Localization of the κ Opioid Receptor in Lipid Rafts

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ABSTRACT

Lipid rafts are microdomains of plasma membranes enriched in cholesterol and sphingolipids in the outer layer. We determined whether κ opioid receptors (KOR) in human placenta and FLAG (DYKDDDDK)-tagged human KOR (FLAG-hKOR) expressed in Chinese hamster ovary (CHO) cells are localized in lipid rafts and whether changes in cholesterol contents affect hKOR properties and signaling. Lipid rafts were prepared from placenta membranes and CHO cells expressing FLAG-hKOR using the Na2CO3 method and fractionation through a sucrose density gradient. The majority of the KOR in the placenta and FLAG-hKOR in CHO cells, determined by [3H]diprenorphine binding and/or immunoblotting with an anti-FLAG antibody, was present in low-density fractions, coinciding with high levels of caveolin-1 and cholesterol, markers of lipid rafts, which indicated that the KOR is localized in lipid rafts. Pretreatment with 2% methyl β-cyclodextrin (MCD) reduced cholesterol content by ~48% and changed the cells from spindle-shaped to spherical. MCD treatment disrupted lipid rafts, shifted caveolin-1 and FLAG-hKOR to higher density fractions, increased the affinity of (−)-(trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (U50,488H) for the hKOR, and greatly increased U50,488H-induced [35S]guanosine 5′-O-(3-thiotriphosphate binding to p42/44 mitogen-activated protein kinase phosphorylation. Cholesterol replenishment reversed all the MCD effects. Caveolin-1 immunoprecipitated with Gαi proteins and MCD treatment reduced caveolin-1 associated with Gαi proteins, which may contribute to the enhanced agonist-induced G protein activation. Caveolin-1 also immunoprecipitated with FLAG-hKOR, but MCD treatment had no effect on the association. Thus, the KOR is located in lipid rafts and its localization in the microdomains greatly affects coupling to G proteins.

Plasma membranes were traditionally viewed as uniform lipid bilayers and G protein-coupled receptors (GPCRs), G proteins, and membrane-bound effectors were randomly distributed in plasma membranes. In recent years, the concept that lipid rafts function as microdomains in plasma membranes to concentrate signaling molecules for regulated activation by related receptors has gained increasing acceptance (Pike, 2003; Chini and Parenti, 2004; Cohen et al., 2004). Lipid rafts are microdomains of cell membranes highly enriched in cholesterol and sphingolipids in the outer layer. Brown and Rose (1992) proposed the following operational definition of lipid rafts: when cell membranes were solubilized with ice-cold nonionic detergents such as Triton X-100 (1% for 1 h at 4°C) followed by sucrose density gradient centrifugation; lipid rafts are resistant to detergent solubilization and float in the lighter fractions, whereas the bulk of the solubilized cellular lipids and proteins are in the high-density fractions (Brown and Rose, 1992). There are two types of lipid rafts: planar lipid rafts and caveolae. Caveolae, a specialized subtype of lipid rafts, are flask-shaped invaginations in the plasma membrane and are enriched in caveolins, 21- to 24-kDa integral membrane proteins (Razani et al., 2002). Many signaling molecules including some GPCRs, Gα proteins, growth factor receptors, protein kinase C, and adenylyl cyclase were found to be enriched in or recruited into lipid rafts (Pike, 2003; Chini and Parenti, 2004). Growing evidence has indicated that lipid rafts are crucial in the regulation of GPCRs in signal transduction and in exocytic and endocytic pathways (Pike, 2003; Chini and Parenti, 2004). Cholesterol has been shown to play a critical role in assem-

ABBREVIATIONS: GPCR, G protein-coupled receptor; MAP kinase, mitogen-activated protein kinase; FLAG epitope, (DYKDDDDK); hKOR, human κ opioid receptor(s); FLAG-hKOR, FLAG-tagged human κ opioid receptor(s); CHO, Chinese hamster ovary; CHO-sFLAG-hKOR, CHO cells stably transfected with FLAG-hKOR cDNA that has a signal peptide preceding the FLAG-hKOR; GTP-γ-S, guanosine 5′-3-O-(thiophosphate; U50,488H, (−)-(trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide; MES, 2-morpholinoethanesulfonic acid; MCD, methyl β-cyclodextrin; CH-MCD, MCD-conjugated cholesterol; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis.
bling microdomains of lipid rafts, binding directly to caveolins and facilitating caveolin integration into membrane (Murata et al., 1995; Li et al., 1996). MCD is used extensively as a cholesterol-depleting reagent, rapidly removing cholesterol from the plasma membrane of cultured cells (Kiladonk et al., 1995) and eventually leading to disruption of lipid rafts (Rothberg et al., 1992; Lawrence et al., 2003).

Caveolins have been demonstrated to interact with many signaling molecules, probably through interactions with specific caveolin-binding motifs in the proteins (Okamoto et al., 1998; Cohen et al., 2004). Thus, caveolins may function as scaffolding proteins to concentrate signaling molecules within caveolea membranes. Caveolins were shown to negatively regulate the activities of many signaling molecules (Okamoto et al., 1998). Caveolin-1 and -2 are widely distributed and coexpressed in most differentiated cells (Cohen et al., 2004).

Opioid drugs and endogenous peptides produce pharmacological and physiological effects by acting on at least three types of opioid receptors, μ, δ, and κ. Stimulation of κ opioid receptors elicits many effects including analgesia, water diuresis, dysphoria and antitussive effects, and attenuation of cocaine craving in addicts (Liu-Chen, 2004, and references therein). Salvinorin A, a potent hallucinogen extracted from the leaves of the plant Salvia divinorum, is a potent and selective agonist of the κ opioid receptor (Roth et al., 2002). κ opioid receptors are also present in non-neuronal tissues, including the human placenta (Porthe et al., 1981; Mansson et al., 1994) and the rat heart (Ventura et al., 1989). In the human placenta, κ opioid receptors regulate secretion of human chorionic gonadotropin (Valette et al., 1983) and release of human growth hormone (HGH) and human prolactin (Valette et al., 1985). In the human placenta (Porthe et al., 1981; Mansson et al., 1994) and the rat heart (Ventura et al., 1989). In the human placenta, κ opioid receptors regulate secretion of human chorionic gonadotropin (Valette et al., 1983) and release of human growth hormone (HGH) and human prolactin (Valette et al., 1985). In the human placenta (Porthe et al., 1981; Mansson et al., 1994) and the rat heart (Ventura et al., 1989). In the human placenta, κ opioid receptors regulate secretion of human chorionic gonadotropin (Valette et al., 1983) and release of human growth hormone (HGH) and human prolactin (Valette et al., 1985).

