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Membrane Cholesterol Affects Stimulus-Activity Coupling in Type 1, but not Type 2, CCK Receptors: Use of Cell Lines with Elevated Cholesterol

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Abstract

The lipid microenvironment of membrane proteins can affect their structure, function, and regulation. We recently described differential effects of acute modification of membrane cholesterol on the function of type 1 and 2 cholecystokinin (CCK) receptors. We now explore the regulatory impact of chronic cholesterol modification on these receptors using novel receptor-bearing cell lines with elevated membrane cholesterol. Stable CCK1R and CCK2R expression was established in clonal lines of 25RA cells having gain-of-function in SCAP [sterol regulatory element binding protein (SREBP) cleavage-activating protein] and SRD15 cells having deficiencies in Insig-1 and Insig-2 enzymes affecting HMG CoA reductase and SREBP. Increased cholesterol in the plasma membrane of these cells was directly demonstrated, and receptor binding and signaling characteristics were shown to reflect predicted effects on receptor function. In both environments, both types of CCK receptors were internalized and recycled normally in response to agonist occupation. No differences in receptor distribution within the membrane were appreciated at the light microscopic level in these CHO-derived cell lines. Fluorescence anisotropy was studied for these receptors occupied by fluorescent agonist and antagonist, as well as when tagged with YFP. These studies demonstrated increased anisotropy of the agonist ligand occupying the active state of the CCK1R in a cholesterol-enriched environment, mimicking fluorescence of the uncoupled, inactive state of this receptor, while there was no effect of increasing cholesterol on fluorescence at the CCK2R. These cell lines should be quite useful for examining the functional characteristics of potential drugs that might be used in an abnormal lipid environment.

Keywords

Cholecystokinin; G protein-coupled receptors; Receptor internalization; Receptor recycling; Receptor regulation

Introduction

Cholesterol is a critically important component of the plasma membrane, affecting its dimensions, organization, and fluidity [1]. These characteristics can have a substantial impact on the structure, function, and regulation of intrinsic membrane proteins, including

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some important membrane receptors [2–4]. Given the strategically important position of receptors within the plasma membrane, where they sense the extracellular environment including the presence of hormones and neurotransmitters and where they activate intracellular signaling events that regulate cellular function, such effects of membrane cholesterol can have profound physiologic implications.

Much of our current understanding of the impact of membrane cholesterol on receptors comes from model cell systems in which the cholesterol composition is acutely modified by chemical means. This is most often accomplished using cholesterol-loaded methyl- β -cyclodextrin (M β CD) or lipoproteins to transfer excess cholesterol into the membrane or, conversely, using M β CD to bind and extract cholesterol from the membrane [5, 6]. While such experimental approaches can demonstrate some effects of this lipid on receptor function, they may not represent adequate models to reflect stable elevated levels of membrane cholesterol, as have been described to exist in cells of patients with hypercholesterolemia and obesity [7, 8], where such an abnormality might affect receptor function, regulation, and trafficking. Receptor-bearing cells with a stable abnormal lipid environment are required to study meaningfully such potentially important regulatory events. Cell lines with elevated levels of membrane cholesterol could also be quite helpful for the analysis of conformational changes induced by different ligands and for the screening of potential receptor-active drugs.

In the current work, two Chinese hamster ovary (CHO) cell lines that have been engineered to exhibit higher than normal levels of plasma membrane cholesterol were utilized [9, 10]. These were further engineered to stably express type 1 and type 2 CCK receptors, providing the opportunity to study CCK receptors in these cellular environments. These cell lines reproduced the functional impact of cholesterol on the type 1 CCK receptor and the lack of functional impact of cholesterol on the structurally closely related type 2 CCK receptor that had been described previously after acute manipulations [5, 6]. They provided the opportunity to examine the effects of an abnormally elevated membrane cholesterol environment on the cellular handling of these receptors and on their regulation. These insights are important in consideration of how to manipulate these hormonal systems in vivo in clinical settings in which membrane cholesterol is abnormally elevated.

