

# Lipid Rafts Are Enriched in Arachidonic Acid and Plasmenylethanolamine and Their Composition Is Independent of Caveolin-1 Expression: A Quantitative Electrospray Ionization/Mass Spectrometric Analysis<sup>†</sup>

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*Received August 8, 2001; Revised Manuscript Received December 11, 2001*

**ABSTRACT:** Lipid rafts are specialized cholesterol-enriched membrane domains that participate in cellular signaling processes. Caveolae are related domains that become invaginated due to the presence of the structural protein, caveolin-1. In this paper, we use electrospray ionization mass spectrometry (ESI/MS) to quantitatively compare the phospholipids present in plasma membranes and nondetergent lipid rafts from caveolin-1-expressing and nonexpressing cells. Lipid rafts are enriched in cholesterol and sphingomyelin as compared to the plasma membrane fraction. Expression of caveolin-1 increases the amount of cholesterol recovered in the lipid raft fraction but does not affect the relative proportions of the various phospholipid classes. Surprisingly, ESI/MS demonstrated that lipid rafts are enriched in plasmenylethanolamines, particularly those containing arachidonic acid. While the total content of anionic phospholipids was similar in plasma membranes and nondetergent lipid rafts, the latter were highly enriched in phosphatidylserine but relatively depleted in phosphatidylinositol. Detergent-resistant membranes made from the same cells showed a higher cholesterol content than nondetergent lipid rafts but were depleted in anionic phospholipids. In addition, these detergent-resistant membranes were not enriched in arachidonic acid-containing ethanolamine plasmalogens. These data provide insight into the structure of lipid rafts and identify potential new roles for these domains in signal transduction.

An emerging theme in the control of cellular signaling and metabolic regulation is the importance of spatial localization of the molecules participating in cell activation. Scaffolding, anchoring, and adapter proteins participate in the localization and regulation of protein function in such divergent processes as ion channel activation and insulin-mediated signaling (1). Recent work has underscored the importance of specialized plasma membrane lipid domains, such as caveolae and lipid rafts, in the regulation of cell signaling. These domains appear to facilitate interactions among the lipid and protein components of signaling pathways, thereby regulating these processes (2, 3).

Caveolae are small, plasma membrane invaginations that were first described by Palade in 1953 (4). Caveolae contain high levels of glycosphingolipids and cholesterol, a characteristic that renders these domains resistant to extraction with Triton X-100 (5, 6). Caveolae possess a striated coat that appears to be comprised largely of caveolin-1 (7), and this protein serves as a specific marker for this subcellular compartment.

Caveolin-1 is a 21 kDa integral membrane protein first identified as a substrate for the tyrosine kinase, pp60<sup>src</sup> (8,

9). It is a member of a family of three homologous proteins termed caveolin-1, caveolin-2, and caveolin-3 (10, 11). Cells that do not express caveolin-1 lack morphologically identifiable caveolae (12, 13). However, expression of caveolin-1 in such cells results in the generation of membrane invaginations indistinguishable from caveolae (14). Thus, caveolin-1 appears to be an essential structural component of caveolae.

Cells that do not exhibit morphologically identifiable plasmalemmal caveolae nonetheless possess low-density, cholesterol-enriched, Triton-resistant membrane domains (12), referred to as detergent-insoluble glycosphingolipid-enriched domains (15), detergent-resistant membranes (DRM), or lipid rafts (16). Lipid rafts cofractionate with caveolae in many different subcellular membrane fractionation procedures because they exhibit physical properties very similar to those of caveolae. Proteins such as the flotillins are useful markers for identifying these low-density domains (17).

Prior studies have demonstrated that a variety of signaling components are highly enriched in caveolae. This includes low molecular weight and heterotrimeric G proteins, src family kinases, EGF receptors, PDGF receptors, endothelin receptors, the phosphotyrosine phosphatase syp, Grb2, Shc, MAP kinase, protein kinase C, and the p85 subunit of PtdIns<sup>1</sup> 3-kinase (2, 18–23). Lipid rafts derived from cells that lack caveolae contain a complement of signaling proteins similar to that reported to be in caveolae (13, 24). Accordingly, caveolae and lipid rafts are thought to be specialized regions of the plasma membrane that facilitate signal transduction.

<sup>†</sup> This work was supported by Grants GM56239–01A1(L.J.P.), PO1HL57278/JDFI 996003 (R.W.G. and X.H.), and RO2 HL 41250–06A1(R.W.G.).

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Consistent with a role of caveolae and lipid rafts in regulating signal transduction, cell signaling is sensitive to the cellular concentration of cholesterol, a major component of lipid rafts. Depletion of cellular cholesterol with either filipin or lovastatin inhibits PDGF-stimulated PtdIns-3 kinase activity (25) and tyrosine kinase activity (26). Furthermore, Roy et al. (27) reported that a dominant negative mutant of caveolin-3 reduces plasma membrane cholesterol content and inhibits Ha-ras signaling. In addition, depletion of cellular cholesterol by treatment with the cholesterol-binding agent, methyl  $\beta$ -cyclodextrin, inhibits EGF- and bradykinin-stimulated PtdIns turnover (28). As lipid rafts are disrupted under conditions of cholesterol depletion (28), these data suggest that a variety of signaling pathways require intact caveolae or lipid rafts for proper function.

A major difference between caveolae and lipid rafts is the presence of the structural protein, caveolin-1, in the former domain. Caveolin-1 is a cholesterol-binding protein (29, 30) that appears to be responsible for inducing the characteristic invaginated structure of caveolae (14). It is also involved in cholesterol transport within cells (31–34). Expression of caveolin-1 in tumor cells significantly reduces the rate of cell growth and inhibits the ability of the cells to grow in soft agar (35, 36). Conversely, inhibition of caveolin-1 expression using antisense RNA leads to cell transformation (37). Thus, in addition to being involved in cholesterol homeostasis, caveolin-1 appears to be a negative regulator of cell growth.

A complete characterization of the protein and lipid components of caveolae and lipid rafts is essential to fully understand how these domains carry out their specialized regulatory functions. ESI/MS of phospholipids is the most sensitive, discriminating and direct method to assess the phospholipid content of biologic samples (38–41). A particular advantage of ESI/MS for lipid analyses is the ability to completely analyze phospholipid classes, subclasses, and individual molecular species from chloroform extracts without the need for multiple steps, such as chromatographic separation or derivatization.

In this paper, we have used ESI/MS to quantitatively compare the phospholipids present in plasma membranes and lipid rafts from caveolin-1-expressing and nonexpressing cells. Caveolae/lipid rafts were isolated using the nondetergent method of Smart et al. (42) which yields a highly purified fraction that contains a full complement of signaling proteins (24). The results were compared with those from low-density, detergent-resistant membranes (DRM) isolated from the same cells. We report here that nondetergent lipid rafts are enriched in cholesterol, sphingomyelin, phosphatidylserine, and arachidonic acid-containing plasmenylethanolamine as compared to the plasma membrane fraction. Expression of caveolin-1 in cells does not significantly change the phospholipid composition of caveolae/lipid raft

fraction but does substantially increase the cholesterol content of the membrane fraction. By comparison, the detergent-resistant membranes contain higher levels of cholesterol and saturated fatty acyl chains than nondetergent rafts. They are depleted in anionic phospholipids and are not enriched in plasmenylethanolamines. These data provide new insight into the structure of caveolae and lipid rafts and identify potential new roles for these domains in signal transduction.

## EXPERIMENTAL PROCEDURES

**Materials.** Phospholipids used as internal standards including 1,2-dipentadecanoyl-*sn*-glycero-3-phosphoethanolamine (15:0–15:0 GroPEtn), 1,2-ditetradec-9'-enoyl-*sn*-glycero-3-phosphocholine (14:1–14:1 GroPCho), and 1,2-ditetradecanoyl-*sn*-glycero-3-phosphoglycerol (14:0–14:0 GroPGro) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The purity of these internal standards was examined by ESI/MS. All solvents were at least HPLC grade and were obtained from Fisher Scientific (Pittsburgh, PA). Opti-Prep was from GibcoBRL (Invitrogen, Carlsbad, CA). The CII cholesterol assay kit was from Wako (Osaka, Japan). Polyclonal anti-caveolin-1 antibody was from Transduction Laboratories (Lexington, KY). Polyclonal anti-Gq antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal anti-EGF receptor antibody was generated by immunization of rabbits with a portion of the intracellular domain of the EGF receptor (43). The enhanced chemiluminescence kit was from Amersham (Cleveland, OH). All other reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO).

**Electron Microscopy.** Cells ( $5 \times 10^4$ ) were grown for 2 days on chromic acid-cleaned 2-mm cut glass coverslips, which were placed inside 60-mm dishes. Cells were rinsed three times with warm mammalian Ringer's and then lysed on the glass coverslips by momentary sonication in mammalian Ringer's. The resulting membranes were immediately fixed for 30 min at 4 °C with 2% glutaraldehyde in 20 mM Hepes, 100 mM KCl, 5 mM MgCl<sub>2</sub>, and 3 mM EGTA (pH 6.8). Each coverslip was rapidly frozen (44). Samples were coated with  $\sim 9$  Å of platinum using an electron gun at an angle of 24°. Replicas were reinforced with  $\sim 120$  Å carbon using an electron gun at an angle of 90° or 50°. Replicas were floated free of the coverslips in 30% HF and digested with Clorox and water for 1 min before being mounted on Formvar-coated grids for standard transmission electron microscopic viewing and 3D photography.

**Preparation of Subcellular Membrane Fractions. (1) Nondetergent Method.** Caveolae/lipid rafts were prepared according to the method of Smart et al. (42). Briefly, cells were scraped into ice cold 0.25 M sucrose, 1 mM EDTA, and 20 mM Tris (pH 7.8) (Buffer A) and pelleted. Two milliliters of Buffer A was added to the cell pellet which was lysed using 20 strokes in a Dounce homogenizer followed by 10 passes through a 21 g needle. The nuclei and cell debris were sedimented by centrifugation for 10 min at 1000g. The supernatant (2 mL) was applied to the top of a tube containing 8 mL of 30% Percoll in Buffer A. Tubes were centrifuged for 30 min at 84 000g. The band of membranes that sediments approximately halfway down the gradient is used as the plasma membrane fraction.

