G_s Signaling Is Intact after Disruption of Lipid Rafts[†]

Yukiko Miura,[‡,§] Kentaro Hanada,[||] and Teresa L. Z. Jones*[,‡]

Metabolic Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, and Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, 1-23-1, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

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ABSTRACT: Membrane microdomains enriched in cholesterol and sphingolipids modulate a number of signal transduction pathways and provide a residence for heterotrimeric G proteins, their receptors, and their effectors. We investigated whether signaling through G_s was dependent on these membrane domains, characterized by their resistance to detergents, by depleting cells of cholesterol and sphingolipids. For cholesterol depletion, rat salivary epithelial A5 cells were cultured under low-cholesterol conditions, and then treated with the cholesterol chelator methyl-β-cyclodextrin. For sphingolipid depletion, LY-B cells, a mutant CHO cell line that is unable to synthesize sphingolipids, were incubated under low-sphingolipid conditions. Depletion of cholesterol or sphingolipid led to a loss or decrease, respectively, in the amount of $G\alpha_s$ from the detergent-resistant membranes without any change in the cellular or membrane-bound amounts of $G\alpha_s$. The cAMP accumulation in response to a receptor agonist was intact and the level slightly increased in cells depleted of cholesterol or sphingolipids compared to that in control cells. These results indicate that localization of $G\alpha_s$ to detergent-resistant membranes was not required for G_s signaling. Analysis of the role of lipid rafts on the kinetics of protein associations in the membrane suggests that compartmentalization in lipid rafts may be more effective in inhibiting protein interactions and, depending on the pathway, ultimately inhibit or promote signaling.

The lipid raft hypothesis postulates that cell membranes contain membrane microdomains that are enriched in cholesterol and sphingolipids (reviews in refs *1*−*3*). The hypothesis is based on research in lipid bilayers where distinct liquid-ordered domains of tightly packed cholesterol and sphingolipids coexist with the loosely packed, disordered state of phospholipids. This property of tight packing of cholesterol and sphingolipid leads to resistance to detergent extraction and buoyancy on sucrose-density gradient centrifugation and has been applied as a method for isolating lipid rafts from cell membranes. Lipid rafts can also be detected in living cells using fluorescence resonance energy transfer (FRET), chemical cross-linking, and other methods (*2*, *3*).

A dominant model for the function of lipid rafts is that they act to bring proteins together to improve the kinetics of signaling (*1*, *2*, *4*). Signal transduction through many pathways, including the T cell receptor, immunoglobulin E, tumor necrosis factor receptor, H-ras, and RET**,** depend on lipid rafts because their disruption significantly impairs signaling (*5*−*13*). Also, activation of some pathways leads to coalescence of rafts that can be detected in living cells.

Heterotrimeric G proteins, receptors, and effectors reside in lipid rafts as do other signaling proteins (*4*, *5*, *14*−*16*). G proteins transduce signals from cell surface receptors to intracellular effectors (*17*, *18*). The heterotrimer consists of an α subunit, which exchanges GDP for GTP upon activation of the receptor, and $\beta\gamma$ subunits, which are tightly bound together. The membrane attachment of G proteins is critical for their interactions with membrane-bound receptors and effectors, but models of random movement and collisions in membranes are insufficient to explain the speed and fidelity of these interactions. Instead, components of G protein signaling are likely to be highly organized at the membrane, but the nature of this organization is not fully understood (*17*, *19*, *20*).

Lipid rafts have been postulated to be signaling centers for G proteins on the basis of the localization of G proteins in detergent-resistant membranes (DRMs),[1] the interactions between caveolin and G proteins, and the role of lipid rafts in other signaling pathways (*4*, *19*−*21*). We investigated whether signaling through G_s was dependent on lipid rafts by depleting cells of cholesterol and sphingolipids and measuring the cAMP response to receptor activation. These conditions led to a reduction in the amount of $G\alpha_s$ in the DRMs but intact signaling, contrary to predictions that the function of lipid raft association is facilitation of signal transduction.