Materials and Methods

Materials

Cholecystokinin octapeptide (identified as CCK-26-33, based on the numbering of CCK-33, also known as CCK-8) was purchased from Peninsula Laboratories (Belmont, CA). The fluorescent CCK analogues representing full agonist, alexa⁴⁸⁸-Gly-(CCK-26-33) (alexa-CCK), and antagonist, alexa⁴⁸⁸-Gly-[(^D-Trp³⁰)CCK-26-32]-phenylethylester (alexa-^D-Trp-OPE), were synthesized and purified to homogeneity in our laboratory [11]. The CCK-like tracer, ¹²⁵I-^D-Tyr-Gly-[(Nle^{28,31})CCK-26-33], was prepared by oxidative radioiodination using iodination beads (Pierce, Rockford, IL), as we have previously described [11]. Cycloheximide, Dulbecco's modified Eagle's medium/F12 medium mixture, Ham's F-12 medium, OptiMEM medium, L-glutamine, lipofectamine LX reagent, and Fura 2-AM, were from Invitrogen (Carlsbad, CA). Fetal clone II tissue culture medium supplement was from Hyclone Laboratories (Logan, UT), and the lipoprotein-deficient serum (LPDS) was obtained from Intracel (Frederick, MD). Filipin, guanosine 5'-[β , γ -imido]triphosphate trisodium salt (GppNHp), 25-hydroxycholesterol, methyl- β -cyclodextrin (M β CD), and probenecid were from Sigma chemicals (St Louis, MO). Costar 96-well black assay plates with clear bottoms and V-bottoms were from Corning (Corning, NY). All other reagents were of analytical grade.

Cell Culture

Chinese hamster ovary (CHO) cell lines engineered to express the human type 1 CCK receptor (CHO-CCK1R) and type 2 CCK receptor (CHO-CCK2R) were used as sources of receptors in a normal membrane environment in this study. These cell lines had previously been fully characterized, establishing the expression of fully functional receptors that bind CCK, signal, and are internalized in a normal manner [12]. CHO cell lines were grown in tissue culture flasks containing Ham's F-12 medium supplemented with 5 % fetal Clone II in a humidified environment containing 5 % carbon dioxide. Two distinct CHO cell variants with elevated levels of cholesterol were also utilized. 25RA cells [9] were a generous gift from Dr. Ta Yuan Chang (Dartmouth Medical School, Hanover, NH), and SRD15 cells [10] were a generous gift from Dr. Russell DeBose-Boyd (University of Texas Southwestern Medical center, Dallas, TX). The 25RA cells have elevated membrane cholesterol secondary to a gain-of-function defect in the SCAP [sterol regulatory element binding protein (SREBP) cleavage-activating protein] gene [9]. These cells were maintained in DMEM/F12 medium supplemented with 10 % fetal clone II, and were treated with 25-hydroxy cholesterol every ten passages to avoid any reversion of the cells. The SRD15 cells have elevated cholesterol as a result of deficiency in both Insig-1 and Insig-2 enzymes that affect HMG CoA reductase and SREBP [10]. These cells were maintained in DMEM/F-12 medium supplemented with 5 % lipoprotein-deficient serum and 25-hydroxy cholesterol. Cells were passaged approximately two times per week.

Stable Expression of CCK Receptor in Model Cell Lines

25RA and SRD15 cell lines that stably express human type 1 or type 2 CCK receptors (25RA-CCK1R, 25RA-CCK2R, SRD15-CCK1R, and SRD15-CCK2R) were prepared by transfection, antibiotic selection, and cloning. The parental cell lines did not express either type of CCK receptor. Cells were seeded in 10-cm dishes 24 h prior to transfections and were allowed to grow to semiconfluence. They were then transfected with 5 μ g of receptor cDNA in pcDNA3.1-Zeo using Lipofectamine LX reagent in OptiMEM medium following manufacturer's instructions. Transfectants were selected with 1.0 mg/ml zeocin and were cloned by limiting dilution. Receptor expression in the cell lines was directly characterized using radioligand binding assays (described below).

Membrane Cholesterol Modification

Receptor-bearing cells were washed with Krebs-Ringers-HEPES (KRH) medium containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM KH_2PO_4 , 1.2 mM MgSO_4 , 0.01 % soybean trypsin inhibitor, and 0.2 % of bovine serum albumin (BSA), and used for binding, biological activity and fluorescence spectroscopy. Membrane cholesterol levels in some cells were modified by depletion of cholesterol during incubation with 7.5 mM M β CD in KRH medium (pH 7.4) for 30 min at 37 °C with slow shaking.

Membrane Cholesterol Determination

Plasma membrane cholesterol levels in intact cells were determined using a flow cytometric assay with a fluorescent, cell impermeable probe recently described, alexa⁶⁶⁰-perfringolysin domain 4 (alexa-PFO domain 4) [6]. For this, the semi-confluent cells in culture were lifted using non-enzymatic cell dissociation solution (Sigma-Aldrich), washed with phosphate-buffered saline (PBS, pH 7.4), and resuspended in PBS supplemented with 0.5 % BSA and 0.01 % sodium azide at a density of 5×10^6 cells/ml. Cells were labeled with alexa-PFO domain 4 (10 μ g protein/ml) at room temperature for 20 min. After incubation, cells were pelleted, resuspended in medium, and then analyzed using a Cyan flow cytometer (Beckman Coulter, Brea, CA). Stained cells were gated on forward and side scatter to eliminate dead cells and debris, and were further gated for receptor expression. Control studies were

performed using unstained CHO cells to establish conditions to gate the forward scatter signal. Of note, specific importance was given to forward-scatter gating to ensure that cells being analyzed for fluorescence were in the appropriate size range. Raw data were analyzed using FloJo software (Tree Star, Ashland, OR).