Two milliliters of plasma membranes were sonicated on ice 6 times for 15 s each at maximum power for a microtip

<sup>1</sup> Abbreviations: ESI, electrospray ionization; GC, gas chromatography; GroPCho, glycerophosphocholine; GroPEtn, glycerophosphoethanolamine; GroPGro, glycerophosphoglycerol; HPLC, high-performance liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; *m:n*, fatty acyl substituent with *m* carbons and *n* double bonds; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PlsEtn, plasmenylethanolamine, PtdGro, phosphatidylglycerol; PtdIns, phosphatidylinositol; PtdH, phosphatidic acid; PtdSer, phosphatidylserine; SM, sphingomyelin.

with a 1 min rest between each sonication. The sonicated membrane fraction was made up to 23% Optiprep by the addition of a 50% Optiprep stock in 20 mM Tris, 0.25 M sucrose, and 1 mM EDTA (pH 7.8) (Buffer C) and placed in the bottom of a centrifuge tube. An 8 mL gradient from 20% to 10% Optiprep was layered on top of the sample which was then centrifuged for 90 min at 52 000g in an SW41 swinging bucket rotor. The top 5 mL of the gradient were collected, mixed with 4 mL of Buffer C, and overlaid with 2 mL of 5% Optiprep in Buffer A. After centrifugation for 90 min at 52 000g, a band of membranes is visible just below the 5% Optiprep layer. This material was collected and used as the lipid raft fraction (or caveolae/lipid raft fraction in the case of KBC cells). Protein content of the plasma membrane and lipid raft fractions was determined using the method of Lowry as modified by Peterson (45).

**(II) Detergent Extraction Method.** KB cells were harvested into phosphate-buffered saline. Cell pellets were mixed with buffer containing 25 mM Hepes (pH 6.8), 150 mM NaCl, and 1% Triton X-100 and homogenized by passage through a 21 gauge needle 10 times. The lysate was mixed with an equal volume of 80% sucrose in 25 mM Hepes (pH 6.8). Six milliliters of a buffer containing 25 mM Hepes (pH 6.8) and 150 mM NaCl plus 30% sucrose and 4 mL of the same buffer containing 5% sucrose were layered on top of the lysate. The tubes were centrifuged for 3 h at 175 000g in an SW41 rotor. The material that collected at the 5%/30% sucrose interface was collected and used for the analysis of DRM.

**Western Blotting.** Aliquots of membrane fractions containing equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were electrophoretically transferred to nitrocellulose. Nitrocellulose was blocked by treatment with 10% powdered milk for 1 h, washed, and incubated with primary antibody for 2 h. Blots were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody, washed, and developed using enhanced chemiluminescence reagents.

**Quantitation of Total Cholesterol of Subcellular Membrane Fractions.** Lipids from triplicate samples containing 75  $\mu$ g of plasma membrane protein or 50  $\mu$ g of lipid raft protein were extracted according to the method of Bligh–Dyer (46). The chloroform layer was dried under vacuum, and the total cholesterol content of the residual lipid pellet was assayed using the CII cholesterol assay kit.

**Preparation of Phospholipid Extracts from Subcellular Membrane Fractions.** Protein assays on each of the isolated membrane fractions were performed prior to the phospholipid extraction. Phospholipids were extracted from the membrane fraction samples containing 200  $\mu$ g of protein by a modified Bligh–Dyer technique (46), utilizing 50 mM LiCl in the aqueous layer in the presence of 15:0–15:0 GroPEtn (20 nmol/mg of protein), 14:1–14:1 GroPCho (70 nmol/mg of protein), and 14:0–14:0 GroPGro (40 nmol/mg of protein). These phospholipid molecular species were used as internal standards for quantitation of ethanolamine-containing, choline-containing, and anionic phospholipids, respectively, as previously described (40, 41). In endogenous cellular phospholipids, these molecular species represent  $\ll$ 1% of lipid mass. The lipid extracts were dried under a nitrogen stream,

dissolved in chloroform, desalted with Sep-Pak columns, and filtered with 0.2  $\mu$ m Gelman acrodisc CR PTFE syringe filters. Lipids were re-extracted utilizing 20 mM LiCl in the aqueous layer and then dried under a nitrogen stream. The final lipid residue was resuspended in 0.2 mL of 1:1 chloroform/methanol for ESI/MS analyses.

**Quantitative Analyses of Subcellular Membrane Phospholipid Molecular Species Using ESI/MS.** ESI/MS analyses were performed utilizing a Finnigan TSQ-7000 spectrometer equipped with an electrospray ion source, as described previously (41, 47). Typically, a 5-min period of signal averaging in the profile mode was employed for each spectrum of a lipid extract. All extracts were diluted to 100-fold in 1:1 chloroform/methanol prior to direct infusion into the ESI chamber using a syringe pump at a flow rate of 1  $\mu$ L/min in this study.

Anionic phospholipids in the diluted chloroform extracts of membrane samples were analyzed by ESI/MS in the negative-ion mode and quantitated by comparisons of the individual ion peak intensity with an internal standard (i.e., 14:0–14:0 GroPGro) after correction for  $^{13}\text{C}$  isotope effects relative to the internal standard. Prior to the analyses of choline and ethanolamine glycerophospholipids in the diluted tissue extracts, LiOH in methanol (50 nmol/mg of protein) was added. Choline glycerophospholipids and sphingomyelins in the diluted tissue extracts were directly quantitated as their lithium adducts by comparison with an internal standard (i.e., lithiated 14:1–14:1 GroPCho after correction for  $^{13}\text{C}$  isotope effects relative to the internal standard in the positive-ion mode). It should be noted that the correction for  $^{13}\text{C}$  isotope effects only represents that part of the total  $^{13}\text{C}$  isotope effects which results from the carbon number difference between the internal standard and the molecular species of interest (see ref 48 for details). Although the  $^6\text{Li}$  isotope accounts for 7.4% of the natural abundance of  $^7\text{Li}$ , this isotope effect is constantly proportional to the  $^7\text{Li}$  ion peak for all molecular species including internal standard. Therefore, this effect is eliminated during direct ion peak comparison. The secondary effect of  $^6\text{Li}$  isotope to the  $^{13}\text{C}$  isotope effect resulting from the carbon number difference is very small and can be neglected.

After rendering the solution mildly basic by the addition of LiOH (50 nmol/mg of protein), ethanolamine glycerophospholipids were directly quantitated by comparison with an internal standard (i.e., 15:0–15:0 GroPEtn) after correction for  $^{13}\text{C}$  isotope effects relative to the internal standard in negative-ion mode. Previously, it has been demonstrated that different molecular species of polar lipids have nearly identical ionization efficiencies after corrections for  $^{13}\text{C}$  isotope effects ( $\pm 10\%$ ) for molecular species containing acyl chains with 14–20 carbons and different numbers of double bonds (39, 71). Accordingly, we utilized ESI/MS instead of ESI/MS/MS because the latter technique results in differential fragmentation rates for individual molecular species containing different acyl constituents which are highly sensitive to the collisional activation energy employed (48).

The selectivity of ESI/MS for direct analysis of distinct phospholipid classes was achieved by exploiting differential ionization propensities in the positive- and negative-ion modes of each phospholipid class (40). Identification of ion peaks was achieved utilizing tandem mass spectroscopic analyses in either molecular ion, neutral-loss, or product ion



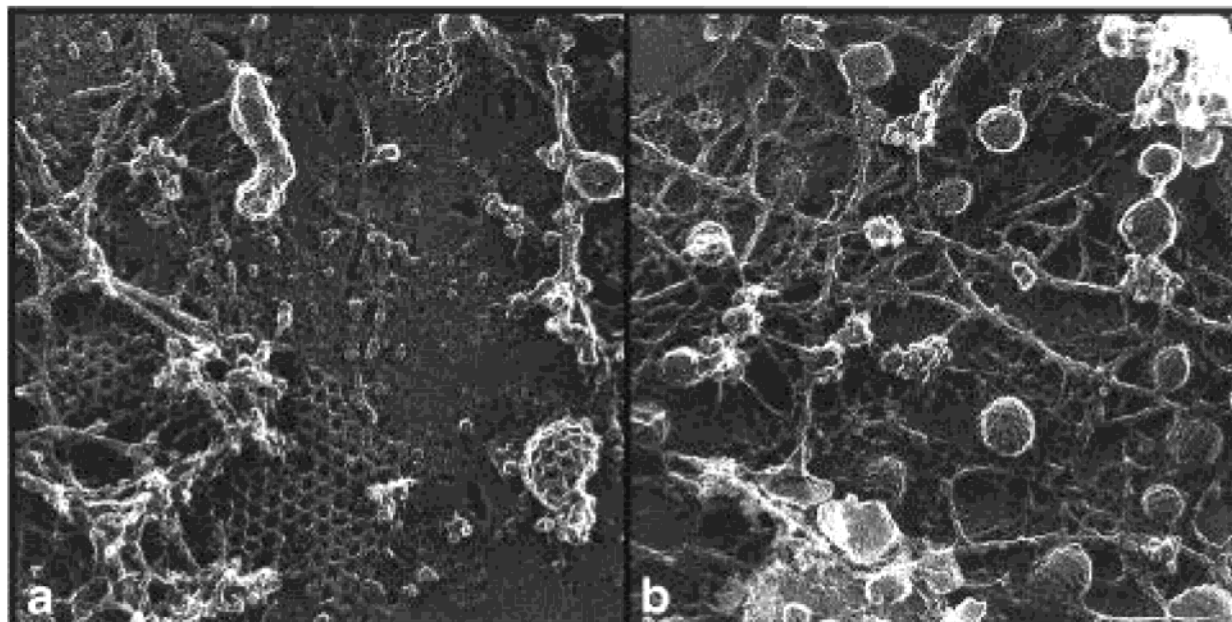


FIGURE 1: Deep-etch electron micrographs of KB and KBC cells. KB and KBC cells were grown on coverslips and lysed by sonication. The resulting “cell bottoms” were fixed and coated with platinum, as described in Experimental Procedures. Replicas were mounted on Formvar-coated grids for standard transmission electron microscopy. (Panel A: KB cells. Panel B: KBC cells.)

scanning mode. Quantification of ion peaks corresponding to multiple individual molecular ions was substantiated utilizing product-ion ESI/MS/MS analyses, as described previously (49, 50). Plasmalogen molecular species were distinguished from alkyl-acyl phospholipid molecular species by treating lipid extracts with acidic vapors prior to mass spectrometric analyses, as described previously (51).

Statistical comparisons of the three data sets were performed using the statistical analysis computer program, Data Desk.