Opioid receptors are mainly coupled to Gs, Ga, and Gq proteins to affect several different effectors, including inhibition of adenylyl cyclase, enhancement of K+ conductance, a decrease in Ca2+ conductance, and activation of p2/p4 MAP kinases (for a review, see Law et al., 2000). In addition, opioid receptors are shown to be coupled to Gz and G16 (Law et al., 2000).

Recently, we have demonstrated that activation of κ opioid receptors stimulates Na+,H−-exchanger activity via Na+,H−-exchanger regulatory factor-1/Ezrin-radixin-moesin-binding phosphoprotein-50 independent of pertussis toxin-sensitive G proteins (Huang et al., 2004). μ, δ, and κ opioid receptors have been cloned. Opioid receptors belong to the rhodopsin subfamily of the GPCR family. All GPCRs are integral membrane proteins and have seven transmembrane domains.

In the present study, we found that κ opioid receptors in human placenta membranes and FLAG-human κ opioid receptor (FLAG-hKOR) expressed in CHO cells were localized in lipid rafts. We then used CHO cells stably transfected with FLAG-hKOR cDNA, which has a signal peptide preceding the FLAG-hKOR (CHO-sFLAG-hKOR) as a model system to investigate whether disruption of lipid rafts by cholesterol reduction affected binding properties and signal transduction of the κ opioid receptor.

Materials and Methods

Materials. [3H]Diprenorphine (58 Ci/mmol) and [35S]GTPγS (1250 Ci/mmol) were purchased from PerkinElmer (Boston, MA). Naloxone was a gift from the former DuPont Merck Pharmaceutical Co. (Wilmington, DE). (−)-(trans)3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneaceticamide (U50,488H) was purchased from Toxis Cookson Inc. (Eliisville, MO). Sodium carbonate, 2-morpholinoethanesulfonic acid (MES), glycerol, EDTA, EGTA, dithiothreitol, phenylmethanesulfonyl fluoride, leupeptin, GDP, GTPγS, methyl-β-cyclodextrin (MCD), cholesterol-methyl-β-cyclodextrin (CH-MCD), and anti-FLAG polyclonal antibody (F7425) were purchased from Sigma Chemical (St. Louis, MO). For phosphate assay, Fisk-SubbaRow reducer and phosphate standard were obtained from Sigma Chemical and ammonium molybdate was purchased from Fisher Scientific (Newark, DE). Ammonium persulfate was purchased from Bio-Rad (Hercules, CA). Anti-caveolin-1 monoclonal antibody (clone 2297) and anti-flotillin-1 monoclonal antibody (clone 18) were obtained from BD Translaction (San Jose, CA). Polyclonal anti-Gαi3, antibody, which recognizes Gαi3, Gαs, and Gαi3, and protein A/G PLUS-agarose were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA) and Immobilon-P transfer membrane from Millipore Corporation (Billerica, MA). PhosphoPlus p44/42 MAP kinase (Thr202/Tyr204) antibody kit and p44/42 MAP kinase antibody kit were obtained from Cell Signaling Technology Inc. (Beverly, MA). Goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) was from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Goat anti-rabbit IgG conjugated with HRP, SuperSignal West Pico Chemiluminescent Substrate Solution, and Restore Western Blot Stripping buffer were from Pierce Chemical (Rockford, IL). Persarin was from Calbiochem (La Jolla, CA). Complete Protease Inhibitor Cocktail was from Hoffman-La Roche (Nutley, NJ).

Preparation of Human Placenta Membranes. Fresh human placenta was obtained immediately after cesarean sections from the labor and delivery room of Temple University Hospital. The study qualifies as exempt under the Code of Federal Regulations and has been approved by Temple University Institutional Review Board. The tissue was washed with ice-cold 0.9% normal saline to remove as much blood as possible. The fetal side was cleared of connective tissues as much as possible and minced with scissors. The tissue was then homogenized in 10 volumes of 10 mM Tris-HCl, 0.32 M sucrose, 10 mM glucose, and 10 μM leupeptin (pH 7.4), first with a Polytron and then with a Teflon pestle-glass homogenizer. After centrifugation at 10000 g for 10 min, the supernatant was saved and centrifuged again at 40000 g for 20 min. The pellet was resuspended in buffer A (5 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, and 10 μM leupeptin, pH 7.4), placed on ice for 30 min, homogenized, and centrifuged at 40000 g for 30 min. The pellet was saved and kept at −80°C for detergent-free lipid raft preparations. All procedures were performed at 4°C or on ice.

Detergent-Free Lipid Raft Preparation. Detergent-free lipid rafts were prepared according to Song et al. (1996). Clonal CHO cells stably expressing FLAG-hKOR (Li et al., 2002) were detached by versene solution (0.54 mM EDTA, 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.46 mM KH2PO4, and 1 mM glucose, pH 7.0), counted by use of a Z1 Coulter Particle Counter and centrifuged at 300g for 2 min.