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* To whom correspondence should be addressed: National Institutes of Health, Building 10/Room 9C101, Bethesda, MD 20892-1802. Phone: (301) 496-8711. Fax: (301) 496-0200. E-mail: tlzj@helix.nih.gov.

[‡] National Institutes of Health.

[§] Present address: Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, 1-23-1, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan.

[||] National Institute of Infectious Diseases.

[1] Abbreviations: G protein, heterotrimeric guanine nucleotide-binding protein; $G\alpha_s$, G protein α subunit associated with adenylyl cyclase stimulation; DRMs, detergent-resistant membranes; FBS, fetal bovine serum; Nu, Nu-serum; CD, methyl-β-cyclodextrin; cpm, counts per minute; SEM, standard error of the mean.

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EXPERIMENTAL PROCEDURES

Cell Culture and Lipid Modification. Rat salivary epithelial A5 cells and the mutant CHO-K1 cells, LY-B and LY-B/cLCB1, were maintained as previously described (*22*, *23*). For cholesterol depletion, A5 cells were cultured in McCoy's 5A medium with 10% Nu-serum IV (Becton-Dickinson) for 17 h and then incubated in serum-free medium with or without 10 mM methyl-β-cyclodextrin (Sigma Chemical Co.) for 30 min. For sphingolipid depletion, LY-B and LY-B/cLCB1 cells were cultured in Nutridoma-BO medium for 2 days at 37 °C as previously described (*23*). COS7 cells and HEK293T cells were maintained in DMEM with 10% FBS.

Isolation of DRMs from Sucrose Gradients. DRMs were prepared as described previously (*24*) with the following modifications. Cells from one 150 mm plate were washed three times with phosphate-buffered saline, pelleted, lysed at 4 °C for 20 min in 0.3 mL of TNE buffer [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM EDTA] containing 1% (w/v) Triton X-100 and protease inhibitors, and passed through a 25 gauge needle 12 times. The homogenates were suspended in TNE buffer containing sucrose to adjust its volume and concentration to 0.6 mL of 40% (w/v) sucrose and placed in the bottom of a centrifuge tube. Next, 1.8 mL of 30% sucrose in TNE buffer and 1.2 mL of 5% sucrose in TNE buffer were layered on top of the sample solution, and the solution was centrifuged for 17 h at 200000*g* and 4 °C. Twelve fractions (0.3 mL each) were collected from top to bottom, and the proteins precipitated with 20% trichloroacetic acid.

Signaling Assays. cAMP accumulation was assessed as previously described (*25*). Briefly, the cells were incubated overnight in [^{3}H]adenine, washed, incubated in serum-free medium with or without 10 mM CD for 30 min, and then incubated for 5, 15, or 45 min in serum-free medium containing agonists and 1 mM 1-methyl-3-isobutylxanthine, a phosphodiesterase inhibitor. [^{3}H]cAMP and [^{3}H]ATP were separated on Dowex and alumina columns and the amounts determined by scintillation counting. The level of cAMP accumulation was calculated as [[^{3}H]cAMP cpm/([^{3}H]cAMP cpm + [^{3}H]ATP cpm)] $\times$ 10^3.

Cell Fractionation. Cells were homogenized in 10 mM Hepes (pH 7.4) containing 50 mM mannitol, 1 mM EDTA, and protease inhibitors and centrifuged for 3 min at 750*g* and 4 °C. The lysates were separated into particulate and soluble fractions by ultracentrifugation for 1 h at 100000*g* and 4 °C (*25*).