**Quantitative Analyses of Lipid Raft Ethanolamine Glycerophospholipids Using Capillary GC.** Bligh and Dyer extracts from lipid rafts containing exogenously added internal standard (i.e., 20:0–20:0 GroPEtn) were loaded on to an Ultrasphere-Si HPLC column (250 × 4.6 mm, 5  $\mu$ m; Beckman). Ethanolamine glycerophospholipids were resolved using a mobile phase comprised of hexane/2-propanol (1:1, v/v) with a linear gradient of 0–6% H<sub>2</sub>O for 45 min at a flow rate of 1 mL/min, as described previously (52). Under these conditions, the ethanolamine glycerophospholipid fraction eluted at approximately 20 min. The collected fraction was dried under a nitrogen stream, and the masses of ethanolamine glycerophospholipids were quantified by capillary GC after acid methanolysis by comparisons with a 20:0–20:0 GroPEtn standard, as described previously (53).

## RESULTS

**Characterization of Cells and Membrane Fractions.** KB cells are a line of human epidermal carcinoma cells that do not express caveolin-1. As a result, they lack morphologically identifiable caveolae (Figure 1A). KB cells were transfected with a construct encoding mouse caveolin-1, and stable transfectants were isolated. The resulting cell line is referred to, hereafter, as KBC cells. In contrast to KB cells, KBC cells possess numerous plasmalemmal vesicles with a striated coat that are readily identifiable as caveolae (Figure 1B). These data indicate that expression of caveolin-1 in KB cells induces the formation of caveolae in the resulting KBC cells.

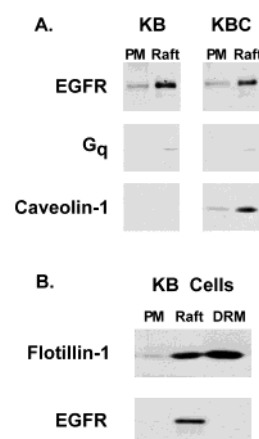


FIGURE 2: Distribution of caveolin-1, EGF receptor and Gq in lipid raft, plasma membrane, and DRM fractions. (Panel A) Ten micrograms of protein from the plasma membrane fraction (PM) and the caveolae/lipid raft fraction (Cav) were analyzed by SDS–PAGE. Proteins were transferred to nitrocellulose and subjected to western blotting with anti-caveolin-1 antibody, anti-EGF receptor antibody, and anti-Gq antibody. (Panel B) Ten micrograms of protein from the plasma membrane (PM), caveolae/lipid raft (Cav), and detergent-resistant membrane fractions from KB cells were analyzed by SDS–PAGE. Proteins were transferred to nitrocellulose and subjected to western blotting with an anti-flotillin-1 antibody and an anti-EGF receptor antibody.

Caveolae/lipid raft membranes were prepared from KB and KBC cells using the method of Smart et al. (42). This low-density fraction prepared *in the absence of detergent* is referred to, hereafter, as caveolae/lipid rafts. These lipid rafts are distinct from low-density membrane fractions prepared by extracting whole cells with 1% Triton X-100. These detergent-resistant membranes will be referred to as DRM.

Equal amounts of protein from the plasma membrane fraction, and the low-density, nondetergent, caveolae/lipid raft fraction derived from that plasma membrane fraction, were analyzed by SDS–PAGE and western blotting. As shown in Figure 2A, no caveolin-1 was detected in any of

Table 1: Lipid Content of Whole KB Cell Extracts<sup>a</sup>

choline-containing phospholipids			ethanolamine-containing phospholipids			anionic phospholipids		
phospholipid	nmol/mg of protein	mol %	phospholipid	nmol/mg of protein	mol %	phospholipid	nmol/mg of protein	mol %
choline glycerophospholipids			diacyl species			PtdH		
D16:1–16:1	1.76	2.1	16:0–18:1	2.9	3.5	18:1–18:1	0.11	0.1
D16:1–16:0	3.93	4.8	16:0–18:2	2.33	2.8	18:0–18:1	0.23	0.3
D16:0–16:0	0.91	1.1	16:0–22:5/18:1–20:4	1.45	1.8	total	0.34	0.4
P16:0–18:1	5.32	6.4	18:0–18:1	1.52	1.8	PtdGro		
D16:0–18:2	3.83	4.6	18:0–20:4	1.69	2.0	16:0–18:2	0.54	0.7
D16:0–18:1	8.64	10.4	18:0–22:5	0.57	0.7	16:0–18:1	0.82	1.0
D18:1–18:2	2.05	2.5	18:0–22:6	0.52	0.6	18:1–18:2	0.27	0.3
D18:0–18:2 D18:1–18:1	8.34	10.1	18:1–18:1	5.97	7.2	18:1–18:1	1.57	1.9
subtotal	34.78	42.0	20:0–20:4	0.63	0.8	18:0–18:1	0.33	0.4
			subtotal	17.58	21.2	subtotal	3.53	4.3
sphingomyelin			plasmalogen species			PtdIns		
N18:1	2.17	2.6	20:0–20:4	0.66	0.8	16:0–18:2	0.16	0.2
N26:2	1.12	1.4	plasmalogen species			16:0–18:1	0.34	0.4
subtotal	3.29	4.0	14:0–18:1	1.67	2.0	18:0–18:2	0.53	0.6
			16:0–18:1	3.73	4.5	18:0–18:1	0.48	0.6
			16:0–18:2	1.6	1.9	18:1–20:4	0.06	0.1
			18:0–18:1	2.01	2.4	18:0–20:4	0.1	0.1
			18:1–18:1/18:0–18:2	2.22	2.7	18:0–20:3	0.12	0.1
			18:0–22:6	0.92	1.1	18:0–20:2	0.26	0.3
			18:1–22:6	0.93	1.1	subtotal	2.05	2.5
			18:2–22:6	1.09	1.3			
			16:0–20:4	3.11	3.8			
			18:1–20:4	2.37	2.9			
			18:0–20:4	0.85	1.0			
			subtotal	20.5	24.8			
total	38.07	46.0	total	38.74	46.8	total	5.92	7.2

<sup>a</sup> Lipids were extracted from intact KB cells and analyzed for phospholipid content using ESI/MS, as described in Experimental Procedures. The first number in each pair refers to the number of carbon atoms in the fatty acid chain. The number after the colon refers to the number of double bonds. The two fatty acid chain designations are separated by a hyphen. The prefix D indicates a diacyl compound. The prefix P indicates a plasmalogen compound. For sphingomyelin, only a single fatty acid chain is designated because the other is the 18:1 aliphatic chain included in the sphingosine backbone. The prefix N indicates that the fatty acid is in an amide linkage with the sphingosine.

the fractions from KB cells. By contrast, caveolin-1 was detectable in all fractions from KBC cells and was highly enriched in the caveolae/lipid raft fraction. Thus, exogenous caveolin-1 fractionates biochemically in a manner identical to that reported for endogenous caveolin-1.

EGF receptors and Gq have also been reported to be localized in caveolae/lipid rafts (23, 28). To determine whether the low-density membrane fractions prepared from KB cells were enriched in markers for caveolae/lipid rafts other than caveolin-1, plasma membranes and lipid raft fractions were screened for the presence of these proteins. The data in Figure 2A demonstrate that the EGF receptor and Gq were similarly enriched in the low-density membrane fractions prepared from KB and KBC cells. These data indicate that the low-density fractions from KB and KBC cells are similar with respect to their content of these signaling proteins but differ in that the former lack caveolin-1 while the latter contain this protein.

Figure 2B compares protein markers in lipid rafts from KB cells made by the nondetergent method with those made by the Triton X-100 extraction method (DRM). The plasma membrane, lipid raft, and DRM fractions were screened for the presence of flotillin-1, a lipid raft marker, as well as the EGF receptor. As compared to plasma membranes, flotillin-1 was enriched in the nondetergent lipid raft fraction as well as the DRM. By contrast, the EGF receptor is present only in the rafts prepared using the nondetergent method. These data are consistent with previous studies that indicate that while DRM and lipid rafts contain traditional raft marker

proteins, the DRM fraction contains only a subset of the signaling proteins that are present in nondetergent lipid raft preparations (24).

**Phospholipid and Cholesterol Content of KB and KBC Cells.** Lipids were extracted from intact KB cells as well as from samples of the plasma membrane fraction and the low-density caveolae/lipid raft fraction derived from KB and KBC cells. The phospholipids in the lipid extracts of the plasma membrane and caveolae/lipid raft fractions derived from KB and KBC cells were quantitatively analyzed using ESI/MS, as described in Experimental Procedures. Table 1 summarizes the phospholipid content of KB cells. Table 2 summarizes the phospholipid and cholesterol content of the plasma membrane and low-density membrane fractions from KB and KBC cells.

The phospholipid content of KB cells is typical of most cells. The ratio of choline-containing phospholipids to ethanolamine-containing phospholipids is approximately 1, and plasmalogens account for about half of the ethanolamine-containing phospholipid species. Anionic phospholipids represent less than 10 mol % of the phospholipids. Interestingly, phosphatidylglycerol was the major anionic phospholipid, whereas phosphatidylserine was in such low abundance that it was not detectable by this method.

With respect to the plasma membrane and lipid raft fractions (Table 2), the most obvious difference between the two was in their total lipid content. Plasma membrane fractions from KB and KBC cells contained ~850 nmol of lipid/mg of protein while the nondetergent caveolae/lipid raft

Table 2: Lipid Content of Rafts and Plasma Membranes<sup>a</sup>

	KB Plasma Membranes			KB Lipid Rafts			KBC Plasma Membranes			KBC Lipid Rafts		
Class/ Subclass	nmol/ mg protein	mol% Total Lipids	mol% Phospho lipids	nmol/ mg protein	mol% Total Lipids	mol% Phospho lipids	nmol/ mg protein	mol% Total Lipids	mol% Phospho lipids	nmol/ mg protein	mol% Total Lipids	mol% Phospho lipids
Ethanolamine glycerophospholipids	302.9 ±33.9		40	680.7 ±33.0		445	283.4 ±22.7		39.2	516.5 ±79		42.4
Choline glycerophospholipids	223.1 ±18.1		29.4	294.8 ±7.9		192	214.8 ±21.3		29.7	238.1 ±15.8		19.6
Phosphatidylinositol	105.6 ±12.3		14	158.4 ±8.6		104	98.2 ±14.0		13.6	126.6 ±10.6		10.4
Phosphatidylserine	8.7 ±2.1		1.1	61.3 ±12.2		39	8.7 ±0.5		1.2	43.7 ±0.9		3.6
Phosphatidic Acid	5.5 ±1.8		0.7	14.5 ±1.7		09	4.4 ±1.8		0.6	16.1 ±1.9		1.4
Sphingomyelin	111.3 ±5.6		14.7	319.8 ±50.2		209	113.5 ±9.5		15.7	277.6 ±51.6		22.9
Cholesterol	110 ±35	12.7	N/A	484 ±93	24.0	N/A	126.4 ±24.0	14.9	N/A	614 ±212	33.5	N/A
Total Phospholipids	757		100	1529		100	723		100	1218		100
Total Lipids	867	100		2013	100		849	100		1832	100	

<sup>a</sup> Plasma membranes and lipid rafts were isolated from KB and KBC cells as outlined in Experimental Procedures. Lipids were extracted from samples containing 200  $\mu$ g of membrane protein and were analyzed for phospholipid content by ESI/MS. Results represent the mean  $\pm$  SD of three separate experiments.

fractions contained more than twice this amount of lipid per mg of protein. The higher lipid/protein ratio of rafts is consistent with the lower density of lipid rafts as compared to plasma membranes.