Cell pellet (~106 cells) or placenta membrane (from ~6 g wet weight) was resuspended in 2 ml of 500 mM sodium carbonate buffer (pH 11) containing 1 mM phenylmethylsulfonyl fluoride and homogenized using a Wheaton loose-fitting glass Dounce homogenizer (10 strokes) followed by sonication (three 20-s bursts) on ice using a Fisher sonicator. Two milliliters of 90% sucrose prepared in MES buffer (20% glycerol, 150 mM NaCl, 2 mM EDTA, and 25 mM MES, pH 6.5) was added to the homogenized samples yielding 45% sucrose in a total volume of 4 ml. A discontinuous sucrose gradient was layered on the top of the 45% fraction with 4 ml of 35% sucrose and 4 ml of 5% sucrose in MES buffer containing 250 mM sodium carbonate. For continuous sucrose gradients, 7 ml of 45% sucrose and 7 ml of 5% sucrose, both in MES buffer with 250 mM sodium carbonate, were used to make the gradient. The homogenized samples were
layered at the bottom of the gradients. Isopycnic ultracentrifugation was then carried out at 40,000 rpm (200,000g) using a SW 41 rotor for 16 to 20 h at 4°C. After ultracentrifugation, 12 1-ml fractions were collected from the bottom of the gradient tube using a peristaltic pump (Rainin Instruments, Woburn, MA).

**Determination of Cholesterol and Phospholipid Contents.** Lipids in intact cells and the 12 fractions isolated from the sucrose density gradient tube were extracted by a chloroform-methanol solvent mixture (2:1, v/v). The chloroform layer was taken out for cholesterol and phospholipid determinations. Cholesterol content was determined using a cholesterol reagent (cholesterol E) obtained from Wako Pure Chemicals (Tokyo, Japan). In brief, upon addition of 300 μl of the color reagent, samples were incubated for 5 min at 37°C followed by spectrophotometric analysis (absorption at 600 nm). In this reaction, cholesterol esters were hydrolyzed to free cholesterol and fatty acid in a reaction catalyzed by cholesterol oxidase and generated hydrogen peroxide. The hydrogen peroxide formed participated in the quantitative oxidative condensation between 3,5-dimethoxy-N-ethyl-N-(2-hydroxy-3-sulphopropyl)-aniline, sodium salt and 4-aminoantipyrine in the presence of peroxidase. The product of the reaction was a blue pigment. The total amount of cholesterol in the test sample was determined by measurement of the absorbance of the blue color at 600 nm using a six-point standard curve generated with known concentrations of cholesterol. In the cholesterol depletion assay, to standardize cholesterol content, phospholipid concentrations were determined using a phosphate assay. The phospholipid concentration was determined with the method of Bartlett (1959). Data are expressed as concentration of cholesterol/concentration of phospholipid.

**Ligand Binding to κ Opioid Receptor.** Binding was performed on each fraction of human placenta or CHO-sFLAG-hKOR preparations immediately after sucrose gradient centrifugation with [3H]diprenorphine (1 nM) in 50 mM Tris-HCl buffer-1 mM EGTA (pH 7.4) (TE buffer) at room temperature for 1 h in duplicate according to our published procedure (Zhu et al., 1997). Nonspecific binding was defined as binding in the presence of naloxone (10 μM).

Saturation binding of [3H]diprenorphine to κ opioid receptors in membranes of CHO-sFLAG-hKOR cells treated with or without 2% MCD was performed with at least six concentrations of [3H]diprenorphine (ranging from 25 pM to 2 nM), and K_d and B_max values were determined (Huang et al., 2001). Competition inhibition with U50,488H of [3H]diprenorphine (0.5 nM) binding to κ opioid receptors was performed in the absence or presence of various concentrations of U50,488H and its K_i value was determined (Zhu et al., 1997).

**Treatment of CHO-sFLAG-hKOR Cells with MCD or MCD followed by CH-MCD.** CHO-sFLAG-hKOR cells were incubated at 37°C for 1 h in a serum-free medium with or without MCD at the indicated concentration, medium was aspirated, and cells were harvested. For a cholesterol repletion experiment, after MCD pretreatment, cells were then incubated for 2 h with CH-MCD in serum-free medium. Control cells were treated with vehicle for 3 h. MCD-treated cells were treated with MCD for the last 1 h.

**[35S]GTP_S Binding.** Determination of [35S]GTP_S binding to G proteins was performed using an attached method of Zhu et al. (1997). CHO FLAG-hKOR cells were incubated with 2% MCD for 1 h at 37°C in the serum-free medium while control cells received serum-free medium alone, cells were then washed with 100 mM phosphate-buffered saline, and membranes were prepared as described previously. Membranes (10 μg of protein) were incubated in reaction buffer (50 mM HEPES, 100 mM NaCl, 5 mM MgCl_2, and 1 mM EDTA) containing [35S]GTP_S (100,000–150,000 dpm, 80–100 pM) and 10 μM GDP with or without U50,488H (10^-10–10^-8 M) in a total volume of 0.5 ml for 60 min at 30°C. Nonspecific binding was determined in the presence of 10 μM GTP_S. Bound and free [35S]GTP_S was separated by spin filters under reduced pressure. Radioactivity was determined by liquid scintillation counting with a counting efficiency of 95%.

**Immunoblotting of FLAG-hKOR, Caveolin-1, and Flotillin-1.** These were carried out on each fraction of sucrose gradients according to our published method (Li et al., 2002). Twenty microliters of 2× Laemmli sample buffer (4% SDS, 0.1 M DTT, 20% glycerol, and 62.5 mM Tris, pH 6.8) were added to each 20-μl sample and subjected to 8% SDS-polyacrylamide gel electrophoresis (PAGE), and protein bands were transferred to Immobilon-P membrane. Membranes were incubated with 5% nonfat dried milk in TBS-T solution (10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.4) for 30 min at room temperature on an orbital shaker to block nonspecific binding and then incubated with one of the following primary antibodies at 4°C overnight on an orbital shaker: rabbit anti-FLAG polyclonal antibody (1:5000 dilution), mouse anti-caveolin-1 monoclonal antibody (1:2000 dilution for placenta; 1:10,000 for CHO cells), or mouse anti-flotillin-1 monoclonal antibody (1:10,000 dilution for CHO cells). After three washes with TBS-T, blots were incubated with goat anti-rabbit IgG conjugated with HRP (1:10,000) or with anti-mouse IgG conjugated with HRP (1:5000) for 1 h at room temperature. Membranes were washed three times with TBS-T and then reacted with SuperSignal West Pico Chemiluminescence Substrate Solution. Images were captured with a FUJIFILM LAS-1000 imaging system.