Cell membrane cholesterol content was also determined using filipin staining of formaldehyde-fixed cells, as previously described [5], as well as a biochemical assay of cholesterol content within cell membranes using the Amplex Red reagent [13]. In short, cells were fixed in 2 % formaldehyde in PBS for 30 min followed by incubation in PBS containing 100 μ g/ml filipin for 30 min at room temperature. The stained cells were washed three times with 0.01 M glycine and three times with PBS. The coverslips were mounted on a slide and visualized using a Zeiss Axiovert 200 M inverted epifluorescence microscope (Thornwood, NY) with DAPI filter setting (excitation 360 nm, dichroic mirror 400 lp, emission 460 nm) (Chroma Technology Corp., Brattleboro, VT). The Amplex Red assay for cholesterol content was performed on a lipid extract from a particulate fraction [14] derived from CCK receptor-bearing cells. Membrane protein content was determined in the same particulate fraction using a BCA kit [15], allowing the cholesterol content to be expressed as micrograms of cholesterol per milligram of protein.

Isolation of Cell Membranes

Membrane fractions enriched in receptor content were isolated from receptor-bearing cell lines, as described previously [16]. Cells reaching 80–90 % confluence were harvested mechanically using a cell scraper and were suspended in PBS. The membrane fraction was isolated using differential sucrose density centrifugation, as described previously [16]. Membranes were suspended in KRH medium containing 0.01 % soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride, and were stored at -80°C until use.

Receptor Binding Assays

Receptor-bearing cells were plated in sterile tissue culture plates 3 days before the radioligand binding assays, allowing them to reach approximately 80 % confluence. The cells or the membrane suspension (20 μ g/tube) were incubated with ~ 1 – 2 pM ($\sim 20,000$ cpm) of the CCK-like radioligand (^{125}I -D-Tyr-Gly-[(Nle^{28,31})CCK-26-33], specific radioactivity 2,000 Ci/mmol) in the absence and presence of increasing concentrations of unlabeled CCK in KRH medium at room temperature for 60 min. For the membrane binding assay, the bound fraction was separated from free radioligand by centrifugation and repeated washing with ice-cold KRH medium. For the cell binding assay, the bound fraction was separated from free radioligand by washing the cells twice with ice-cold KRH medium, followed by lysis with 0.5 M NaOH. The radioactivity in the lysate was quantified by gamma counter. In both types of assays, the radioactivity bound in the presence of 1 μ M unlabeled CCK, considered as non-specific binding, was <15 % of total radioligand bound. Data were analyzed using the LIGAND program of Munson and Rodbard [17], and were graphed using the nonlinear least-squares curve-fitting routine in the Prism 4 suite of programs (GraphPad, San Diego, CA).

Intracellular Calcium Assays

Agonist-stimulated biological activity was assessed by measuring the intracellular calcium concentrations in response to CCK. Receptor-bearing cells were plated in sterile clear-bottom black 96-well tissue culture plates 24 h before the assay. The assay was initiated by washing the semi-confluent cells with modified KRH medium (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1.5 mM CaCl_2 , 1.0 mM KH_2PO_4 , 1.2 mM MgSO_4 , 1.2 mM MgCl_2) containing 2.5 mM probenecid and 0.2 % BSA, and incubating them with Fura 2-AM (1.5 μ M in medium) for 1 h at 37°C in the dark. The incubation was terminated by aspirating the

medium and washing the cells. The assay was performed in a Flexstation 3.0 (Molecular Devices, Sunnyvale, CA) using robotic addition of CCK (1 pM to 100 nM). The intracellular calcium responses were measured at 37 °C by measuring the ratio of intensities of emission at 510 nm arising from excitation at 340 and 380 nm, with data collection every 4 s over a 120 s period. Data were analyzed and plotted using the Prism program.