Cholesterol content was a second major difference between lipid rafts and the plasma membrane fraction. In KB cells, the lipid raft fraction contained nearly twice as much cholesterol as the plasma membrane fraction on a mole percent basis. In KBC cells, the cholesterol content of the caveolae/lipid raft fraction was 225% that of the corresponding plasma membrane fraction. Because there was a slight increase in the cholesterol content of the KBC plasma membrane fraction relative to that of KB cells, the amount of cholesterol in the caveolae/lipid raft fraction of KBC cells was 40% higher on a mole percent basis than that seen in KB cells. This difference was significant at the  $p < 0.01$  level. With the exception of this difference in the cholesterol content of lipid rafts derived from KB and KBC cells, none of the other differences in lipid content of like membrane fractions isolated from KB and KBC cells were statistically significant.

Because the substantial differences in cholesterol content between the plasma membrane and lipid raft fractions differentially affected the relative content of phospholipids in rafts versus plasma membranes, the amount of each phospholipid is reported as the mole percent of phospholipids (as opposed to the mole percent of total lipids for cholesterol) in Table 2. As can be seen from this table, ethanolamine glycerophospholipids were the primary phospholipid present

in each of the four membrane fractions, representing about 40% of the total phospholipid. Phosphatidylcholine was the next most abundant lipid, accounting for  $\sim 30\%$  of the total phospholipid in the KB and KBC plasma membrane fractions. By contrast, the phosphatidylcholine content was significantly diminished in the caveolae/lipid raft fraction of both of these cells to  $\sim 20\%$  of the total phospholipids. Also reduced in the caveolae/lipid raft fraction was the content of phosphatidylinositol, which declined from about 14% of the total phospholipid in KB and KBC plasma membranes to  $\sim 10\%$  in the corresponding caveolae/lipid raft fraction.

These decreases in phospholipid content of lipid rafts as compared to plasma membrane were counterbalanced by increases in the levels of other phospholipids. For example, there was approximately a 3-fold increase in the amount of phosphatidylserine in caveolae/lipid rafts as compared to plasma membrane fractions. In addition, the amount of sphingomyelin was increased by  $\sim 50\%$  in the low-density fraction over that in the corresponding plasma membrane fraction. The increase in sphingomyelin in lipid rafts offset the decrease in phosphatidylcholine content of these domains so that the content of choline-containing phospholipids remained fairly constant at  $\sim 40\text{--}45\%$  mol % and in approximately a 1:1 ratio with ethanolamine glycerolipids.

*Analyses of Individual Molecular Phospholipid Species.* Figure 3 shows representative negative-ion ESI/MS spectra of lipids from KB cell plasma membrane and lipid raft fractions. These spectra show distinct differences in the

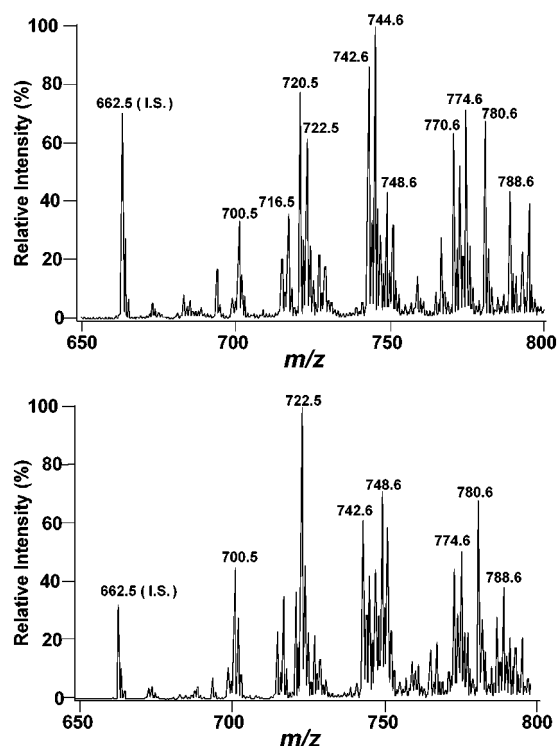


FIGURE 3: Negative-ion ESI/MS spectra of ethanolamine glycerophospholipids in lipid extracts of the plasma membrane fraction and the low-density caveolae/lipid raft fraction derived from KB cells. Subcellular membrane fractions were isolated using the nondetergent method of Smart et al. (42). Phospholipids were extracted by a modified method of Bligh and Dyer (46). Aliquots of the chloroform extracts were directly infused into the ESI ion source using a Harvard syringe pump at a flow rate of 1  $\mu$ L/min after the addition of LiOH (50 nmol/mg of protein) to the extracts. Negative-ion ESI/MS of lipid extracts of the plasma membrane fraction (panel A) and the low-density caveolae/lipid raft fraction (panel B) were performed as described in Experimental Procedures. Individual molecular species were identified using tandem mass spectrometry and are tabulated in Table 2. The internal standard (IS) is 15:0–15:0 GroPEtn ( $m/z$ : 662.5).

abundance of the various ethanolamine glycerophospholipid species in the two membrane preparations. These differences are summarized in Table 3, which presents the average results from three separate experiments. In plasma membranes from KB and KBC cells, major molecular species were present at  $m/z$  of 720.5, 722.5, 770.6, and 774.6, corresponding to ethanolamine plasmalogens containing the combinations of alkenyl-acyl of (16:1–20:4), (16:0–20:4), (18:2–22:6), and (18:0–22:6), respectively. Collectively, ethanolamine plasmalogens account for approximately 21% of the total phospholipid in the plasma membrane fraction of KB and KBC cells. Phosphatidylethanolamine species account for ~18% of the total phospholipids in these membranes.

In lipid rafts, the major ethanolamine-containing phospholipid species is the plasmalogen containing 16:0–20:4 substituents ( $m/z$ : 722.5). This species is present at a level twice that seen in the plasma membrane fraction. Other plasmenylethanolamine molecular species are also enriched in the lipid raft fraction, resulting in an ~30% increase in the total amount of plasmalogens in lipid rafts from both KB and KBC cells. This enrichment of plasmalogens in lipid rafts is depicted graphically in Figure 4 (top panel) which plots the mole percent of each molecular species of ethanolamine-containing phospholipid in lipid rafts against its mole

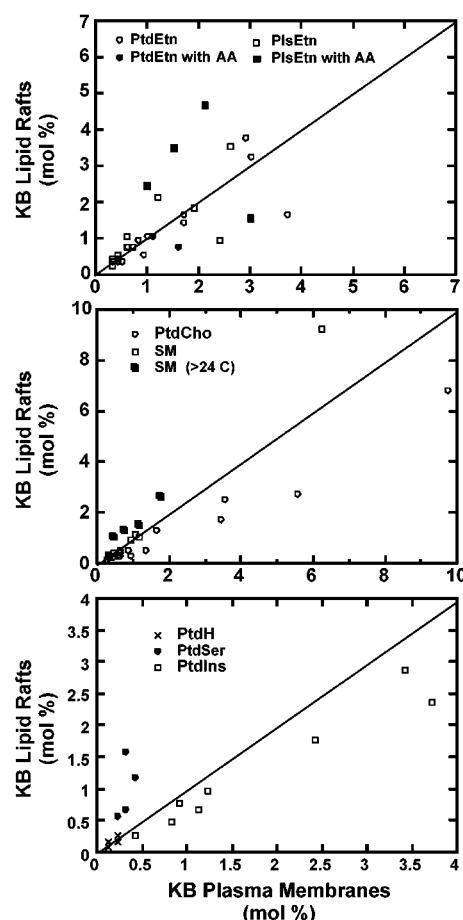


FIGURE 4: Comparison of molecular species of phospholipids in KB lipid rafts versus plasma membranes. Data from Tables 3–5 were used to plot the mole percent of each phospholipid species in lipid rafts versus the mole percent of that species in plasma membranes derived from KB cells. The line shows the region of identity.

percent in plasma membranes. The line represents the region of identity. As can be seen from the figure, the concentration of most of the individual molecular species of ethanolamine plasmalogen (indicated by squares) is higher in lipid rafts than in plasma membranes.

Surprisingly, lipid rafts were found to be enriched in the highly unsaturated arachidonic acid. As shown in the top panel of Figure 4, lipid rafts contained significantly higher levels of ethanolamine plasmalogens containing arachidonic acid than the corresponding plasma membrane fraction (filled squares in Figure 4). By contrast, diacyl PtdEtn species containing arachidonic acid were not enriched in lipid rafts (filled circles). The data in Table 3 indicate that lipid rafts isolated from KB and KBC cells had ~30% more arachidonic acid than the corresponding plasma membranes. This increase in arachidonic acid is due almost exclusively to an increase in the amount of arachidonic acid-containing ethanolamine plasmalogens.

