptor in SPB-1-CCKR cells at 33, 40, and at 40 °C after being fed sphingomyelin. In the recycling assay, the cell surface receptors were first saturated by incubation with 50 nM nonfluorescent CCK at 4 °C for 1 h, followed by washing with buffer at 4 °C. Internalization was then initiated by warming the cells to 37 °C for different times. At each time point, the cells were cooled to 4 °C, and the receptor on the cell surface was labeled with Alexa 488-CCK. The control cells that were not preincubated with CCK illustrate the starting density of receptors. Bar, 20 μm.
observed on the internalization and sorting of the CCK receptor. Because membrane cholesterol and sphingolipids are often co-regulated, it is possible that influence on other lipid components of the plasma membrane might have an additional role in affecting CCK receptor function. Depletion of cellular sphingolipids modified agonist-stimulated CCK receptor endocytosis and trafficking, with these abnormalities reversed upon replenishment of the sphingolipids. In contrast to the cholesterol studies, there was no effect of modulation of sphingolipids on agonist ligand binding affinity or initiation of signaling by CCK in these cells. It is interesting that this manipulation also resulted in a modification of the fluorescence characteristics of the probe from the control state, but this does not suggest that the conformational change implied by this was the same as that observed after manipulation of membrane cholesterol. Indeed, the observation that one lipid modification affected ligand binding without having an effect on trafficking, whereas the other affected trafficking without having an effect on ligand binding, is consistent with differences in receptor conformation induced by the two lipid modifications.

Most experimental focus to date has been on the cholesterol composition of the membrane to affect CCK receptor function (6, 7, 9, 11). We now see that other lipid components are important as well. Here we chose sphingolipids to study because they parallel the general cellular distribution of cholesterol. Both are enriched in the plasma membrane and are reduced in concentration in intracellular membranes. Both are substantially enriched in specialized domains within the plasma membrane known as rafts. The raft domains likely reflect the strong capacity of lipids to organize themselves, with immiscibility driving phase separation and yielding domains of unique composition and biophysical properties. There is even a preferential interactivity between sphingolipids and sterols, held together by Van der Waals forces. Despite the active membrane trafficking between the plasma membrane and endocytic compartments of the cell, the unique lipid compositions of each compartment are maintained.

In addition to the structural studies defining the differences in lipid composition of various organelles and domains, there are also functional studies that support unique roles for specific lipids. The plasma membrane rafts are an excellent example of this. These domains have been implicated in compartmentalization, regulation, and integration of signaling events, facilitated by such a platform (2, 4, 48). This is also a focus for studies of different routes of endocytosis and exocytosis. Caveolae likely originate in raft domains (48). Similarly, SNARE proteins are concentrated there and provide sites for docking and fusion of exocytic vesicles (49).

It is unclear whether the observed effects of modification of cholesterol and sphingolipids reflect events occurring in the bulk phase of the plasma membrane or within rafts. However, these insights add a new dimension to our understanding of CCK receptor biology. We know that this receptor can function by guest on August 16, 2017 http://www.jbc.org/ Downloaded from

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