CCK Receptor Internalization and Recycling

CCK-stimulated receptor internalization was assayed morphologically using alexa-CCK, as we have previously described [5]. This fluorescent ligand is a full agonist that binds to the CCK receptor with high affinity [11]. For studies of receptor internalization, cells grown on coverslips were incubated with 100 nM of fluorescent ligand for 1.5 h at 4 °C to saturate surface receptors. Cells were then warmed to 37 °C for various periods of time (5–30 min) before washing, and were fixed with 2 % paraformaldehyde. Cell surface fluorescence was visualized using a Zeiss Axiovert 200 M inverted epifluorescence microscope with YFP filter setting (excitation 480 nm, dichroic mirror Q515 lp, emission 525 nm; 40 × 1.4 numerical aperture). Images were captured using a monochromatic ORCA-12ER CCD camera (Hamamatsu, Bridgewater, NJ) with QED InVivo (version 2.039) acquisition software (Media Cybernetics, Silver Spring, MD). The final images were processed using Metamorph 6.3 (Molecular Devices, Sunnyvale, CA) and assembled using Photoshop 7.0 (Adobe Systems, Mountain View, CA).

For studies of receptor recycling, the internalized receptors trafficking back to the cell surface were monitored as described previously [5]. CCK receptor-bearing cells were grown on coverslips and were incubated with 100 nM non-fluorescent CCK in PBS containing 0.08 mM CaCl₂ and 0.1 mM MgCl₂ for 2 h at 4 °C to saturate cell surface receptors. Cycloheximide (150 µg/ml) was added during the last 30 min of the incubation and was maintained in the subsequent incubations. The cells were then warmed for various periods of time (30–120 min). After each time point, the cells were cooled to 4 °C and then incubated with 100 nM alexa-CCK for 1.5 h before being washed with ice cold PBS and fixed using 2 % paraformaldehyde. Fluorescence of recycled cell surface receptors was visualized using a Zeiss Axiovert 200 M epifluorescence microscope, as described above.

Fluorescence Spectroscopy

The steady state fluorescence spectroscopy of the alexa-CCK bound to receptor-bearing CHO, 25RA, and SRD15 cells was determined as described previously [18]. The fluorescence measurements were recorded in a SPEX Fluoromax-3 spectrofluorometer equipped with Datamax 2.2 software. Semi-confluent cells were lifted using non-enzymatic cell dissociation solution and were washed with KRH medium. Samples were prepared by incubating the cells (50,000 cells/sample) or isolated membranes (40 µg protein/sample) with 50 nM alexa-CCK at room temperature for 30 min in KRH medium. For assays using cells, bound and free ligand were separated by washing twice using centrifugation at low speed for 5 min at 4 °C. For assays using membranes, bound and free ligand were separated by centrifugation at 14,000 rpm for 5 min. Fluorescence measurements in cell- and membrane-bound fractions were acquired as rapidly as possible in a 1 ml quartz cuvette. The effects of background fluorescence and light scatter were corrected using unlabeled cells or membranes. Static emission intensities were collected between 500 and 600 nm.

Fluorescence Anisotropy

Fluorescence anisotropy was measured at 4 and 20 °C, as described previously [18], using an L-format-based single channel Fluoromax-3 spectrofluorometer equipped with a thermostatically adjusted cuvette holder and an automatic polarizer. The excitation and emission polarizing filters were aligned at 55° and 0°, respectively. The excitation

wavelength was fixed at 482 nm and the emission wavelength was fixed at the optimal wavelength of 521 nm. Data were collected using the constant wavelength analysis profile with 10 s integration times and three sets of acquisition after a delay time of 10 s. Anisotropy was calculated as described previously [18].

Statistical Analysis

Differences in receptor binding and biological activity data between various experimental conditions were evaluated using one-way analysis of variance and Dunnett's post test. Fluorescence data in the multiple cell lines were analyzed using one-way analysis of variance and the Tukey multiple comparison test. Differences were considered to be significant at $P < 0.05$ (Prizm 5, Graphpad, San Diego, CA).

Results

Membrane Cholesterol Levels in Model Cell Systems

Figure 1 illustrates the elevated cell membrane cholesterol levels in 25RA and SRD15 cell lines. Cellular cholesterol content was examined morphologically by staining the membrane cholesterol with filipin. Staining was higher in both 25RA and SRD15 cells relative to that in the parental, normal CHO cells (Fig. 1a). The cholesterol content of the plasma membrane in these cells was quantitatively determined using a flow cytometric assay with alexa-PFO domain 4, following the procedures previously established and validated [6]. Figure 1b shows the fluorescence profiles, which demonstrate a shift to the right reflecting more intense membrane staining for the 25RA and SRD15 cell lines relative to the CHO cell line (1.4 and 1.7-fold, respectively). Levels of membrane cholesterol are also illustrated in the bar graph, reflecting both the flow assay fluorescence intensity values and the quantitative Amplex Red assay for cholesterol. Both 25RA and SRD15 cell lines have cholesterol levels higher than present in CHO cell lines, with the levels highest for the SRD15 cell lines (2.0-fold that of CHO controls) and intermediate for the 25RA cell lines (1.7-fold that of CHO controls). In addition, CHO cells were treated with M β CD-cholesterol to acutely increase their membrane cholesterol to a level similar to that observed in these cell lines [6]. This treatment yielded a 1.9-fold increase in membrane cholesterol (5.9 μ g/mg protein).