The enrichment of plasmalogens and arachidonic acid in lipid rafts observed using ESI/MS was confirmed using conventional methods of HPLC and GC, as described in Experimental Procedures. The ethanolamine phospholipid pool was separated from other phospholipid classes by straight phase HPLC prior to derivatization in acidified methanol and quantitation by capillary GC. Figure 5 shows



Table 3: Ethanolamine-Containing Phospholipids<sup>a</sup>

assignment	KB plasma memb			KB lipid rafts			KBC plasma memb			KBC lipid rafts		
	(nmol/mg of protein)			(nmol/mg of protein)			(nmol/mg of protein)			(nmol/mg of protein)		
	mean	SD	mol %	mean	SD	mol %	mean	SD	mol %	mean	SD	mol %
Phosphatidylethanolamine												
16:0–16:1	13.1	1.7	1.7	22.8	0.9	1.5	16.8	1.5	2.3	19.5	0.9	1.6
18:0–18:1	27.8	5.1	3.7	25.4	8.5	1.7	20.9	9.8	2.9	22.4	8.5	1.8
16:0–18:2	5.9	0.4	0.8	15	1	1.0	7.5	0.6	1.0	10.7	0.8	0.9
18:1–18:1	23	1.1	3.0	50.5	6.7	3.3	36.4	3.9	5.0	41.8	7.1	3.4
18:1–18:2	3.1	0.4	0.4	5.8	0.3	0.4	2.8	0.3	0.4	4.2	0.5	0.3
16:0–22:5/18:1–20:4	4.4	0.1	0.6	11.9	0.3	0.8	4	0.1	0.6	7.8	0.3	0.6
16:0–22:6	2.5	0.7	0.3	5.7	1.6	0.4	1.6	0.3	0.2	3.7	0.4	0.3
16:1–22:6	4.1	0.5	0.5	6	0.5	0.4	2.8	0.9	0.4	5.8	0.3	0.5
18:0–22:4	7.5	0.1	1.0	17.3	0.4	1.1	5	0.1	0.7	15.5	2.6	1.3
18:0–22:5	7.1	0.3	0.9	8.5	1.7	0.6	5.4	0.9	0.7	6.6	0.8	0.5
18:0–22:6	5.6	2.6	0.7	11.5	5.5	0.8	5.2	2.1	0.7	6.3	2.9	0.5
18:1–22:6	12.8	1.5	1.7	25.5	3	1.7	5.6	0.9	0.8	18.2	3	1.5
16:0–20:4	3	0.6	0.4	7	2	0.5	2.4	0.6	0.3	6	2	0.5
18:0–20:4	8.6	0.3	1.1	16.3	0.3	1.1	7.6	0.1	1.1	10.6	0.9	0.9
20:0–20:4	12	2.8	1.6	11.8	3.6	0.8	8.9	2.4	1.2	10.2	2.7	0.8
total PtdEtn with 20:4			3.1			2.3			2.6			2.2
total PtdEtn			18.6			15.8			18.4			15.5
Plasmanylethanolamine												
20:0–20:4	22.1	0.3	2.9	58.5	1.8	3.8	14.6	0.5	2.0	43	9.4	3.5
Plasmenylethanolamine												
									0.0			
14:0–18:1	2.1	0.2	0.3	4.7	0.4	0.3	2.4	0.3	0.3	3.9	0.9	0.3
16:0–18:1	9.1	0.3	1.2	33.6	4.6	2.2	12.4	2.5	1.7	33.4	8.9	2.7
18:0–18:1	4.5	0.2	0.6	12.6	0.7	0.8	6.3	0.8	0.9	11.6	2.4	1.0
16:0–18:2	2.5	0.2	0.3	6.9	1.1	0.5	2.7	0.2	0.4	3.7	0.5	0.3
18:1–18:1/18:0–18:2	4.3	0.4	0.6	16.5	3.3	1.1	7.1	1.1	1.0	14.6	3.8	1.2
18:1–18:2	2.7	0.2	0.4	8.8	1.8	0.6	2.5	0.2	0.3	5.3	0.8	0.4
18:0–22:5	5.4	1	0.7	12.9	0.2	0.8	5.3	0.5	0.7	7.4	0.4	0.6
18:0–22:6	20	4.10	2.6	54.3	1.9	3.6	19.4	4.9	2.7	44.5	4.6	3.7
18:1–22:6	14.7	3.6	1.9	28.7	1.9	1.9	9.5	1.9	1.3	19.8	2.6	1.6
18:2–22:6	18.2	3.4	2.4	15.5	4	1.0	12.8	5	1.8	13.3	3.1	1.1
16:0–20:4	15.6	3	2.1	71.6	19.1	4.7	20.2	5.1	2.8	51.1	13.7	4.2
16:1–20:4	22.8	6.5	3.0	23.9	6.2	1.6	13.4	5	1.9	16	2.5	1.3
18:0–20:4	7.4	1	1.0	38.1	4	2.5	7.8	1.4	1.1	24.4	6.8	2.0
18:1–20:4	11.1	2	1.5	53.1	13	3.5	14.2	4.5	2.0	35.2	9.9	2.9
total PlsEtn with 20:4			10.4			16.0			9.7			13.9
total PlsEtn			21.5			28.8			20.8			26.9
grand total	302.9	23.8	40.0	680.7	25.2	44.5	283.4	16.5	39.2	516.5	53.1	42.4

<sup>a</sup> Lipids were extracted from plasma membrane and lipid raft fractions derived from KB and KBC cells. Extracts were analyzed for ethanolamine glycerophospholipid content using negative-ion ESI/MS, as described in Experimental Procedures. Results represent the mean  $\pm$  SD of three separate experiments. The first number in each pair refers to the number of carbon atoms in the fatty acid chain. The number after the colon refers to the number of double bonds. The two fatty acid chain designations are separated by a hyphen. If the  $m/z$  cannot provide a unique assignment, both possibilities are given separated by a back slash.

a representative GC profile of the ethanolamine glycerophospholipids from lipid rafts. Analysis of the data indicated the presence of  $\sim 514$  nmol of ethanolamine glycerophospholipids/mg of protein, slightly lower than what was found using ESI/MS ( $\sim 680$  nmol/mg of protein). Of this, 375 nmol/mg of protein or  $\sim 73\%$  of the molecules contained a dimethyl acetal aliphatic species indicative of the vinyl ether linkage present in plasmalogens. This is in good agreement with the value of 65% plasmalogens in lipid rafts determined by ESI/MS and is consistent with a significant enrichment of ethanolamine plasmalogens in these domains. Arachidonic acid accounted for approximately 33% of the *sn*-2 acyl chains as determined by GC, which is comparable to the results obtained using ESI/MS (41%). Thus, two different analytical techniques provide independent evidence for an enrichment of plasmalogens and arachidonic acid in nondetergent lipid rafts.

Choline glycerophospholipids and sphingomyelins in membrane fractions from KB and KBC cells were quanti-

tatively analyzed by both ESI/MS and ESI/MS/MS in positive-ion mode in the presence of LiOH. Positive-ion ESI/MS/MS spectra of lipids present in KB plasma membrane and lipid raft fractions are shown in Figure 6. The average results from three separate analyses are given in Table 4. Predominant PtdCho molecular species for all membrane preparations were observed at  $m/z$  of 738.6, 766.6, and 792.6, corresponding to lithiated 16:0–16:1 GroPCho, 16:0–18:1 GroPCho, and 18:0–18:2/18:1–18:1 GroPCho, respectively. However, the overall choline glycerophospholipid content of lipid rafts was found to be about one-third lower than that of plasma membrane fractions. As shown in the Table 4 and the middle panel of Figure 4, this decrease is spread relatively evenly across all of the species present.

An overall  $\sim 40\%$  increase in sphingomyelin species was documented in lipid rafts as compared to the plasma membrane fraction of KB and KBC cells (Figure 6 and Table 4). A large part of this increase was due to the enrichment of a single major sphingomyelin molecular species at  $m/z$



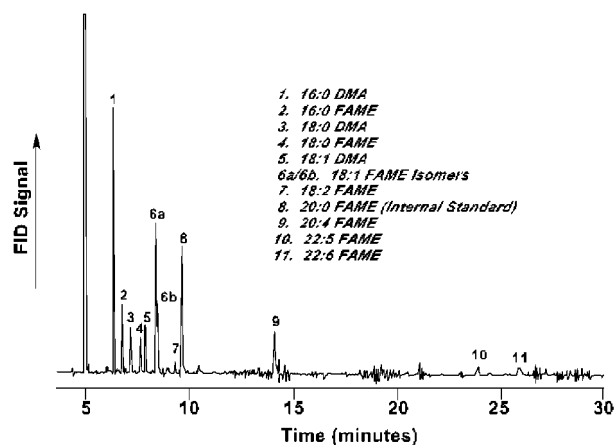


FIGURE 5: Gas chromatograph of lipid raft ethanolamine glycerophospholipids. Raft lipid extracts were prepared as described in Experimental Procedures. Ethanolamine glycerophospholipids were isolated by employing an Ultrasphere-Si HPLC column, and raft ethanolamine glycerophospholipids were subjected to acid-catalyzed methanolysis and analyzed by capillary gas chromatography, as described in Experimental Procedures. The identities of peaks on the chromatograph were determined by comparison of their retention times with those of authentic compounds under identical experimental conditions. DMA denotes dimethyl acetal from plasmalogen species, and FAME denotes fatty acid methyl ester from acyl-linked species.

of 709.5, corresponding to lithiated SM containing an 18:1 fatty acid (see middle panel, Figure 4). The remainder of the increase appears to be due to increases in species containing acyl chains with 24 or more carbon atoms (Figure 4, middle panel).

Anionic phospholipids were analyzed by ESI/MS in negative-ion mode without the addition of LiOH (Figure 7 and Table 5). Predominant peaks were observed for both plasma membrane and lipid raft fractions at  $m/z$  of 835.6, 861.6, 863.6, and 889.7. Tandem mass spectroscopic analyses demonstrated that these ions corresponded to 16:0–18:1 GroPIs, 18:0–18:2 GroPIs, 18:0–18:1 GroPIs, and 18:0–20:2 GroPIs, respectively (spectra not shown).

The anionic phospholipids, phosphatidic acid, phosphatidylserine, and phosphatidylinositol, account for approximately 15% of the total phospholipids in both plasma membrane and lipid raft fractions. However, as shown in Table 5 and the bottom panel of Figure 4, the distribution among these three classes differs with lipid rafts containing significantly more phosphatidylserine but less phosphatidylinositol than plasma membranes. These differences appear to be broadly distributed among all of the lipid species rather than restricted to a individual molecular species.

**Comparison of Phospholipids in Nondetergent Lipid Rafts and Detergent-Resistant Membranes (DRM).** Lipids were extracted from the DRM fraction from KB cells and analyzed for phospholipid content by ESI/MS. Cholesterol content was determined enzymatically. The results are shown in Table 6. As compared to nondetergent lipid rafts, DRM are cholesterol-enriched and phospholipid-poor. Cholesterol accounts for approximately 60% of the total lipid in DRM but only 24% in nondetergent lipid rafts. Conversely, phospholipids accounted for only 40% of the total lipid content in DRM but 76% in rafts.