**p42/p44 MAP Kinase Phosphorylation.** Cells were left untreated or treated with MCD or MCD followed by CH-MCD, suspended by Versene solution and washed with phosphate-buffered saline buffer twice. Cells were incubated in a serum-free medium for 20 min at 37°C and then treated with or without U50,488H at 37°C for 5 min. Cells were lysed with 2× Laemmli sample buffer and subjected to 8% SDS-PAGE. Phosphorylated MAP kinase and total p44/p42 MAP kinase were detected by immunoblotting as we described previously (Huang et al., 2004). The amounts of phosphorylated and total MAP kinases were quantified with ImageGauge software. For each condition, phosphorylated MAP kinases were normalized against total MAP kinases, and data were expressed as fold stimulation over the control basal level.

**Colloimmunoprecipitation of the FLAG-hKOR and Caveolin-1.** CHO cells stably expressing FLAG-hKOR were treated with vehicle or 2% MCD at 37°C for 1 h and harvested. Cells were solubilized in the solubilization buffer (2% Triton X-100, 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4, and Complete Protease Inhibitor Cocktail) for 1 h at 4°C on a rocking shaker and then centrifuged at 100,000g for 1 h. The supernatant was incubated for 1 h at 4°C with rabbit polyclonal antibody against FLAG at 3 μg/ml or with the antibody preincubated with FLAG peptide (15 μg/ml, 30 min) as the control. Twenty microliters of Pansorbin was then added to precipitate the immunocomplex for 1 h at 4°C. The mixture was centrifuged, and the pellets were washed three times by centrifugation and resuspension. Immunoprecipitated materials were dissolved in 2× Laemmli sample buffer, and subjected to 8% SDS-PAGE. Caveolin-1 was detected by Western blot using an anti-caveolin-1 monoclonal antibody (1:1000) as described above. The membranes were then stripped at 37°C for 10 min with Restore Western Blot Stripping buffer, rinsed three times with TBS-T, and blotted with M2 mouse monoclonal antibody against FLAG followed by goat anti-mouse IgG conjugated with HRP and chemiluminescence reagents to determine the amount of FLAG-hKOR immunoprecipitated.

**Colloimmunoprecipitation of Ga_i and Caveolin-1.** CHO cells stably expressing FLAG-hKOR were incubated with vehicle or 2% MCD at 37°C for 1 h and harvested. Cells were solubilized in the solubilization buffer at 4°C for 1 h and centrifuged at 100,000g and 4°C for 30 min. Supernatants were collected and incubated with or without 10 μl of rabbit anti-Gα_i at 4°C for 1 h and then 20 μl of resuspended protein A/G PLUS-agarose for 1 h at 4°C on a rocker platform. The agarose pellets were collected, washed with 1.0 ml of TBS-T four times, and resuspended in 40 μl of 2× Laemmli sample buffer. Twenty microliters of each samples were separated on SDS-PAGE and caveolin-1 was detected using an anti-caveolin-1 mono-
Results

Detergent-Free Lipid Raft Preparation. The human placenta has a high level of \( \kappa \) but not of \( \mu \) or \( \delta \) opioid receptors (Porthe et al., 1981; Mansson et al., 1994), which are present in brush border membranes of microvilli. Placenta membranes were sonicated, and the mixture was fractionated in a 5%/35%/45% discontinuous sucrose density gradient by ultracentrifugation. From the sucrose density gradient, 12 fractions were collected. Cholesterol levels peaked in fraction 4, which was in 5% sucrose and near the interface of 5%/35% sucrose in the gradient (Fig. 1A). Caveolin-1 immunoreactivity also peaked in fraction 4 (Fig. 1C). Because low density and high levels of cholesterol and caveolins are characteristics of lipid raft microdomains (Okamoto et al., 1998), these results validate the detergent-free lipid raft preparation method we used. It should be noted that this method does not distinguish between planar lipid rafts and caveolae. The term lipid rafts will be used in this article.

\( \kappa \) Opioid Receptors in the Human Placenta Are Localized in Lipid Rafts. Placenta membranes were subject to lipid raft preparation procedures. The highest level (\(-50\%) of binding of \( [\text{H}] \)) diprenorphine, a nonselective opioid antagonist, was detected in fraction 4 (Fig. 1B), coinciding with the peaks of cholesterol and caveolin-1. The rest of the binding activities were spread among other fractions (Fig. 1B). Therefore, \( >50\% \) of the receptor was present in fractions 1 to 5. These results indicate that the majority of \( \kappa \) opioid receptors in placenta membranes are localized in lipid rafts.

FLAG-hKOR Expressed in CHO Cells Is Localized in Lipid Raft Microdomains. Because the human placenta is not amenable to biochemical manipulation, we determined whether FLAG-hKOR stably expressed in CHO cells is localized in lipid rafts. CHO cells were subject to the detergent-free lipid raft preparation procedure as described for placenta membranes. A narrow milky band appeared in the low-density region (fractions 4 and 5). High cholesterol levels were found in fractions 1 to 5, with the highest level in fractions 4 or 5 (Fig. 2A), probably due to slight variations in fraction collection. The majority of \( [\text{H}] \) diprenorphine-specific binding to the KOR and FLAG and caveolin-1 immunoreactivities were located in fractions 1 to 5, with the highest level in fractions 4 or 5 (Fig. 2, C and D). Figure 2 shows representative results with a peak in fraction 4. In contrast, the majority of proteins were in higher density fractions, particularly fractions 10 to 12 (Fig. 2B). These results indicate that the KOR in CHO cells is located in lipid raft microdomains of plasma membranes. We thus used CHO cells stably expressing FLAG-hKOR for further study.