Functional Characterization of CCK Receptors Expressed on 25RA and SRD15 Cell Lines

CCK-like radioligand binding was characterized for the receptor-bearing cell lines (Fig. 2). CCK radioligand binding to receptors on all cell lines was saturable and specific. The binding affinity for the type 1 CCK receptor expressed on SRD15 cells was found to be fivefold higher than that on parental CHO cells ($P < 0.05$), while that for receptors expressed on 25RA cells tended to be higher than that of parental cells (1.4-fold), but did not reach statistical significance (Fig. 2a; Table 1). In contrast, CCK binding affinities were not different from that on parental CHO cells for either of the high cholesterol cell lines expressing the type 2 CCK receptors (Fig. 2b).

Figure 2 also illustrates the CCK stimulated intracellular calcium responses in the receptor-bearing cells. CCK stimulated intracellular calcium responses in a concentration-dependent manner in all CCK receptor-bearing cells. Results show that 25RA and SRD15 cells expressing type 1 CCK receptors exhibited decreases in the potency of CCK-stimulated intracellular calcium responses relative to those in parental CHO cells (6.5- and 3-fold, respectively) (Fig. 2c). Again, the cell lines with elevated cholesterol that expressed the type 2 CCK receptors exhibited intracellular calcium responses to CCK that were not different from the control responses in the CHO cell environment (Fig. 2d).

GTP Sensitivity of CCK Receptors Expressed on 25RA and SRD15 Cell Lines

CCK receptors are known to couple to heterotrimeric G proteins (Gq), and to exhibit a shift toward lower affinity in the presence of GppNHp, a non-hydrolyzable analogue of GTP [19]. Figure 3 shows the effects of increased concentrations of GppNHp on CCK binding for both types of CCK receptors expressed in receptor-bearing 25RA, SRD15, and CHO cell lines. The effect of GppNHp on CCK radioligand binding was greater for the type 1 CCK receptor (reduction in level of saturable binding by 77 %) than for the type 2 CCK receptor (reduction by 40 %). Of note, the impact of high membrane cholesterol content on CCK binding, as demonstrated in the setting of 25RA and SRD15 cells, was significant for the type 1 CCK receptor, with less binding inhibition by GppNHp observed than in the control CHO cell environment. In contrast, there was no significant impact of the cholesterol environment on CCK binding inhibition by GppNHp for the type 2 CCK receptor.

Trafficking of CCK Receptors Expressed on 25RA and SRD15 Cell Lines

Agonist-induced receptor internalization is an important mechanism for receptor desensitization. The CCK receptor is known to be internalized predominantly via clathrin-dependent endocytosis after agonist binding [20, 21]. Internalization processes for the type 1 and type 2 CCK receptors expressed on 25RA and SRD15 cell lines were examined (Fig. 4). Both receptors expressed in the cell lines with elevated cholesterol were found to internalize normally, having time-courses similar to that observed in parental CHO cells. Similarly, recycling of both types of CCK receptor back to the cell membrane was also found to be normal in these cells (Fig. 5).

Fluorescence Studies

Anisotropy (rotational mobility) of alexa-labeled CCK ligands bound to CCK receptors was studied while the receptors were expressed in a normal CHO cell environment and in the high cholesterol environments provided by the 25RA and SRD15 cell lines (Fig. 6). Similar effects were observed at both 4 °C (top panels) and 20 °C (bottom panels). The high cholesterol environment resulted in increased anisotropy of the fluorescent agonist ligand bound to the type 1 CCK receptor, but had no significant effect on the anisotropy of the same ligand bound to the type 2 CCK receptor (panel A). Of note, occupying the type 1 CCK receptor in the CHO cell environment with an analogous fluorescent antagonist ligand also resulted in increased anisotropy than that which was observed with the agonist ligand (panel B). In contrast, the anisotropy of the antagonist ligand bound to the type 1 CCK receptor in the high cholesterol environment provided by the 25RA and SRD15 cells was not significantly different from that of the same ligand in a parental CHO cell environment (panel B). Therefore, the anisotropy of the antagonist probe was not affected by the cholesterol environment of the type 1 CCK receptor, while the anisotropy of the agonist probe bound to this receptor was clearly affected. When the SRD15 and 25RA cells had their membrane cholesterol reduced by extraction using M β CD, the anisotropy of the fluorescent agonist was reduced to control values, while that of the fluorescent antagonist remained elevated (panel B).