Like nondetergent rafts, the DRM fraction exhibited a ratio of  $\sim 1$  between choline-containing and ethanolamine-contain-

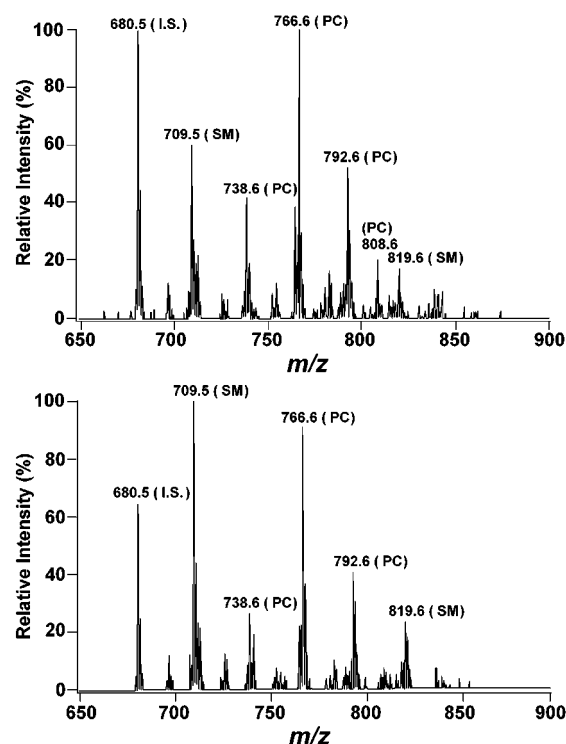


FIGURE 6: Positive-ion electrospray ionization tandem mass spectra of choline glycerophospholipids in lipid extracts of the plasma membrane fraction and the low-density caveolae/lipid raft fraction derived from KB cells. Subcellular membrane fractions and their lipid extracts were prepared as described in the legend of Figure 3. Aliquots of the chloroform extracts were directly infused into the ESI ion source using a Harvard syringe pump at a flow rate of 1  $\mu$ L/min after the addition of LiOH (50 nmol/mg of protein) to the extracts. Positive-ion ESI/MS/MS in the neutral loss of 183 (corresponding to a phosphocholine derivative) from the lipid extracts of the plasma membrane fraction (panel A) and the low-density caveolae/lipid raft fraction (panel B) was performed as described in Experimental Procedures. Individual choline glycerophospholipid molecular species were identified using tandem mass spectrometry in the product-ion mode and are tabulated in Table 4. The internal standard (IS) is 14:1–14:1 GroPCho ( $m/z$ : 680.5). PC represents phosphatidylcholine, and SM represents sphingomyelin. Some minor peaks were sodiated choline-containing phospholipid molecular species.

ing phospholipids. Thus, the excess of cholesterol did not appear to alter the balance between these two major classes of phospholipids. However, unlike the lipid rafts, DRM were not enriched in ethanolamine plasmalogens. Plasmalogens accounted for only 51% of the ethanolamine-containing phospholipids in DRM but represented 65% of the ethanolamine glycerophospholipids in nondetergent lipid rafts.

Interestingly, the DRM appeared to be relatively depleted in anionic phospholipids. Negatively charged lipids accounted for only  $\sim 5$  mol % of the total phospholipid in DRM as compared to  $\sim 15$  mol % in nondetergent rafts. The mole percent of phosphatidylserine and phosphatidic acid in DRM was 3- to 6-fold less than what was observed in the lipid raft fraction (see Table 5) and was similar to the levels seen in KB plasma membranes. Strikingly, phosphatidylinositol accounted for less than 2 mol % of the phospholipids in DRM but 10.4 mol % in lipid rafts. Of interest is the observation that PtdGro was present at  $\sim 3$  mol % in DRM but was undetectable in lipid rafts. Because PtdGro is depleted in plasma membranes but rich in the membranes

Table 4: Choline-Containing Phospholipids<sup>a</sup>

assignment	KB plasma memb			KB lipid rafts			KBC plasma memb			KBC lipid rafts		
	(nmol/mg of protein)			(nmol/mg of protein)			(nmol/mg of protein)			(nmol/mg of protein)		
	mean	SD	mol %	mean	SD	mol %	mean	SD	mol %	mean	SD	mol %
Choline Glycerophospholipids												
D16:1–16:0	26.5	4.1	3.5	39.3	7.9	2.6	22	3.7	3.0	22.4	7.9	1.8
D16:0–16:0	11.9	1.4	1.6	21.6	0.7	1.4	13.1	6.2	1.8	22.8	1.5	1.9
P16:0–18:1	1.6	0.6	0.2	2.7	0.8	0.2	1.4	0.1	0.2	1.6	0.6	0.1
A16:0–18:1	4.6	1.8	0.6	7.4	0.6	0.5	4.1	1.3	0.6	5.2	0.2	0.4
D16:0–18:1	73.6	4.7	9.7	105	5.7	6.9	68.1	11.6	9.4	83.3	14.7	6.8
D16:0–18:2	26.1	5.2	3.4	27.6	3.2	1.8	21.3	4.4	2.9	21.4	3.2	1.8
D16:1–16:1	2.8	0.2	0.4	4.2	0.8	0.3	2.2	0.6	0.3	3.7	1.3	0.3
D18:1–18:2	9.7	1.7	1.3	9.9	2	0.6	7.5	2.2	1.0	7.5	1.7	0.6
D18:0–18:2/D18:1–18:1	41.5	2	5.5	42.8	5.7	2.8	51.1	6.5	7.1	42.2	0.7	3.5
D18:0–18:1	4.9	0.3	0.6	9.1	2.5	0.6	6.5	0.9	0.9	8.7	2.2	0.7
D16:0–22:6	1.8	1.2	0.2	3.6	0.8	0.2	2.7	1.3	0.4	2.5	0.1	0.2
D16:0–20:4	6.4	1.1	0.8	8.5	2	0.6	4.1	1.3	0.6	5.6	1	0.5
D18:1–20:4	6.8	0.5	0.9	6.1	0.2	0.4	6	0.9	0.8	5.1	1.4	0.4
D18:0–20:4	4.9	1.3	0.6	6.8	1.6	0.4	4.9	2.5	0.7	6	1.2	0.5
total with 20:4			2.4			1.4			2.1			1.4
grand total	223.1	18.1	29.5	294.8	7.9	19.3	214.8	21.3	29.7	238.1	15.8	19.5
Sphingomyelin												
N16:1	3.4	1.5	0.4	7	1.2	0.5	2.7	1.1	0.4	4.8	1.5	0.4
N16:0	4	1.9	0.5	6	1.4	0.4	3.4	1.6	0.5	3.8	1.2	0.3
N18:1	47.1	1.4	6.2	141.8	31.5	9.3	54.6	2.9	7.6	125.2	37.7	10.3
N18:0	7.9	1.3	1.0	17.8	4.4	1.2	8.1	2.0	1.1	19.4	8.0	1.6
N20:1	8.2	1.8	1.1	16.5	6.2	1.1	5	2.1	0.7	11.8	7.1	1.0
N24:1	3.3	0.3	0.4	17.4	0.6	1.1	2.7	0.4	0.4	13.3	1.8	1.1
N18:2	7	1.3	0.9	15.4	2.1	1.0	4.6	0.2	0.6	12.4	2.8	1.0
N20:2	2	0.5	0.3	4.1	2.8	0.3	1.5	0.2	0.2	2.9	1.0	0.2
N24:3	2.1	0.3	0.3	6.2	1.2	0.4	1.8	0.4	0.2	6.3	0.2	0.5
N24:2	8.3	1.7	1.1	24.8	2.6	1.6	9.3	2.6	1.3	22	1.7	1.8
N26:3	5.1	0.2	0.7	21	4.0	1.4	7.4	2.4	1.0	16.4	2.5	1.3
N26:2	12.8	1.6	1.7	41.8	5.8	2.7	12.4	2.8	1.7	39.4	4.7	3.2
total SM	111.3	5.6	14.7	319.8	50.2	20.9	113.5	9.5	15.7	277.6	51.6	22.8

<sup>a</sup> Lipids were extracted from plasma membrane and lipid raft fractions isolated from KB and KBC cells. Extracts were analyzed for choline-containing phospholipid content using positive-ion ESI/MS, as described in Experimental Procedures. Results represent the mean  $\pm$  SD of three separate experiments. The first number in each pair refers to the number of carbon atoms in the fatty acid chain. The number after the colon refers to the number of double bonds. The two fatty acid chain designations are separated by a hyphen. If the  $m/z$  cannot provide a unique assignment, both possibilities are given separated by a back slash. The prefix D indicates a diacylglycerol compound. The prefix P indicates a plasmalogen compound. The prefix A indicates a plasmalogen compound. For sphingomyelin, only a single fatty acid chain is designated because the other is the 18:1 aliphatic chain included in the sphingosine backbone. The prefix N indicates that the fatty acid is in an amide linkage with the sphingosine.

of some intracellular organelles, this finding is consistent with the possibility that at least some of the material in the DRM fraction is derived from intracellular membranes.

**Distribution of Saturated and Mono-Unsaturated Acyl Chains.** An analysis of the content of saturated and mono-unsaturated acyl chains in the plasma membrane, lipid raft and DRM fractions from KB or KBC cells was performed. The content of saturated or mono-unsaturated acyl chains was 39.6 mol % in plasma membranes from KB cells and 37.5 mol % in lipid rafts prepared from these cells. Similarly, the content of saturated or mono-unsaturated acyl chains was 40.4 mol % in plasma membranes from KBC cells and 40.6 mol % in lipid rafts from these cells. Thus, there is no evidence of an enrichment of lipids containing saturated and mono-unsaturated acyl chains in rafts isolated from these cells using a nondetergent method. By contrast, DRM are enriched in saturated acyl chains. The content of saturated and mono-unsaturated acyl chains was  $\sim$ 57% in DRM from KB cells, indicating that the detergent extraction procedure does lead to an enrichment of the membrane fraction in acyl chains with a low degree of unsaturation. As a result, the levels of the major arachidonic acid containing-species were significantly reduced in DRM as compared to lipid rafts. In DRM, only 19% of the ethanolamine glycerophospholipids

contained arachidonic acid, whereas 43% of the ethanolamine-containing phospholipids in nondetergent lipid rafts contained arachidonic acid.