Lipid Analysis. The cells were washed three times with phosphate-buffered saline and harvested by scraping. Lipids were extracted by the method of Bligh and Dyer (*26*). The amount of cellular cholesterol was determined using an enzymatic method (Cholesterol CII assay kit, Wako). For sphingolipid analysis, lipids were separated by thin-layer chromatography on Silica Gel 60 (Merck) with a solvent of chloroform, methanol, and acetic acid (65/25/10, v/v) and were stained by a modification of the method of Nakamura and Handa (*27*). Briefly, after air-drying, the chromatography plates were immersed in 25% methanol containing 0.1 M NaCl and 0.05% Coomassie Blue for 5 min, gently washed three times in 25% ethanol containing 0.1 M NaCl, and dried under air. Amounts of the lipids were determined by densitometric analysis of the stained plates, using a calibration curve made with a standard of sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, or phosphatidylethanolamine (Sigma Chemical Co.). The lipid amounts were corrected for protein concentration.

Growth Rate. A5 cells (1×10^4 cells/dish) were transferred into 100 mm dishes and incubated for 6 h for adherence of the cells to dishes. The medium was changed to 10 mL of McCoy's 5A medium with 10% fetal bovine serum or 10% Nu serum IV. The cells were cultured at 37 °C for 1, 2, 3, and 5 days and then harvested by trypsinization. The cell counts were determined with a hemocytometer after staining with trypan blue. The doubling time of the cells was calculated using a growth curve.

Receptor Binding. The particulate fractions (20 μg of protein/tube) were resuspended in binding buffer [50 mM Tris buffer (pH 7.4) containing the protease inhibitors] supplemented with 10 nM [^{3}H]dihydroalprenolol (American Radiolabeled Chemicals). The level of nonspecific binding was determined in the presence of 10 nM [^{3}H]dihydroalprenolol and 50 μM ($\pm$)-alprenolol. The mixture was incubated for 90 min at room temperature with shaking at 200 rpm. Bound [^{3}H]dihydroalprenolol was separated by vacuum filtration through GF/C filters, followed by three washes with ice-cold Tris buffer. The amount of filter-bound radioactivity was determined by liquid scintillation counting. Nonspecific binding was less than 10% of the total binding.

Miscellaneous. For immunoblotting, a caveolin antibody (Transduction Laboratories) and the RM antibody to the carboxy-terminal decapeptide of $G\alpha_s$ were used as previously described (*25*). Enhanced chemiluminescence (Amersham) was used as the detection system. For transient transfection, LY-B and LY-B/cLCB1 cells were transfected with a pcDNA3.1 vector containing the cDNA for the β_2-adrenergic receptor using the Lipofectamine reagent (Life Technologies Inc.). The Allfit software (*28*) was used to calculate the EC_{50} from the dose−response curves. Densitometry was performed using a UMAX scanner, model UTA-II, and Scion Image software.

RESULTS

Disruption of Detergent-Resistant Membranes (DRMs) after Depletion of Cellular Cholesterol. For cholesterol depletion, A5 cells were incubated overnight in either medium with 10% fetal bovine serum (FBS), as the control, or 10% Nu-serum and then for 30 min in the presence or absence of 10 mM methyl-β-cyclodextrin (CD), a cholesterol chelator. The concentration of cholesterol in Nu-serum is ~25% of the concentration in FBS. The level of cellular cholesterol was decreased by ~30% with treatment with Nu-serum alone and ~40 and ~60% in the presence of CD (Figure 1).

Analysis of the lipid composition of cells treated with the different cholesterol-lowering conditions by thin-layer chromatography showed no differences in the amount of sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, or phosphatidylethanolamine compared to the control cells (data not shown). These conditions did not decrease cell viability as determined by trypan blue staining (data not shown). The growth rate for cells incubated under the Nu-serum conditions was not significantly different from that for the control cells. The doubling time calculated after

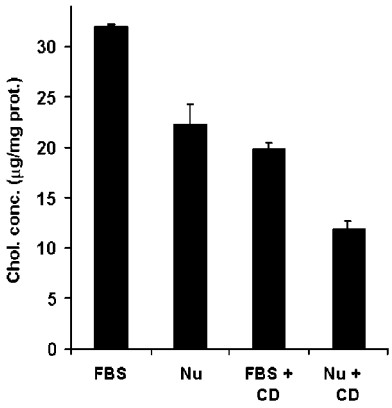

FIGURE 1: Cellular cholesterol concentration under low-cholesterol conditions. A5 cells were cultured in McCoy's 5A medium with 10% FBS or 10% Nu-serum for 17 h and then incubated in serum-free medium in the presence or absence of 10 mM CD for 30 min. The level of cellular cholesterol was determined by an enzymatic method. Values are means ± the standard error of three experiments performed in triplicate.