Use of GppNHp to shift the affinity of the type 1 CCK receptor to the lower affinity state resulted in higher anisotropy of the bound fluorescent agonist, both in the setting of the parental CHO cell and in the higher cholesterol environment provided by the 25RA cells (Fig. 7).

Effects of Reducing Membrane Cholesterol in Model Cell Systems

Reducing the level of membrane cholesterol in 25RA and SRD15 cells using M β CD treatment returned the CCK binding affinities toward control levels in the CHO cell

environment in both CCK1R-expressing 25RA cells and SRD15 cells (Fig. 8). M β CD treatment did not alter the CCK binding affinities of type 2 CCK receptors expressed in these cellular environments (Fig. 8; Table 1). Calcium responses in these cells were also studied after reducing membrane cholesterol with M β CD. The calcium-response curves in the type 1 CCK receptor-bearing cells were shifted to the right, while those in the type 2 CCK receptor-bearing cells were not significantly affected (Fig. 8). It is important to note that this treatment of the type 1 CCK receptor-bearing cells resulted in increased disruption of signaling, rather than normalization of signaling, analogous to what was observed with the CCK radioligand binding. It is possible that the M β CD treatment had a disruptive effect on the membrane organization and did not allow normal coupling with the Gq.

An additional important set of controls represents the effects of increasing membrane cholesterol in the parental CHO cell environment. This has previously been studied [6] to demonstrate a 3.2-fold increase in binding affinity and a 2.5-fold reduction in potency to stimulate intracellular calcium responses through the type 1 CCK receptor that were both significant, while no significant changes in these parameters for the type 2 CCK receptor were observed.

Discussion

Plasma membrane receptors play a critical role in mediating the regulation of target cells by circulating hormones and neurotransmitters. Essentially every regulatable cell of the body has such receptors, with the largest number representing guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs). The current work is focused on two closely related members of the class A family of GPCRs, the type 1 and 2 cholecystokinin (CCK) receptors. The type 1 CCK receptor is present on gallbladder muscularis, exocrine pancreas, smooth muscle and nerves along the digestive tract, and selected brain nuclei, where it plays important roles in regulating nutritional homeostasis [22]. It achieves this by stimulating gallbladder contraction and pancreatic exocrine secretion, regulating gastric emptying and gut transit, and inducing post-cibal satiety. The type 2 CCK receptor is present on gastric parietal cells and throughout the brain, where it helps to stimulate gastric acid secretion and has roles in anxiety and has been implicated in panic attacks [23]. Given the roles for cholecystokinin receptors in nutritional homeostasis, including appetite regulation, and the common associations between obesity, hypercholesterolemia, and gallstone disease, it becomes particularly important to understand the effects of membrane cholesterol on cholecystokinin receptor function.

Of note, the two subtypes of CCK receptors have been described to be differentially sensitive to the acute manipulation of plasma membrane cholesterol composition, with the type 1 CCK receptor having its binding and biological activity affected, while the type 2 CCK receptor was refractory to functional impact [5, 6]. These insights are timely, with recent observations of membrane cholesterol effects on a series of GPCRs, including receptors for oxytocin, serotonin, galanin, opioid receptors, and rhodopsin [24–28]. These effects are not generalized throughout a family or structurally related group of these receptors, and the basis for the cholesterol effects is not well understood. It is quite interesting that recently solved crystal structures of several class A GPCRs has demonstrated conserved regions of cholesterol association [29, 30], and consensus sequences have been reported for cholesterol association with GPCRs [29, 31]. Unfortunately, the presence of these sequences does not separate the cholesterol-sensitive from the cholesterol-insensitive members of this superfamily.

Most of the studies of effects of cholesterol on GPCRs have been performed using reconstitution in artificial membranes or by the acute manipulation of cholesterol using

cholesterol chelators or liposomal delivery of excess cholesterol [26]. While these systems are adequate to demonstrate the impact of changes in the lipid bilayer on receptor binding and signaling, they are less useful for the evaluation of the impact on receptor regulation, such as might be important in a living organism. To evaluate receptor regulatory processes such as internalization and recycling, a stable cell system with abnormal lipid composition is required. Indeed, that is what was achieved and studied in the current work.