## DISCUSSION

**Effects of Caveolin-1 Expression on Lipid Content.** KB cells do not express caveolin-1 and do not exhibit cell surface caveolae. Nonetheless, when these cells are subjected to subcellular fractionation procedures designed to separate caveolae and other low-density membranes, a light membrane fraction is readily isolated. We have termed these membranes lipid rafts. This lipid raft fraction is enriched in EGF receptors and Gq, two proteins that are typically found in the light membrane fraction of cells that express caveolin-1 and exhibit cell surface caveolar invaginations. The lipid rafts are also enriched in cholesterol, a marker for caveolae-like membranes.

Expression of caveolin-1 in KB cells led to the production of morphologically identifiable caveolae in the KBC cells. Subcellular fractionation of KBC cells generated a low-density membrane fraction that, as anticipated, was enriched in caveolin-1. It was also enriched in EGF receptors, Gq, and cholesterol. The enrichment of EGF receptors and Gq

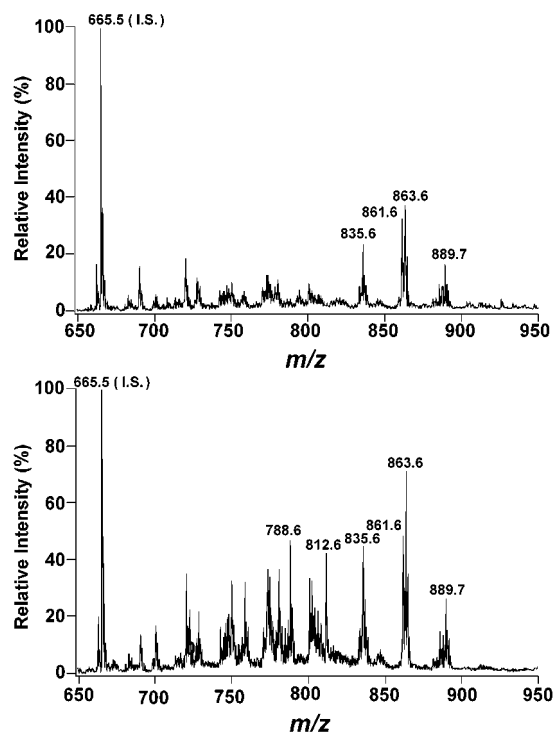


FIGURE 7: Negative-ion ESI/MS spectra of anionic phospholipids in lipid extracts of the plasma membrane fraction and the low-density caveolae/lipid raft fraction derived from KB cells. Subcellular membrane fractions and their lipid extracts were prepared as described in the legend of Figure 3. Phospholipids were extracted by a modified method of Bligh–Dyer, as described in Experimental Procedures. Aliquots of the chloroform extracts were directly infused into the ESI ion source using a Harvard syringe pump at a flow rate of 1  $\mu$ L/min. Negative-ion ESI/MS of lipid extracts of the plasma membrane fraction (panel A) and the low-density caveolae/lipid raft fraction (panel B) were performed as described in Experimental Procedures. Different classes of anionic phospholipids and their individual molecular species were identified using tandem mass spectrometry and are tabulated in Table 5. The internal standard (IS) is 14:0–14:0 GroPGro ( $m/z$ : 665.5). Unlabeled peaks were mainly ethanolamine glycerophospholipid molecular species, which were analyzed under different experimental conditions.

in the caveolar/lipid raft fraction of KBC cells was similar to that observed in KB cells, suggesting that the expression of caveolin-1 did not substantially alter the localization of these two signaling proteins. This suggests that these proteins can localize to lipid rafts via a mechanism that is independent of caveolin-1. Because caveolin-1 expression did not further enhance this localization, the results imply that caveolin-1 is not substantially involved in the localization of these proteins to the low-density membrane fraction in these cells.

Lipid rafts isolated from KB and KBC cells contained substantially higher levels of cholesterol and sphingomyelin than the corresponding plasma membrane fractions. This is consistent with previous reports, demonstrating that lipid rafts are enriched in these compounds (5). The lipid raft fraction from KBC cells exhibited a significantly higher cholesterol content than that of lipid rafts isolated from KB cells. Thus, the expression of caveolin-1, a cholesterol-binding protein, appears to lead to an increase in the cholesterol content of the specific compartment in which it is localized.

Increases in cholesterol levels often lead to concomitant increases in sphingomyelin levels because the two lipids interact strongly (54–58). Thus, it is of interest that

caveolin-1 expression increases cholesterol levels but does not change sphingomyelin content. A similar bias toward increased cholesterol without increased sphingomyelin is found in synaptic vesicles. This is associated with the presence in the synaptic vesicles of synaptophysin, a cholesterol-binding protein (59). Direct binding of all the excess cholesterol to synaptophysin was deemed unlikely (59). Similarly, our calculations indicate that caveolin-1 could not directly bind all the excess cholesterol present in the caveolae/lipid raft fraction. However, it may direct the organization of a structure that facilitates cholesterol enrichment using less sphingomyelin. In any event, the extra cholesterol must either be sequestered in some fashion or restricted to the inner leaflet of the plasma membrane so that it does not alter the balance of cholesterol and sphingomyelin at the cell surface.

**Plasmenylethanolamines and Arachidonic Acid.** Analyses of the phospholipid content of lipid raft and plasma membrane fractions identified several distinct features. First, in all membrane fractions analyzed, ethanolamine glycerophospholipid was the major phospholipid accounting for 40% of the total phospholipids. Second, ethanolamine plasmalogens were significantly enriched in the lipid raft fraction of both KB and KBC cells. Third, the plasmenylethanolamines in lipid rafts were highly enriched in arachidonic acid. This gives rise to an overall increase in the amount of arachidonic acid-containing phospholipids in lipid rafts as compared to the plasma membrane fraction. Cholesterol and sphingomyelin tend to be enriched in the outer leaflet of the plasma membrane, whereas ethanolamine glycerophospholipids tend to partition into the inner leaflet of the plasma membrane (60). If the topology of these lipids in caveolae/lipid rafts is similar to that in plasma membranes, the present results suggest that arachidonic acid-containing plasmenylethanolamine may represent a third to a half of the phospholipids on the cytosolic face of lipid rafts.

The enrichment of such arachidonic acid-containing lipids in rafts suggests that these membrane domains may represent a focus for the production of free arachidonic acid during cellular signaling. Arachidonic acid-containing plasmenylethanolamines are the major source of released arachidonic acid in multiple different cell types, as determined by both HPLC and mass spectroscopic approaches (41, 61, 62). Lipid rafts have previously been shown to be the source of PtdInsP<sub>2</sub> used in EGF and bradykinin-stimulated phosphatidylinositol turnover (63). It will be important to determine whether these domains also serve as an enriched source of arachidonic acid-containing phospholipids for any of the phospholipase A<sub>2</sub> enzymes.

**Phosphatidylserine and Anionic Phospholipids.** The increases in cholesterol and sphingomyelin content of lipid rafts as compared to the plasma membrane fractions were largely counterbalanced by compensatory decreases in PtdCho and PtdIns. The relative lack of PtdIns in lipid rafts is consistent with our previous studies of [<sup>3</sup>H]myo-inositol-labeled cells that demonstrated that PtdInsP<sub>2</sub> and, to some extent, PtdInsP were enriched in low-density membrane fractions while PtdIns itself was not (28, 63, 64). In all fractions, the major species of PtdIns were those containing 18:0–18:1 and 18:0–18:2. This finding was surprising because it was anticipated that arachidonic acid-containing species of PtdIns would predominate. However, the fact that this distribution



Table 5: Anionic Phospholipids<sup>a</sup>

assignment	KB plasma memb			KB lipid rafts			KBC plasma memb			KBC lipid rafts		
	(nmol/mg of protein)			(nmol/mg of protein)			(nmol/mg of protein)			(nmol/mg of protein)		
	mean	SD	mol %	mean	SD	mol %	mean	SD	mol %	mean	SD	mol %
Phosphatidic Acid												
16:0–18:1	1	0.3	0.1	3.3	0.4	0.2	0.9	0.3	0.1	3.8	0.5	0.3
16:0–18:0	0.8	0.6	0.1	0.8	0.5	0.1	0.6	0.4	0.1	0.8	0.5	0.1
18:0–18:1	1.6	0.3	0.2	5.3	0.9	0.3	1.3	0.1	0.2	6.7	1.9	0.6
16:0–18:2	0.7	0.6	0.1	1.3	1	0.1	0.5	0.5	0.1	0.9	0.6	0.1
18:1–18:1	1.4	0.2	0.2	3.8	0.6	0.2	1.1	0.6	0.2	3.9	0.2	0.3
total PtdH	5.5	1.8	0.7	14.5	1.7	0.9	4.4	1.8	0.6	16.1	1.9	1.3
Phosphatidylserine												
16:0–18:1	1.3	0.4	0.2	9.2	2.9	0.6	1.3	0.3	0.2	6.1	0.9	0.5
18:0–18:1	2.4	0.8	0.3	23.7	4.6	1.6	3.3	0.5	0.5	18.1	0.7	1.5
18:0–18:2	3	0.3	0.4	17.9	2.5	1.2	3.1	0.4	0.4	14.2	1	1.2
18:0–20:4	2.1	0.7	0.3	10.5	5.4	0.7	1.1	0.3	0.2	5.3	1.1	0.4
total PtdSer	8.7	2.1	1.1	61.3	12.2	4.0	8.7	0.5	1.2	43.7	0.9	3.6
Phosphatidylinositol												
16:0–18:1	18.2	2.4	2.4	27.9	4	1.8	13.3	0.8	1.8	18.1	2.7	1.5
18:0–18:1	26.1	4.5	3.4	43.6	3.1	2.9	27.1	4.5	3.7	36.1	2.9	3.0
18:0–18:2	28.2	5.4	3.7	37	0.9	2.4	25	4.8	3.5	28.8	4.7	2.4
16:0–18:2	8	0.9	1.1	11	1.4	0.7	5.3	0.5	0.7	7.9	0.7	0.6
18:0–20:3	6	0.7	0.8	8.2	1	0.5	6.8	1.3	0.9	7.9	1	0.6
18:0–20:2	9.2	0.3	1.2	15.1	2.1	1.0	9	0.7	1.2	12.6	0.5	1.0
18:1–20:4	3.2	0.7	0.4	4.1	1	0.3	3.4	0.5	0.5	3.9	0.5	0.3
18:0–20:4	6.7	0.8	0.9	11.5	2.5	0.8	8.3	3	1.1	11.3	2.6]	0.9
total PtdIns with 20:4			1.3			1.0			1.6			1.2
total PtdIns	105.6	12.3	13.9	158.4	8.6	10.4	98.2	14	13.6	126.6	10.6	10.4
total anionic lipids	119.9	9.6	15.8	234.2	5	15.3	111.3	12.5	15.4	186.4	10.5	15.3

<sup>a</sup> Lipids were extracted from plasma membrane and lipid raft fractions isolated from KB and KBC cells. Extracts were analyzed for anionic phospholipid content using negative-ion ESI/MS, as described in Experimental Procedures. Results represent the mean  $\pm$  SD of three separate experiments. The first number in each pair refers to the number of carbon atoms in the fatty acid chain. The number after the colon refers to the number of double bonds. The two fatty acid chain designations are separated by a hyphen. All species are diacylglycerol compounds.

was found in all fractions tested, including those from whole cells, suggests that this cell type maintains a nontraditional ratio of PtdIns species.