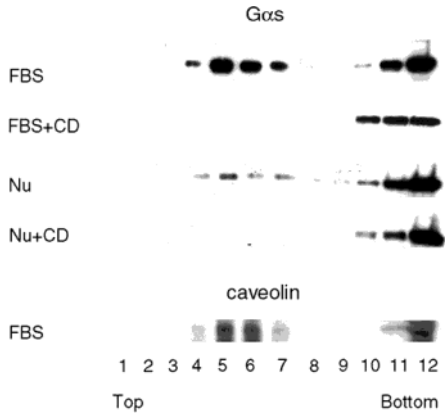

FIGURE 2: Effect of cholesterol depletion on the partitioning of $G\alpha_s$ to DRMs. A5 cells were cultured in McCoy's 5A medium with 10% FBS or 10% Nu-serum for 17 h and then incubated in serum-free medium in the presence or absence of 10 mM CD for 30 min. The cells underwent lysis in TNE buffer containing 1% Triton X-100 at 4 °C, homogenization, and sucrose gradient centrifugation. The proteins of each fraction were subjected to SDS−PAGE and immunoblotting with antibodies to $G\alpha_s$ or caveolin. The $G\alpha_s$ immunoblots were prepared concurrently with the same exposure times.

incubation for 5 days was 10.1 h for cells under the Nu-serum conditions and 8.7 h for cells under the FBS control conditions.

The preparation of detergent-resistant membranes (DRMs), which resist solubilization by nonionic detergents and float to low density on sucrose gradient centrifugation, is one method for determining components of lipid rafts (*2*). In A5 cells, $G\alpha_s$ and caveolin, a resident protein of lipid rafts, were found in fractions 4−7 after solubilization of cells with 1% Triton X-100 at 4 °C and gradient centrifugation (Figure 2). Treatment with CD alone or CD and prior incubation in Nu-serum led to a loss of $G\alpha_s$ in the buoyant fractions (Figure 2). The loss of DRM localization correlated with the level of cholesterol depletion (Figure 1) because treatment with Nu-serum alone led to a partial loss of $G\alpha_s$ from fractions 4−7 (Figure 2). These cholesterol-lowering conditions did not change the membrane-bound or total amount of $G\alpha_s$ (Figure 3). Treatment with CD also led to a loss of caveolin from the bouyant fractions (data not shown), indicating a

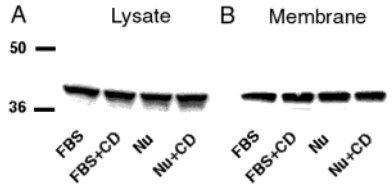

FIGURE 3: $G\alpha_s$ in the cell lysate and particulate fraction after cholesterol depletion. A5 cells were cultured in McCoy's 5A medium with 10% FBS or 10% Nu-serum for 17 h and then incubated in serum-free medium in the presence or absence of 10 mM CD for 30 min. (A) A cell lysate was obtained after homogenization and low-speed centrifugation. (B) The crude particulate fraction was separated from the lysate after ultracentrifugation. $G\alpha_s$ was detected by immunoblotting with a $G\alpha_s$ specific antibody and the enhanced chemiluminescence detection system.