Much has been learned about cholesterol biosynthesis and regulation [32, 33]. Two CHO cell lines with defects that result in elevated membrane cholesterol were utilized in the current project, 25RA cells and SRD15 cells, where they were compared with a parental unmodified CHO cell line. All of these cells were engineered to express type 1 or type 2 CCK receptors. The 25RA cells have elevated membrane cholesterol as a function of their gain-of-function of the SCAP (steroid regulatory element-binding protein (SREBP) cleavage-activating protein) gene. This facilitates the export of SREBP from the endoplasmic reticulum to the Golgi, where S1P and S2P proteases release the transcriptionally active domain of SREBP that then moves to the nucleus where it can stimulate the synthesis of the first four enzymes in the cholesterol synthetic pathway. This includes effects on 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, which is known to be the rate-limiting enzyme in cholesterol synthesis. The SRD15 cells have defects in both Insig-1 and Insig-2, thereby reducing the feedback inhibition of the synthesis of cholesterol. It achieves this through abolishing the sterol-accelerated degradation of HMG CoA reductase and the sterol-mediated inhibition of SREBP processing. It is important to recognize, however, that while these cell lines have abnormally high membrane cholesterol, they also likely have other lipid abnormalities as effects of the modifications of these synthetic and metabolic pathways. However, most of the effects would be expected in the composition of various sterols, with cholesterol the predominant lipid affected in the plasma membrane.

We believe that the elevated membrane cholesterol environment provided by these cell lines more accurately reflects the elevated membrane cholesterol levels observed in cells of patients with hypercholesterolemia and morbid obesity than the chemical methods that have typically been employed in similar studies. Following stable transfection of CCK receptor constructs, these cell lines reproduced the acute effects of cholesterol elevation on the binding and biological activity of the type 1 CCK receptor, while demonstrating no effect of these changes in cholesterol on the function of the type 2 CCK receptor. Additionally, they exhibited normal agonist-induced internalization and recycling of both types of CCK receptors. There was no negative impact of this cholesterol-rich enrichment on these desensitization and resensitization mechanisms.

It is important to note that the levels of membrane cholesterol in the 25RA and SRD15 cells used in this study are comparable to those described for cells in patients having hypercholesterolemia [7]. Our observations in 25RA and SRD15 cells, particularly the reduced potency of CCK to stimulate a biological response from activation of the type 1 CCK receptor strongly suggest that decreased signaling from the CCK1R in the presence of elevated membrane cholesterol could contribute to obesity [8] and cholesterol gallstones [34, 35]. The cell lines established in this work thus provide a valuable resource for the screening of potential drugs that might be effective in restoring CCK function in these clinical settings.

It is interesting and important to recognize that acute manipulations of membrane cholesterol in these cell lines had some unexpected effects. While ligand binding after extracting some of the membrane cholesterol generally followed the predicted effects, moving toward normal, the biological effects (CCK-stimulated signaling) were not

normalized by this manipulation of these cells. This likely reflects the disruption of the organization of the membrane with resultant interference with the ability of G proteins to couple with this receptor in a normal manner. It could also reflect the impact of other lipid abnormalities in these cells. While this does not invalidate the use of these cells in a drug screening and development program, it needs to be appreciated and kept in mind regarding the limitations in the use of such cells.

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Abbreviations

CCK1R	Type 1 cholecystokinin receptor
CCK2R	Type 2 cholecystokinin receptor
CHO	Chinese hamster ovary
DMEM	Dulbecco's modified Eagle's medium
Fura-2AM	Fura-2-acetoxymethyl ester
GPCR	G protein-coupled receptor
KRH	Krebs-Ringer's-HEPES
LPDS	Lipoprotein-deficient serum
MβCD	Methyl- β -cyclodextrin
PFO	Perfringolysin θ
SREBP	Sterol regulatory element-binding protein

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Fig. 1.

Cellular cholesterol levels. Shown are the microscopic images of cells stained with filipin to reflect cholesterol levels (a), flow cytometry fluorescence profiles of CCK receptor-expressing CHO, 25RA and SRD15 cell lines labeled with alexa-PFO domain 4 (b), and biochemical assay of membrane cholesterol using Amplex Red (b). The images were collected for each cell line using fixed exposure conditions. Both 25RA and SRD15 cell lines exhibited increased filipin staining relative to control CHO cells. This was confirmed with the alexa-PFO domain 4 flow data, as well as the Amplex Red assay for membrane cholesterol. The cholesterol content of each of the cell lines was significantly higher than that of the parental CHO cells, $P < 0.05$. Bar 20 μm

Fig. 2. CCK receptor characteristics in 25RA and SRD15 cell lines. Shown are the CCK competition-binding curves for receptor-bearing CHO, 25RA and SRD15 cell lines. Type 1 CCK receptor-bearing cells are shown in **a**, and type 2 CCK receptor-bearing cells are shown in **b**. Shown also are the concentration-dependent CCK-stimulated intracellular calcium response curves for the type 1 CCK receptor (**c**) and type 2 CCK receptor (**d**) expressed on CHO, 25RA and SRD15 cell lines. Values represent means $\pm$ SEM of data from three to eight independent experiments performed in duplicate