Despite the fact that phosphatidylinositol levels decreased in lipid rafts relative to the plasma membrane fraction, the overall content of anionic phospholipids in all fractions remained constant. This was largely due to a 3- to 4-fold increase in the amount of PtdSer in lipid rafts relative to plasma membranes. This represents the largest-fold difference in any phospholipid species observed between lipid rafts and plasma membranes. PtdSer and arachidonic acid are activators of protein kinase C (65–68). Enrichment of these lipids in lipid rafts where diacylglycerol, the second activator of protein kinase C, is also generated through localized PtdIns turnover (63) may potentiate the ability of hormones to recruit specific isoforms of protein kinase C into rafts and stimulate the activity of this kinase. PtdSer may also be important for binding other cytoplasmic proteins.

Annexins are a family of Ca<sup>2+</sup> and anionic phospholipid binding proteins that exhibit a high affinity for phosphatidylserine (69). Annexin II binds strongly to cell membranes, apparently in complex with several proteins of the cortical cytoskeleton (70). Interestingly, this complex can be released from the cell membrane by sequestration of cholesterol. This implies a role for cholesterol-enriched membrane domains in this phenomenon. Our data suggest that this might occur through concentration of the PtdSer ligand in lipid rafts.

*Comparison of Nondetergent Lipid Rafts with Detergent Resistant Membranes.* A number of significant differences were observed between lipid rafts made using the nondetergent method (42) and those made using a Triton X-100

extraction procedure. The most obvious is the greater enrichment of cholesterol in DRM (~60 mol % of total lipids) as compared to lipid rafts (~25 mol %). Thus, while both types of low-density preparations showed similar ratios of total lipid-to-protein, the DRM were largely composed of cholesterol, while the lipid rafts had a preponderance of phospholipids. A second major difference was the level of saturation in the fatty acyl chains of the phospholipids. The content of saturated and mono-unsaturated acyl chains was quite high in DRM (~60%) versus lipid rafts (~40%). This is consistent with previous reports (71), demonstrating a higher degree of saturation in the acyl chains of phospholipids in DRM as compared to plasma membranes. Other notable differences between the lipids present in lipid rafts versus DRM are the relative deficiency of anionic phospholipids and the lack of an enrichment of plasmalogens in DRM, particularly those that contain arachidonic acid. Thus, inclusion of detergent in the isolation of low-density membranes substantially alters the character of the resulting membranes. The fraction is enriched in cholesterol and uncharged phospholipids and contains an abundance of saturated acyl chains. Whether this composition accurately reflects the composition of a “core” of lipid rafts *in vivo* is not known.

It is possible that lipid rafts isolated in the absence of detergent contain, as their core, the same DRM material but in addition, include an annulus of more loosely associated lipids that are enriched in anionic phospholipids, plasmalogens, and arachidonic acid-containing molecular species. Alternatively, lipid rafts could be a homogeneous mixture of phospholipids and cholesterol. Detergent extraction could

Table 6: Phospholipid Content of Detergent-Resistant Membranes<sup>a</sup>

choline-containing phospholipids			ethanolamine-containing phospholipids			anionic phospholipids		
choline glycerophospholipids	nmol/mg of protein	mol %	phospholipid	nmol/mg of protein	mol %	phospholipid	nmol/mg of protein	mol %
D16:1–16:1	4.4 ± 0.4	0.5	diacyl species			PtdH		
D16:1–16:0	35.2 ± 16	4.0	16:0–18:1	55.7 ± 11	6.4	18:1–18:1	0.31 ± 0.01	0.04
D16:0–16:0	23.9 ± 4.4	2.7	16:0–18:2	13.5 ± 4.8	1.6	18:0–18:1	0.89 ± 0.02	0.1
P16:0–18:1	4.4 ± 3.3	0.5	16:0–22:5/ 18:1–20:4	6.6 ± 1.2	0.8	subtotal	1.19 ± 0.03	0.13
D16:0–18:2	14.8 ± 0.1	1.7	18:0–18:1	34.2 ± 0.5	3.9	PtdSer		
D16:0–18:1	82.7 ± 10.0	9.5	18:0–20:4	21.8 ± 10	2.5	16:0–18:1	3.15 ± 1.07	0.36
D18:1–18:2	12.2 ± 6.3	1.4	18:0–22:5	4.8 ± 0.04	2.7	18:0–18:2	1.61 ± 0.33	0.19
D18:0–18:2/ D18:1–18:1	43.9 ± 11.6	5.0	18:0–22:6	8.5 ± 0.1	1.0	18:0–18:1	6.02 ± 0.88	0.7
subtotal	221.3 ± 48.9	25.3	18:1–18:1	44.0 ± 5.5	5.1	18:0–20:4	0.81 ± 0.17	0.09
			20:0–20:4	7.5 ± 2.2	0.9	subtotal	11.60 ± 0.01	1.34
			subtotal	196.6 ± 13.5	24.7			
sphingomyelin			plasmalogen species			PtdGro		
N18:2	9.0 ± 1.1	1.0	20:0–20:4	4.4 ± 0.1	0.5	16:0–18:2	1.42 ± 0.16	0.16
N18:1	73.4 ± 17.9	8.5	plasmalogen species			16:0–18:1	6.64 ± 0.84	0.76
N18:0	12.3 ± 2.7	1.4	14:0–18:1	18.7 ± 0.23	2.1	18:1–18:2	0.85 ± 0.27	0.1
N20:2	4.8 ± 4.1	0.5	16:0–18:1	61.3 ± 23.2	7.1	18:1–18:1	3.38 ± 1.12	0.39
N20:1	8.7 ± 3.9	1.0	16:0–18:2	14.5 ± 0.01	1.7	18:0–18:1	1.06 ± 0.42	0.12
N24:3	3.2 ± 2.5	0.4	18:0–18:1	29.2 ± 3.9	3.4	subtotal	13.35 ± 2.81	1.54
N24:2	12.2 ± 0.3	1.4	18:0–22:6	8.43 ± 0.03	1.0	PtdIns		
N24:1	8.2 ± 1.4	0.9	18:1–18:1/ 18:0–18:2	18.5 ± 0.8	2.1	16:0–18:2	0.66 ± 0.03	0.08
N26:3	7.5 ± 1.1	0.9	18:1–22:6	7.1 ± 1.5	0.8	16:0–18:1	3.97 ± 0.33	0.46
N26:2	37.1 ± 8.5	4.2	18:2–22:6	7.9 ± 1.1	0.9	18:0–18:2	2.41 ± 0.74	0.28
subtotal	176.4 ± 5.7	20.3	16:0–20:4	33.2 ± 2.3	3.8	18:0–18:1	5.72 ± 0.70	0.66
			18:0–20:4	11.7 ± 0.83	1.3	18:0–20:3	0.28 ± 0.13	0.03
			18:1–20:4	20.2 ± 6.1	2.3	18:0–20:2	1.00 ± 0.62	0.12
			subtotal	230.8 ± 15.7	26.6	18:1–20:4	0.41 ± 0.20	0.05
						18:0–20:4	0.79 ± 0.47	0.09
						subtotal	15.23 ± 1.82	1.75
total	397.7 ± 54.7	45.6	total	431.8 ± 1.2	51.8	total	41.36 ± 4.6	4.78
cholesterol	1265 ± 7	N/A						

<sup>a</sup> Lipids were extracted from DRM fractions isolated from KB cells analyzed for phospholipids using ESI/MS, as described in Experimental Procedures. Cholesterol was determined by enzymatic assay. Results represent the mean ± SD of two separate experiments. The first number in each pair refers to the number of carbon atoms in the fatty acid chain. The number after the colon refers to the number of double bonds. The two fatty acid chain designations are separated by a hyphen. If the *m/z* cannot provide a unique assignment, both possibilities are given separated by a back slash. The prefix D indicates a diacylglycerol compound. The prefix P indicates a plasmalogen compound. For sphingomyelin, only a single fatty acid chain is designated because the other is the 18:1 aliphatic chain included in the sphingosine backbone. The prefix N indicates that the fatty acid is in an amide linkage with the sphingosine.

selectively remove phospholipids from these domains, leaving behind the cholesterol-enriched residue. The fact that the EGF receptor is associated with nondetergent lipid rafts but not with DRM suggests that this extra phospholipid may contribute to the ability of rafts to harbor transmembrane proteins. Additional work is needed to determine the role of the specialized subset of lipids present in nondetergent rafts in the organization and function of signal transduction.

In summary, these results demonstrate that nondetergent lipid rafts exhibit a lipid composition that is distinct from that seen in isolated plasma membrane fractions. The enrichment in lipid rafts of arachidonic acid-containing phospholipids implies that these domains may represent a localized pool of substrate for the generation of free arachidonic acid in response to cell activation. Further, PtdSer is substantially enriched in this compartment, suggesting that it may be important for the function of these domains. Finally, the expression of caveolin-1 in otherwise caveolin-1-negative cells induces the formation of caveolae and increases the content of cholesterol in the lipid raft fraction but does not substantially change the phospholipid composition of these domains. These findings indicate that the physical and chemical properties of lipid rafts are dramatically different from those of the surrounding plasma membrane and that this is likely to contribute to the specialized functions of these lipid domains.

## ACKNOWLEDGMENT

The authors are grateful to the Washington University Mass Spectrometer Facility Center for allowing them to use the electrospray ionization mass spectrometer.

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BI0156557