Cholesterol Reduction by MCD Pretreatment and Replenishment by CH-MCD. The cholesterol-binding reagent MCD was used to extract cholesterol from plasma membranes of intact cells. Pretreatment of CHO-FLAG-hKOR cells with MCD for 1 h at 37°C reduced cholesterol content in a dose-dependent manner with 0.5, 1, and 2% MCD causing reductions of 32, 43, and 48%, respectively (Fig. 3A). Pretreatment with 2% MCD followed by different concentrations of CH-MCD restored and then increased cell cholesterol contents in a dose-dependent manner (Fig. 3B). In the following experiments, 2% MCD was used for cholesterol reduction and 2% MCD followed by 1 mg/ml CH-MCD was used for cholesterol replenishment, unless indicated otherwise.

Effects of Pretreatment with MCD or MCD/CH-MCD on Cell Morphology. The majority of control cells are spindle-shaped (Fig. 4A). All cells treated with 2% MCD for 1 h became spherical (Fig. 4B). After MCD treatment, CH-MCD dose dependently restored shape of the cells toward the control state (Fig. 4, C–E). These results indicate that cholesterol plays a key role in maintaining the morphology of CHO cells.

MCD Treatment Disrupted Lipid Rafts and Shifted FLAG-hKOR and Caveolin-1 to Higher Density Fractions, and the Effects Were Reversed by CH-MCD. CHO-FLAG-hKOR cells were left untreated or were treated with 2% MCD or 2% MCD followed by 1 mg/ml CH-MCD, and subjected to detergent-free lipid raft preparation procedures. To facilitate detection of the shift caused by MCD and MCD/
CH-MCD treatments, we used continuous sucrose gradients (5–45%) instead of discontinuous sucrose gradients for these experiments.

In control cells, high cholesterol contents were found in fractions 1 to 4 with the peak at the second fraction (Fig. 5Ai). High levels of \([3H]\)diprenorphine binding and FLAG immunoreactivities were found in fractions 1 to 3 with the peak at the second fraction (Fig. 5A, ii and iii). Caveolin-1 was distributed in fractions 1 to 5 with the highest in the second and third fractions (Fig. 5Aiii). Flotillin-1, another protein residing predominantly in lipid rafts (Bickel et al., 1997; Volonte et al., 1999) had its highest levels in fractions 1 to 3 (Fig. 5Aiii).

In 2% MCD-treated cells, cholesterol levels were greatly reduced in fractions 1 to 4 (Fig. 5Bi), indicating that MCD treatment disrupts lipid rafts. After MCD treatment, high levels of \([3H]\)diprenorphine binding were shifted to fractions 5 to 7 with the peak at the sixth fraction (Fig. 5Bii), and FLAG-hKOR immunoreactivity peaked at the sixth fraction (Fig. 5Biii). The highest levels of caveolin-1 were found in the sixth and seventh fractions (Fig. 5Biii). However, flotillin-1 remained in low-density fractions. Thus, FLAG-hKOR and caveolin-1 are shifted to fractions of higher density after MCD pretreatment.

Fig. 2. FLAG-hKOR expressed in CHO cells is localized in low-density membrane microdomains as caveolin-1. CHO cells stably expressing FLAG-hKOR were subjected to detergent-free lipid raft preparation using 500 mM sodium carbonate buffer (pH 11) and sonication. Cells were then fractionated through a discontinuous sucrose gradient (5%/35%/45%). Twelve 1-ml fractions were collected, and each fraction was examined for (A) determination of cholesterol contents. Data are expressed as the ratios of [cholesterol (Chol) in each fraction]/[total phospholipids (PL)]. B, SDS-PAGE, transfer to membranes, and protein staining with Ponceau S. C, \([3H]\)diprenorphine (~1 nM) binding using naltrexone (10 μM) to define nonspecific binding. Data are expressed as percentage of the sum of specific \([3H]\)diprenorphine binding for each fraction. In this experiment, 1.09 × 10⁶ cells were used and 100 μl of a 1-ml fraction was used in binding experiments in duplicate. The counts of \([3H]\)diprenorphine specific binding in fraction 4 was 5,626 dpm/100 μl. D, immunoblotting of FLAG-hKOR and caveolin-1 with polyclonal anti-FLAG antibody and anti-caveolin-1 monoclonal antibody, respectively. Unfractionated cells were used as the control. The figures are the representative results of the three experiments performed with similar results.
In cells treated with 2% MCD followed by 1 mg/ml CH-MCD, high cholesterol contents were restored in fractions 1 to 4 (Fig. 5C(iii)) with a pattern comparable with that in control cells. High levels of \(^{3}H\)Diprenorphine binding and FLAG immunoreactivities were found in fractions 1 to 4 with the third being highest (Fig. 5C, ii and iii). Caveolin-1 was distributed in fractions 1 to 6 with the third being highest (Fig. 5C, ii and iii). Thus, MCD treatment followed by CH-MCD restored the patterns of distribution of cholesterol, hKOR and caveolin-1, resembling those of control cells. MCD and MCD-CH treatment appeared not to change caveolin-1 levels.

**Effects of Pretreatment with MCD and MCD/CH-MCD on Ligand Binding.** As shown in Table 1, 2% MCD treatment did not significantly change \(K_d\) and \(B_{\text{max}}\) values of \(^{3}H\)Diprenorphine binding to hKOR expressed in CHO cells. However, it did significantly reduce the \(K_d\) value of U50,488H. Cholesterol repletion after MCD treatment returned the \(K_d\) value of U50,488H to the control levels.

**Effect of Pretreatment with MCD and MCD/CH-MCD on p42/p44 MAP Kinase Phosphorylation.** Pretreatment of cells with MCD enhanced phosphorylation to extents similar to those in control cells. MCD and MCD-CH treatment appeared not to change caveolin-1 levels.

**Stimulation of \(\kappa\) opioid receptors has been previously demonstrated to result in enhanced p42/p44 MAP kinase phosphorylation (Li et al., 1999; Bohn et al., 2000), which is mediated by \(\beta\) subunits of pertussis-sensitive G proteins (Bohn et al., 2000).** Pretreatment of cells with MCD enhanced the extent of U50,488H-promoted p42/p44 MAP kinase phosphorylation (Fig. 7). In addition, in cells pretreated with MCD/CH-MCD, U50,488H stimulated p42/p44 MAP kinase phosphorylation to extents similar to those in control cells (Fig. 7).