Fig. 3. GTP sensitivity of CCK binding in 25RA and SRD15 cell lines. Shown are the effects of GppNHp on saturable CCK radioligand binding to type 1 CCK receptors (*top panel*) and type 2 CCK receptors (*bottom panel*) expressed on CHO, 25RA and SRD15 cell lines. Data reflect means $\pm$ SEM from four independent experiments performed in duplicate

Fig. 4. CCK receptor trafficking in 25RA and SRD15 cell lines. Shown are representative epifluorescence images of alexa-CCK-induced internalization of type 1 CCK receptors (*top panel*) and type 2 CCK receptors (*bottom panel*) expressed on CHO, 25RA and SRD15 cell lines. Fluorescent ligand-occupied receptors were internalized in a time- and temperature-dependent manner. The microscopic images are representative of three independent experiments. *Bar* 20 μm

Fig. 5. CCK receptor recycling in 25RA and SRD15 cell lines. Shown are representative epifluorescence microscopic images to reflect the recycled type 1 CCK receptors (*top panel*) and type 2 CCK receptors (*bottom panel*) on the surface of receptor-bearing CHO, 25RA and SRD15 cell lines. The controls at time zero reflect cell surface receptors before internalization. The *images* shown are representative of three independent experiments. Bar 20 μm

Fig. 6.

Fluorescence anisotropy of alexa-CCK analogues bound to CCK receptors on 25RA and SRD15 cell lines. The *graphs on the left (a)* reflect the anisotropy values for alexa-CCK (agonist) bound to type 1 CCK receptors and type 2 CCK receptors in the normal CHO cell or in the environment of elevated cholesterol provided by 25RA and SRD15 cells at 4 °C (*top*) and 20 °C (*bottom*). The *graphs on the right (b)* compare the effects on anisotropy of agonist and antagonist (alexa-D-Trp-OPE) probes, as well as studying the effects on the cell lines having elevated membrane cholesterol that was depleted using MβCD. Data represent means ± SEM from four to eight independent experiments performed in duplicate. Level of significance * $P < 0.05$

Fig. 7.

Effect of GppNHp on fluorescence anisotropy. The *graphs* show the anisotropy values for YFP-tagged type 1 CCK receptors expressed on CHO cells and 25RA cells in the presence or absence of GppNHp (10 μ M). Data represent means $\pm$ SEM from four to eight independent experiments performed in duplicate. ** $P < 0.001$ compared with the same condition in the absence of GppNHp

Fig. 8. Effect of MβCD on CCK receptor function in model cell systems. Shown are the effects of reducing membrane cholesterol using MβCD on CCK1R function (*left panels*) and CCK2R function (*right panels*), with the effects on receptor binding shown in the *upper panels* and the effects on intracellular calcium responses shown in the *lower panels*. Results reflect significant rightward shifts in CCK binding affinities and biological activities for the type 1 CCK receptor expressed on SRD15 and 25RA cells, but no significant shifts for the type 2 CCK receptor expressed in the same cellular environments. Data represent means ± SEM of data from three to six independent experiments performed in duplicate

Table 1

Functional characterization of CCK receptors expressed on cell lines

Cells	Condition	Binding affinity K_i (nM)	Binding sites (sites/cell $\times 10^5$)	Calcium responses EC_{50} (nM)
CCK1R				
CHO	Normal	4.0 ± 0.8	1.8 ± 0.4	0.02 ± 0.01
	M β CD	19.1 ± 3.5^a	1.6 ± 0.2	0.27 ± 0.05^a
25RA	Normal	2.9 ± 0.3	1.9 ± 0.3	$0.13 \pm 0.04^*$
	M β CD	8.3 ± 2.4^a	1.2 ± 0.1	0.58 ± 0.24^a
SRD15	Normal	$0.8 \pm 0.1^*$	1.0 ± 0.2	$0.06 \pm 0.01^*$
	M β CD	1.5 ± 0.1^a	1.1 ± 0.1	0.51 ± 0.12^a
CCK2R				
CHO	Normal	0.8 ± 0.2	1.4 ± 0.2	0.09 ± 0.01
	M β CD	0.6 ± 0.2	1.0 ± 0.2	0.09 ± 0.02
25RA	Normal	1.0 ± 0.3	1.2 ± 0.2	0.06 ± 0.02
	M β CD	1.1 ± 0.4	1.1 ± 0.1	0.06 ± 0.01
SRD15	Normal	1.0 ± 0.2	0.9 ± 0.1	0.06 ± 0.01
	M β CD	1.0 ± 0.3	1.1 ± 0.2	0.10 ± 0.03

Data are expressed as means $\pm$ SEM of values from three to eight independent experiments performed in duplicate* $P < 0.05$ relative to analogous condition in CHO cell lines^a $P < 0.05$ relative to same cell line in the absence of M β CD treatment (without depleting cholesterol)