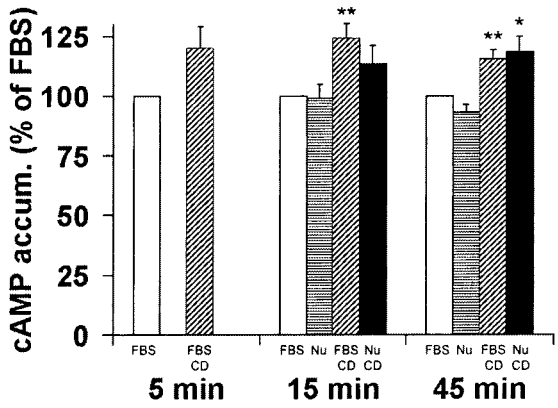

FIGURE 4: cAMP accumulation by isoproterenol stimulation after cholesterol depletion. A5 cells were cultured in McCoy's 5A medium containing [^{3}H]adenine with 10% FBS or 10% Nu-serum for 17 h and then incubated in serum-free medium in the presence or absence of 10 mM CD for 30 min (white, FBS; horizontal lines, Nu-serum; diagonal lines, FBS and CD; and black, Nu-serum and CD). The cells were treated with 5 μM isoproterenol and a phosphodiesterase inhibitor for 5, 15, and 45 min, and the level of cAMP accumulation was determined. The values shown are the means ± the standard error of three experiments for the 5 min incubation time, 8−10 experiments for the 15 min incubation time, and 10−15 experiments for the 45 min incubation time. All experiments were performed in triplicate. Significant differences are indicated as follows: $p < 0.03$ (one asterisk) and $p < 0.003$ (two asterisks) compared to the value for the FBS condition.

general disruption of lipid rafts that was not specific for $G\alpha_s$. Other investigators have found that treatment with CD under similar conditions leads to a significant loss of adenylyl cyclase from caveolar and DRM fractions (*29*, *30*).

G_s Signaling after Cholesterol Depletion. We investigated whether these changes in DRM localization of $G\alpha_s$ would affect its ability to respond to activation by the β-adrenergic receptor and to stimulate adenylyl cyclase to increase the level of intracellular cAMP accumulation. In a whole cell assay, the cAMP accumulation after isoproterenol treatment was the same as in the control cells for the Nu-serum condition (Figure 4). For both of the CD treatment conditions, the level of cAMP accumulation was slightly but significantly increased after isoproterenol treatment compared to that in control cells (Figure 4). All changes were reversible with overnight treatment in FBS (data not shown). The cholesterol-lowering conditions did not shift the dose response to isoproterenol (Table 1).

Treatment with cholera toxin or forskolin to directly activate G_s or adenylyl cyclase, respectively, also showed

Table 1: Dose Response to Isoproterenol[a]

	EC_{50} (μM)		EC_{50} (μM)
FBS	0.40 ± 0.06	FBS and CD	0.37 ± 0.06
Nu-serum	0.33 ± 0.01	Nu-serum and CD	0.36 ± 0.04

[a] The level of cAMP accumulation in A5 cells was determined as described in the legend of Figure 4 except the cells were exposed to a range of isoproterenol concentrations (0−0.1 mM). The dose halfway between the maximal and minimal response (EC_{50}) was calculated using the Allfit software from data from three to four separate experiments performed in triplicate. The means ± SEM are shown.

Table 2: cAMP Accumulation after Treatment with Cholera Toxin or Forskolin[a]

	cholera toxin (%)	forskolin (%)
FBS	100.0	100.0
Nu-serum	122.7 ± 13.3	108.5 ± 6.4
FBS and CD	98.3 ± 2.1	115.3 ± 4.9
Nu-serum and CD	83.5 ± 6.8	140.0 ± 7.4

[a] The level of cAMP accumulation in A5 cells was determined as described in the legend of Figure 4 except the cells were exposed to 1 μg/mL cholera toxin or 5 μM forskolin. The values are the means ± SEM of 6−12 experiments performed in triplicate.

that the cholesterol-lowering conditions led to equivalent or higher levels of cAMP accumulation compared to those of control cells (Table 2).

We also tested the effect of cholesterol depletion on G_s signaling in two other cell lines. In COS7 cells treated with 10 mM CD for 30 min, the cAMP accumulation after isoproterenol stimulation for 45 min was 98.7 ± 4.8% (mean ± SEM of six experiments) compared to the control COS7 cells. In HEK293T cells, the cholesterol-depleted cells exhibited 93.1% (mean of two experiments) of the cAMP response to isoproterenol compared to control cells.