We have performed \(^{35}S\)GTP\(\gamma\)S binding using low-density CHO-sFLAG-hKOR membranes isolated with sucrose gradients. However, we were not able to detect any agonist-stimulated \(^{35}S\)GTP\(\gamma\)S binding. This is probably due to the harsh condition used for preparation (0.5 M sodium carbonate buffer, pH 11) that may disrupt G protein structure and/or receptor-G protein coupling.

We have also examined the effects of cholesterol reduction on U50,488H-induced inhibition of forskolin-stimulated adenyl cyclase. However, 2% MCD pretreatment greatly reduced forskolin-stimulated adenyl cyclase activity (data not shown), which made it difficult to assess U50,488H-induced inhibition. Varga et al. (1998, 1999) detected adenyl cyclase types 6 and 7 mRNAs and type 6 protein in CHO cells. Studies have suggested that Ca\(^{2+}\)-sensitive adenyl cyclases (particularly isoforms 5, 6, and 8) are enriched in lipid rafts, whereas Ca\(^{2+}\)-insensitive adenyl cyclases (isoforms 2, 4, and 7) are excluded (Crossthwaite et al., 2005). It is plausible that adenyl cyclases in CHO cells are in lipid rafts and cholesterol depletion by MCD treatment attenuates their activity.

**Coimmunoprecipitation of Caveolin-1 with FLAG-hKOR and Effect of MCD Treatment.** Couet et al. (1997) found that the caveolin-1 (82–101) peptide bound to two binding
motifs with the sequences of $\phi X\phi XXX\phi$ and $\phi XXXX\phi\phi$, where $\phi$ is an aromatic residue (Trp, Phe, or Tyr). The $\kappa$ opioid receptor has the $\phi X\phi XXX\phi$ motif in the intracellular end of the seventh transmembrane helix (TM7) and the beginning of the C-terminal domain: $Y^{7.53(330)}AFLDENF^{7.60(337)}$. We thus examined whether the FLAG-hKOR coimmunoprecipitated with caveolin-1.

CHO-sFLAG-hKOR cells were solubilized with 2% Triton...
TABLE 1
Effects of MCD and MCD/CH-MCD pretreatment on $K_d$ and $B_{\text{max}}$ values of [35S]GTPγS binding and $K_i$ value of U50,488H binding to FLAG-hKOR stably expressed in CHO cells

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<th>$K_d$ (nM)</th>
<th>$B_{\text{max}}$ (fmol/10^7 cells)</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.14 ± 0.02</td>
<td>312.9 ± 9.2</td>
<td>1.30 ± 0.10</td>
</tr>
<tr>
<td>2% MCD</td>
<td>0.13 ± 0.01</td>
<td>342.8 ± 18.9</td>
<td>0.41 ± 0.01**</td>
</tr>
<tr>
<td>MCD + CH-MCD</td>
<td>0.19 ± 0.04</td>
<td>327.4 ± 13.9</td>
<td>1.18 ± 0.12</td>
</tr>
</tbody>
</table>

** P < 0.01, compared with the control as determined by Student’s t test.

Fig. 6. Effects of cholesterol reduction and repletion on U50,488H-stimulated [35S]GTPγS binding. A, CHO cells stably transfected with FLAG-hKOR were incubated with vehicle, 2% MCD, or 2% MCD and then 1 mg/ml CH-MCD. Membranes were prepared, and U50,488H-stimulated [35S]GTPγS binding to membranes was carried out with different concentrations. Nonspecific binding was determined in the presence of 10 μM cold GTPγS. Data were calculated as percentage of the basal [35S]GTPγS binding. Pretreatment with MCD or MCD and then CH-MCD did not affect basal binding. Data are shown as means ± S.E.M. from three or four experiments. B, displacement of [35S]GTPγS binding with the indicated concentrations of unlabeled GTPγS was performed in the presence and absence of 1 μM U50,488H. Data represent the differences between stimulated and basal levels in control and 2% MCD-treated cells. The figure represents one of the three experiments performed with similar results.

X-100 and centrifuged at 100,000g for 1 h. [Note: In the literature, the Triton X-100 concentration used to solubilize nonlipid raft membranes and, thus, define lipid rafts, ranged from 0.1 to 1% (Brown and Rose, 1992).] To assess whether the FLAG-hKOR was still present in low-density fractions after 2% Triton X-100 solubilization, we fractionated the 100,000g supernatant in a 0 to 45% sucrose gradient. The vast majority of FLAG-hKOR immunoreactivities were detected in fractions 8 to 12 (Fig. 8A). Thus, the receptor is no longer localized in lipid rafts, indicating that the receptor is solubilized from lipid rafts. The supernatant was immunoprecipitated with FLAG antibodies. Immunoprecipitated materials were resolved on SDS-PAGE and immunoblotted with caveolin-1 antibodies. As shown in Fig. 8B, caveolin-1 immunoprecipitated with FLAG-hKOR and MCD pretreatment did not affect coimmunoprecipitation. When the FLAG antibody was preincubated with the FLAG peptide, little FLAG-hKOR or caveolin-1 was precipitated.

Coimmunoprecipitation of Caveolin-1 with $G_\alpha_i$ Proteins and Effect of MCD Treatment. CHO-sFLAG-hKOR cells were solubilized with 2% Triton X-100 and centrifuged at 100,000g for 1 h. $G_\alpha_i$ Subunits of $G_\alpha_i$ proteins were immuno-precipitated from the supernatant with antibodies against the $G_\alpha_i$ proteins but not with preimmune serum (Fig. 9). Caveolin-1 immunoprecipitated with $G_\alpha_i$ proteins and MCD pretreatment reduced the amount of caveolin coimmunoprecipitated with $G_\alpha_i$ proteins (Fig. 9).
We have found that \( \kappa \) opioid receptors in human placenta and the hKOR expressed in CHO cells are localized in low-density fractions, which are enriched in cholesterol and caveolin-1, characteristic of lipid raft microdomains. In addition, opioid receptors in rat brain membranes were found to localize in lipid rafts microdomains; however, brain lipid rafts contain a low level of caveolin-1 (P. Huang, W. Xu, S. I. Yoon, P. L.-G. Chong, and L. Y. Liu-Chen, manuscript in preparation). Thus, KOR is constitutively localized in lipid rafts. Because the KOR coimmunoprecipitates with caveolin-1 in CHO cells, it is likely that the KOR resides in caveolae in these cells. Caveolae were defined morphologically as flask-shaped invaginations in plasma membranes at the ultrastructural level. However, because we have no morphological evidence, we use the term “lipid rafts.”

Recently, the \( \mu \) opioid receptor in adult cardiac myocytes and the nociceptin/orphanin FQ receptor expressed in HEK293 cells were found to reside in membrane rafts independent of agonist stimulation (Butour et al., 2004; Head et al., 2005). In addition, a number of GPCRs are localized in lipid rafts, either constitutively or induced by agonists (for reviews, see Pike, 2003; Chini and Parenti, 2004).

**Discussion**

**Constitutive Compartmentalization of the hKOR in Lipid Rafts**

In addition to receptor binding, we monitored FLAG immunoreactivity for \( \kappa \) opioid receptor in the placenta is localized in lipid rafts. The direct effect of MCD on cells is extraction of cholesterol in the outer layer of plasma membranes. After cholesterol was extracted from plasma membranes, cholesterol in the other intracellular compartment may efflux to plasma membranes. Because cholesterol is much more abundant in plasma membranes than in other cellular compartments, MCD effects are most significant on plasma membranes. The \( \kappa \) opioid receptor construct used in the study contains a signal peptide that enhances insertion of the protein in endoplasmic reticulum membranes and thus promotes cell surface expression (Guan et al., 1992). We have found that the majority of \( \kappa \) opioid receptor observed from whole cell studies are probably due to its effects on plasma membranes.

Cholesterol is important for localization of the hKOR in lipid rafts, maintaining cell shape and constraining receptor-G protein coupling: atomic force microscopy revealed that lipid rafts in model lipid bilayers were patch-like and that MCD treatment resulted in a reduction in size and eventual dissolution of lipid rafts in a time-dependent manner (Law-
It is conceivable that similar processes occur in plasma membranes upon cholesterol reduction, and the processes are reversed during cholesterol repletion. MCD treatment shifted the KOR and caveolin-1 to medium-density fractions, changed cell shape, and enhanced \( \kappa \) receptor-mediated-G protein activation and p42/p44 MAP kinase phosphorylation. Cholesterol repletion after MCD treatment restored high cholesterol contents in low-density fractions and returned the KOR and caveolin-1 to these fractions, restored the spindle-like cell shape, and brought the signaling back to the control levels. Taken together, these results indicate the importance of cholesterol in all of these processes. In addition, MCD effects were reversed by cholesterol repletion, indicating that its effects are due to a reduction in cholesterol. Because the MCD effects under the conditions used in this study were reversed by subsequent cholesterol replenishment, these results suggest that MCD does not affect cell viability.

After MCD treatment, flotillin-1 was still localized in fractions of low sucrose density. Our results are similar to those of Rajendran et al. (2003), who showed that after MCD treatment of Jurkat T cells and U937 cells, flotillin-1 remained in low-density fractions. In the contrast, Urano et al. (2005) reported that flotillin-1 was shifted from low-density fractions to high-density fractions upon MCD treatment in human SH-SY5Y neuroblastoma cells. The reason for the differences is not clear. MCD effects on flotillin-1 location in membranes are likely to be cell type-dependent. Alternatively, there may be heterogeneous populations of lipid rafts, at least in some cells (Pike, 2004).

The mechanisms underlying changes in cell shape by cholesterol reduction remained to be investigated. Cholesterol depletion inhibits chemoattractant-induced neutrophil polarization and migration by abrogating prolonged activation of Rac and sustained actin polymerization (Pierini et al., 2003). Thus, cholesterol reduction may affect the cytoskeleton.

Cell lines or tissues may have different cholesterol contents in membranes. Under certain physiological or pathological conditions, cholesterol contents in cells may vary. For example, synapse formation requires cholesterol delivery from astrocytes to neurons (Mauch et al., 2001). At the time of delivery, membrane cholesterol contents increased sharply. In animals having very high serum cholesterol levels, membrane cholesterol levels are likely to be increased. It is possible that varying cholesterol contents in plasma membranes may affect opioid receptor signaling in vivo.

### Cholesterol Reduction Affects hKOR-G Protein Signaling in CHO Cells

Disruption of lipid rafts significantly enhances coupling of the hKOR to \( G_{i/o} \) and cholesterol replenishment returns it to the control level. Our results are in accord with those of some studies in the literature, for example, those of Bari et al. (2005), Rybin et al. (2000), and Xiang et al. (2002). Bari et al. (2005) reported that in C6 glioma cells, MCD pretreatment profoundly increased CB1 cannabinoid receptor-mediated \([^{35}S]GTP_\gamma S \) binding, p42/p44 MAP kinase phosphorylation, and inhibition of forskolin-stimulated adenylate cyclase. In cardiomyocytes, cholesterol reduction augments \( \beta \)-adrenergic agonist-stimulated cAMP accumulation (Rybin et al., 2000) and an increase in contraction rate (Xiang et al., 2002). However, in the majority of reports, cholesterol reduction resulted in a decrease or no change in signaling or downstream effects mediated by GPCRs compartmentalized in lipid rafts (for a review, see Pike, 2003, and references therein). Effects of cholesterol reduction on GPCR signaling may depend on the receptor and cells and may be related to cholesterol contents in lipid rafts.