G_s Signaling in Cells Deficient in Sphingolipids. We tested G_s signaling in a mutant CHO cell that was unable to synthesize sphingolipids because we wanted to alter lipid rafts independent of cholesterol depletion. Sphingolipid depletion does not effect the detergent solubility of proteins to the same extent as cholesterol depletion (*31*−*33*), but the high concentration of sphingolipids in DRMs (*24*) suggests that their depletion would alter at least the organization of lipid rafts. LY-B cells lack the LCB1 protein, a component of serine palmitoyl transferase that initiates sphingolipid biosynthesis from serine and palmitoyl CoA. Incubation of LY-B cells in a sphingolipid-deficient medium leads to an ~85% loss of cellular sphingolipid that can be restored to control levels by stable transfection of LY-B cells with the LCB1 protein (LY-B/cLCB1 cells) (*23*, *34*). After incubation for 2 days in Nutridoma-BO, a sphingolipid-deficient medium, the total amount of $G\alpha_s$ or the amount of $G\alpha_s$ in the crude membrane fraction did not differ between the LY-B and LY-B/cLCB1 cell lines (Figure 5A,B). The LY-B cells showed a reduced amount of $G\alpha_s$ in the DRM fractions, 72.7 ± 15.4% (mean ± SEM of four experiments) of the level in the LY-B/cLCB1 cells (Figure 5C).

To test signaling, we needed to transfect the LY-B and LY-B/cLCB1 cells with the β-adrenergic receptor. After transfection, 0.67 ± 0.11 pmol of receptor binding sites/mg of membrane protein (mean ± SEM) was present on the LY-B cells and 1.41 ± 0.37 on LY-B/cLCB1 cells. The level of cAMP accumulation in response to isoproterenol was greater in the sphingolipid-deficient LY-B cells than in the

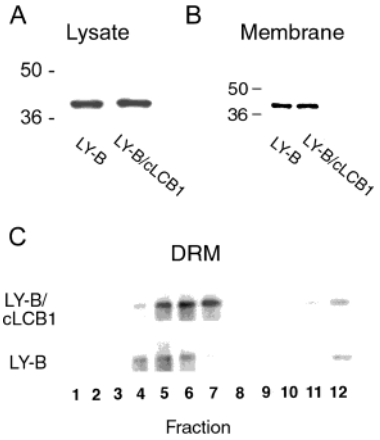

FIGURE 5: Membrane and DRM localization of $G\alpha_s$ after sphingolipid depletion. LY-B and LY-B/cLCB1 cells were cultured in Nutridoma-BO medium for 2 days at 37 °C. The cells were homogenized, and total cell lysates (A), crude membrane fractions (B), and DRMs (C) were prepared as described in Experimental Procedures. $G\alpha_s$ was detected by immunoblotting with a $G\alpha_s$ specific antibody.

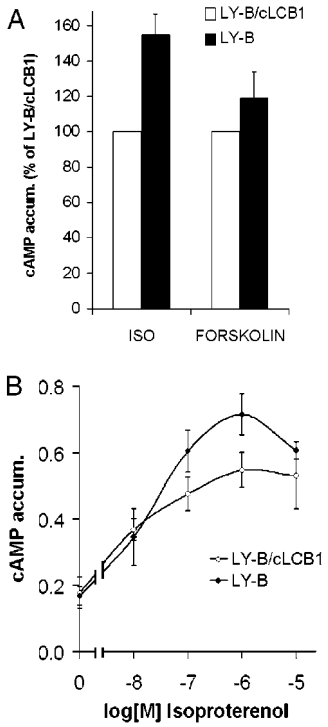

FIGURE 6: cAMP accumulation in cells after sphingolipid depletion. LY-B and LY-B/cLCB1 cells were transfected with the cDNA for the β-adrenergic receptor and cultured in Nutridoma-BO medium for 2 days at 37 °C. (A) The cells were treated with 5 μM isoproterenol or 5 μM forskolin and a phosphodiesterase inhibitor for 45 min, and the level of cAMP accumulation was determined. The values are the means ± the standard error of six experiments. (B) The cells were treated with the indicated concentrations of isoproterenol and a phosphodiesterase inhibitor for 45 min. The values are the means ± the standard error of six experiments.