**Possible Mechanisms Underlying the Enhancement of \( \kappa \) Opioid Signaling by MCD Treatment**

The augmented agonist-induced \([^{35}S]GTP_\gamma S \) binding is due to elevated \( B_{max} \) of \([^{35}S]GTP_\gamma S \) binding without changing its \( K_D \), indicating that more G proteins are activated by the hKOR. There may be two possibilities: one related to caveolin-1 and the other to membrane packing.

**Caveolin-1.** Caveolin-1 has been shown to interact, through its scaffolding domain, with many signaling molecules and inhibit signaling in most cases, including G proteins (for a review, see Razani et al., 2002). Purified caveolin-1 was found to bind to purified \( \alpha \) subunits of \( G_{s/o} \), \( G_{i} \), and \( G_{o} \) proteins and potently inhibit activities of \( G_{i} \) proteins (Li et al., 1995). We have found that caveolin-1 coimmunoprecipitates with \( G_{i} \) proteins, and MCD pretreatment reduced the amount of caveolin-1 coimmunoprecipitated with \( G_{i} \) proteins. The reduced association, which presumably releases some inhibitory effect of caveolin-1 on the activities of \( G_{i} \) proteins, results in enhanced agonist-stimulated G protein activities (\([^{35}S]GTP_\gamma S \) binding and p42/p44 MAP kinase phosphorylation). The role of caveolins is currently being further investigated using siRNA targeting caveolins. Our findings complement those of Toselli et al. (2001), who showed that in NG108-15 cells, which lack endogenous caveolins, stable expression of caveolin-1 or -3 attenuated \( G_{i} \) protein-mediated activities (inhibition of N-type voltage-gated Ca\(^{2+}\) channels mediated by \( \delta \) opioid receptor or direct stimulation of G proteins). The observation that caveolin-1 coimmunoprecipitates with \( G_{i} \) proteins is consistent with several studies, for example, De Weerd and Leeb-Lundberg (1997) and Murthy and Makhloof (2000).

Our finding that the hKOR coimmunoprecipitated with caveolin-1 is in accord with the observations that caveolins coimmunoprecipitate with some GPCRs, for example, endothelin\(_1\) (Chun et al., 1994), 5-hydroxytryptamine\(_{2X}\) (Bhatnagar et al., 2004), and \( \mu \) opioid receptors (Head et al., 2005). MCD pretreatment did not affect coimmunoprecipitation of the hKOR with caveolin-1, indicating that enhanced receptor-G protein coupling after MCD treatment is not related to the relationship between the hKOR and caveolin-1.

**Membrane Packing.** Cholesterol reduction by MCD significantly enhanced the affinity of U50,488H for the hKOR, which was reversed by cholesterol repletion, demonstrating that the membrane environments have an impact on receptor conformation. Although cholesterol enrichment shifted rhodopsin and the cholecystokinin\(_1\) receptor toward an inactive uncoupled state, cholesterol reduction favored active conformations (Niu et al., 2002; Harikumar et al., 2005). It has been shown that high cholesterol inhibits rhodopsin activation by reducing the free volume of phospholipid acyl chain packing (Niu et al., 2002).

**Sterol Affects Binding Properties of Opioid Receptors**

Cholesterol has been shown to modulate the binding properties of opioid receptors. Here we demonstrated that chole-
terol reduction by MCD enhanced the affinity of U50,488H for the hKOR but did not affect that of [3H]diprenorphine. Increasing membrane cholesterol in N1E-115 neuroblastoma cells reduced [3H]Met-enkephalin binding activity at the δ opioid receptor (Rao and Murphy, 1984). Sterol was reported to play roles in modulating the μ opioid receptor functions in yeast: cholesterol appeared to constrain the μ opioid receptor in an active state, whereas ergosterol held it in an inactive state (Lagane et al., 2000).

Lipid Raft Preparation Methods

Three methods and their variations have been used, and each has its supporters and detractors (Pike, 2004). The first, developed by Brown and Rose (1992), involves solubilization of membranes with a nonionic detergent such as Triton X-100 (1% at 4°C for 1 h) followed by sucrose gradient centrifugation. The second, described first by Song et al. (1996) and used in our studies, involves sodium carbonate and brief sonication to solubilize membranes followed by sucrose gradient centrifugation. The third, described by Smart et al. (1995), uses Percoll gradient to isolate plasma membranes from lysed cells, which are then sonicated, followed by Opti-Prep gradient centrifugation to obtain lipid rafts. We opted not to use the detergent method for two reasons. First, we found that Triton X-100 at 0.3 to 1.0% destroyed opioid receptor binding activity, which made it impossible to investigate endogenous opioid receptors in tissues and cells. Second, in the literature, the concentration of Triton X-100 for the hKOR but did not affect that of [3H]diprenorphine. We therefore used the sodium carbonate method, which we were able to detect receptor binding activity. We also showed that low-density fractions contain high levels of cholesterol in preparations from NG108-15 cells and rat brain (P. Huang, W. Xu, S. I. Yoon, P. L.-G. Chong, and L. Y. Liu-Chen, manuscript in preparation) and rat heart, thus validating the method, in addition to CHO cells and human placenta. We routinely monitor cholesterol contents in fractions of sucrose gradient centrifugation for quality control purposes.

CHO Cells as a Model

Opioid receptors are distributed mainly in the central and peripheral nervous systems, which have low or no caveolins (Galliati et al., 1998). However, opioid receptors are also present in non-neuronal tissues. The κ opioid receptor is present in human placenta (Porthe et al., 1981; Mansson et al., 1994) and rat heart myocytes (Ventura et al., 1989; Wong et al., 1990). Caveolins are widely distributed in peripheral tissues, including heart and placenta. We believe that opioid receptors expressed in CHO cells serve as a good model for opioid receptors in peripheral tissues.

In conclusion, we found that κ opioid receptors reside in lipid rafts. Compartmentalization of the κ opioid receptor in the lipid raft microdomain is regulated by cholesterol contents in a reversible manner and has a significant impact on κ opioid receptor-mediated G protein activation and downstream p42/p44 MAP kinase phosphorylation.

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References