LY-B/cLCB1 cells (Figure 6A) despite the lower level of expression of the β-adrenergic receptor. The dose response to isoproterenol was similar between the LY-B and LY-B/cLCB1 cells (Figure 6B). After treatment with forskolin, the level of cAMP accumulation was slightly higher for the LY-B than for LY-B/cLCB1 cells (Figure 6A). Therefore, for both sphingolipid and cholesterol depletion, cAMP accumulation in response to a receptor agonist was intact and the level slightly increased.

DISCUSSION

G proteins sit in discrete membrane microdomains called lipid rafts that are enriched in cholesterol and sphingolipids (*4*, *5*, *14*−*16*). Lipid rafts are critical for signaling through the IgE, T cell, and other receptors with a hypothesis predicting that lipid rafts act by concentrating proteins at the membrane to facilitate interactions (*1*, *2*, *4*, *21*). With this as a background, we anticipated finding that disruption of lipid rafts would impair G_s protein signaling. However, we found that receptor-mediated cAMP accumulation was intact and slightly improved after cholesterol or sphingolipid depletion.

One explanation for our results is that we did not adequately disrupt lipid rafts. G proteins reside on the cytoplasmic face of the membrane. The dynamics of lipids and proteins on the inner leaflet, and their relationship to the outer leaflet is poorly understood due to limitations in current methodologies. We cannot exclude the possibility that our conditions could have disrupted the localization of $G\alpha_s$ to DRMs, but the G_s signaling components still had some weak affinity for raftlike domains. However, the use of two different methods indicates that treatment with 10 mM CD for 30 min, as in this study, significantly impairs lipid raft formation. Pralle and colleagues found that cholesterol extraction accelerates the diffusion of raft-associated proteins to the rate of nonraft proteins in living cells using a laser trap (*35*). Cholesterol depletion also abrogated the patching of raft proteins upon cross-linking (*36*). In addition, treatment with CD, under conditions similar to those used in this study, disrupts signaling by IgE (*9*), T cell receptors (*8*), and the glial cell line-derived neurotrophic factor (*7*).

Another explanation is that the kinetics of G_s signaling with its slow inactivation rate, on the order of seconds, and abundance of proteins did not require compartmentalization in lipid rafts. Signal transduction occurs through a series of protein interactions. An equation that describes the number of associations between two proteins (A and B) is

$$\text{associations} = \eta_c \frac{4(D_A + D_B)}{S} ABt$$

where η_c is the coupling efficiency or the probability that a collision between A and B will result in their association, D is the diffusion coefficient, S is the cell surface area, A and B are the total numbers of proteins, and t is time (*37*). The primary effect of lipid rafts is likely to be a decrease in the surface area for interactions (S), but it could also effect the protein diffusion rates (D), though this has not been measured within lipid rafts, or, less likely, the coupling efficiency (η_c), if the lipid environment could alter the efficiency of protein collisions that form associations. Estimates of the fraction of the cell surface covered by lipid rafts range from 13 to 45%, depending on the cell type (*2*, *38*), but may be higher (*39*). If A and B are maximally concentrated in lipid rafts, then the number of associations increases by a factor of about two to seven. For signaling pathways with unfavorable kinetics, this gain may be critical, whereas in those pathways with favorable kinetics, such as G_s, the small gain in the level of protein association may be relatively unimportant. G_s signaling occurs through a series of bimolecular interactions of abundant proteins over a time scale of seconds. Signal transduction that has been shown to require lipid rafts, e.g., IgE and T cell receptors, occurs through multiprotein complexes. As a result, the kinetics for this signaling are generally less favorable than those of bimolecular interactions because at any time they require several proteins to form and maintain an association.

Lipid rafts may be more effective in inhibiting protein associations. The positive effect of lipid rafts on the association rate with a decrease in area (S) is limited by the relatively large fraction of the cell surface covered by rafts and countered by a decrease in the level of proteins (A and B) in the lipid rafts based on their relative affinities for the rafts. Instead, if proteins A and B have opposite affinities for lipid rafts, then their association would be completely blocked. Our results and results from the Steinberg laboratory (*30*) showing an increase in the extent of G_s signaling after cholesterol or sphingolipid depletion may be due to differences in the affinity of the receptor, G proteins, adenylyl cyclase, or other proteins for lipid rafts. For example, in a study using liposomes that mimic the lipid composition of lipid rafts, $G\beta\gamma$ had a poor affinity for DRMs and restrained acylated $G\alpha_i$ from associating with the DRMs (*40*). If lipid rafts decrease the levels of protein association, then their net effect on signaling could be positive if they inhibit associations with negative regulators such as phosphatases. Compartmentalization of phosphatases out of lipid rafts would promote signaling because phosphorylation is an integral part of signaling for many of the pathways located in lipid rafts. In contrast, inactivation of G_s occurs primarily through the intrinsic GTPase activity [though adenylyl cyclase accelerates this process (*41*)] rather than protein interactions.

How can we reconcile the finding that G proteins are in DRMs but that this localization is not critical for promoting G_s signaling? As discussed above, lipid rafts may inhibit G_s signaling. Another possibility is that lipid rafts may support another function of G_s besides cell surface signaling. Both G_s and lipid rafts are involved in apical sorting of proteins and endocytosis (*42*−*45*). Interestingly, isoproterenol stimulates endocytosis with a shift of plasma membrane proteins, such as G_s and adenylyl cyclase, to a low-density membrane fraction enriched in endosomes (*46*, *47*) and the β-adrenergic receptor to noncoated vesicles in the endosomal pathway (*48*). If lipid rafts facilitate internalization and retain proteins in endocytic compartments, then disruption of lipid rafts would lead to more receptors, G proteins, and adenylyl cyclase at the plasma membrane. An increase in the levels of G_s and adenylyl cyclase on the cell surface could also explain the small increase in the extent of cAMP accumulation that others and we found with forskolin stimulation (Table 2) (*30*).

Last, lipid rafts may be more important in providing a specific lipid environment than bringing G protein signaling components together. Cholesterol is critical for receptor-stimulated adenylyl cyclase activity in membranes (*49*, *50*) and the function of G protein-coupled receptors (*51*, *52*) and may directly affect the activity of adenylyl cyclase (Table 2). The lipid environment has been an important factor in the few studies that have addressed the functional effects of lipid rafts on G protein signaling. For G_i signaling, cholesterol depletion with CD abolished signaling by the integrin-associated protein (CD47), but cholesterol binding rather than raft localization appears to be the critical factor (*53*). The level of G_q signaling through the EGF and bradykinin recep-

tors decreases after depletion of cellular cholesterol, but it is unclear whether the decrease in the extent of phosphoinositide turnover is due to a decrease in the level of the lipid substrate, phosphatidylinositol bisphosphate, from the DRMs or the delocalization of the receptor and G_q (*5*). This question also applies to a study that found a decrease in the rate of IP3-dependent release of calcium after carbachol stimulation (*6*).

In conclusion, our results suggest that lipid rafts are not essential for G_s signaling and are not the organizing centers for G_s signaling, as they are for other signaling cascades. Inherent differences in the kinetics between the GTPase cycle for G_s signaling and the formation of signaling complexes in these other cascades may be the reason. A general model of lipid rafts bringing proteins together to facilitate interactions is at odds with our findings that disruption of the DRMs did not significantly change cAMP accumulation. Lipid rafts and the heterogeneity of lipids within the cell membrane may prove to have complex roles in many cellular processes.

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