Smooth muscle raft-like membranes

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Abstract **We developed a method for extracting raft-like, liquid-ordered membranes from the particulate fraction prepared from porcine trachealis smooth muscle. This fraction, which contains most of the plasma membrane in this tissue, was homogenized in the presence of cold 0.5% Triton X-100. After centrifugation, membranes containing high contents of sphingomyelin (SM) and cholesterol and low phosphatidylcholine (PC) contents remained in the pellet. Thirty-five millimolar octyl glucoside (OG) extracted 75% of these membranes from the Triton X-100-resistant pellet. These membranes had low buoyant densities and accounted for 28% of the particulate fraction lipid. Their lipid composition, 22% SM, 60% cholesterol, 11% phosphatidylethanolamine, 8% PC, <1% phosphatidylinositol, and coisolation with 5′-nucleotidase and caveolin-1 suggest that they are liquid-ordered membranes. We compared characteristics of OG and Triton X-100 extractions of the particulate fraction. In contrast to Triton X-100 extractions, membranes released from the particulate fraction by OG were mainly collected in low buoyant fractions at densities ranging from 1.05 to 1.11 g/ml and had phospholipid and cholesterol contents consistent with a mixture of liquid-ordered and liquid-disordered membranes. Thus, OG extraction of apparent liquid-ordered membranes from Triton X-100-resistant pellets was not due to selective extraction of these membranes. Low buoyant density appears not to be unique for liquid-ordered membranes.**—Baron, C. B., and R. F. Coburn. **Smooth muscle raft-like membranes.** ***J. Lipid Res.*** **2004.** 45: **41–53.**

Supplementary key words sphingomyelin • cholesterol • phosphatidylcholine • liquid-ordered membranes • detergent-resistant membranes

Plasma membrane lipids are nonrandomly distributed and are compartmentalized into liquid-ordered and liquid-disordered microdomains (1–5). Liquid-ordered microdomains enriched in sphingolipids and cholesterol, known as lipid rafts, are characterized by tight packing due to the interactions of lipids that contain saturated acyl chains and cholesterol, and hydrogen bonding between sphingolipid head groups (6–8). The presence of rafts in living cells has been demonstrated using different approaches and techniques (2, 3, 9–16). There are multiple types of rafts in cellular membranes (12, 17) including some that are associated with the actin cytoskeleton (12, 18, 19). Rafts, including caveolae, a subset of rafts (20), are platforms for a number of signaling and trafficking events (1–5). Due to their tight packing and structure, liquid-ordered membranes selectively attract and concentrate proteins that have been acylated with saturated lipids via myristoylation or palmitoylation (21–23). Some signal transduction proteins are known to bind directly to caveolin-1 (Cav-1) (24). The question has been raised whether raft membrane proteins migrate to areas rich in saturated lipids or whether certain membrane proteins, because of their preferred interaction with saturated lipids and/or cholesterol, are the starting points of raft formation (5).

The earliest report of low buoyant density membranes (LBDMs) appears to be that of Mescher, Jose, and Balk (25), who produced them from a detergent (both Nonidet P-40 and Triton X-100) -insoluble fraction. Isolation of raft-like material using cold Triton X-100 extraction followed by collection of LBDMs using isopycnic centrifugation is still in general use in studies of compartmentalization of various signal transduction proteins in raft-like membranes (26–29). (These references are recent and reflect only a small fraction of total published studies that utilized isopycnic centrifugation to collect "raft-like" membranes.) In many studies, LBDMs were not collected, and detergent-resistant membranes were equated with raft-like membranes. Triton X-100 solubilizes liquid-disordered membranes by forming micelles effected by binding of the detergent to phosphatidylcholine (PC), where there are higher lipid-detergent affinities than lipid-lipid affinities, and by disrupting lipid-lipid and lipid-protein interactions (30–32). The detergent resistance of liquid-ordered membranes at 4°C is due in part to the tight packing of saturated acyl chains, which restricts penetration of detergents into these structures (7, 8, 33, 34). That liquid-ordered membranes have low buoyant densities has been explained by their low ratios of protein to lipid (2).

Single tracking studies have provided evidence that in vivo rafts contain detergent-resistant liquid-ordered membranes (11, 14, 19). The use of cold Triton X-100 extraction to isolate liquid-ordered membranes is supported by

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the finding (35) that liquid-ordered membranes evoked in a model system by physiological concentrations of cholesterol and sphingolipid could be quantitatively extracted in the cold as detergent-resistant membranes. However, different nonionic detergents may extract different subsets of raft-like membranes (4, 5, 36).

Our goal in this study was to develop a method of isolating liquid-ordered, raft-like membranes from intact smooth muscle so that we could investigate the characteristics of these membranes. We were able to develop a method for isolating liquid-ordered membranes from a particulate fraction of porcine trachealis muscle where we used octyl glucoside (OG) to extract these membranes from the Triton X-100-resistant pellet. This method should be applicable to studies on other tissues in which membranes are tightly assembled so that homogenization is not able to separate membrane particles sufficiently prior to isopycnic centrifugation. We report some basic characteristics of smooth muscle particulate fraction membranes; i.e., the percentage of total membranes that are liquid-ordered, their phospholipid and cholesterol composition, their buoyant density profiles, and evidence that these membranes have homogenous protein-lipid ratios. Most previous studies of detergent-resistant membranes have been performed using cultured cells. Because some smooth muscle signal transduction systems are muscle length dependent (37, 38), studies of compartmentalization of signal transduction proteins need to be performed using intact smooth muscle, in which length can be controlled. There have been previous studies of detergent-resistant membranes that were isolated from gizzard microsomes (39) and from a cultured smooth muscle cell line (40). These studies utilized detergent resistance as a criterion for raft-like membranes.

A secondary goal in our study was to define differences between Triton X-100 and OG extractions of membranes from a smooth muscle particulate fraction. This goal followed from the finding that OG, unlike Triton X-100, was apparently able to extract liquid-ordered membranes.

MATERIALS AND METHODS

Materials

Lipid standards were from Avanti Polar Lipids, Inc. (Alabaster, AL). [^{3}H] *myo*-inositol was obtained from Amersham Biosciences Corp. (Piscataway, NJ). OptiPrep and all the protease inhibitors were obtained from Sigma (St. Louis, MO). OG and Triton X-100 were obtained from Boehringer Mannheim (Indianapolis, IN). All other chemicals were of reagent grade. Rabbit polyclonal anti-Cav-1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Tissue preparation

Porcine tracheas were obtained from a local meat packing company. Porcine tracheal smooth muscle (PTSM) was finely dissected and cut into 15 to 20 × 8 mm, 1 mm-thick strips each containing 10–15 mg muscle. Strips were incubated for 1 h in tubes containing Krebs solution [114 mM NaCl, 5.9 mM KCl, 11.5 mM glucose, 1.2 mM NaH_2PO_4, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 22 mM $NaHCO_3$ (pH 7.35)] bubbled with 85%O_2-5%CO_2-10%N_2 (v/v/v) at 37°C to allow for "recovery." **Figure 1** illustrates the cross-sectional appearance of the smooth muscle preparation used in this study.

Labeling of tissue with [^{3}H]*myo*-inositol, setting muscle length at 1.0 Lo, freezing, and powdering frozen muscle

Figure 2 lists various steps in the membrane isolation procedure. Muscle strips, in 37°C Krebs solution, were incubated with [^{3}H] *myo*-inositol as described previously (41) to allow measurement of inositol phospholipids. After labeling, muscle strips were transferred to an apparatus (38) that set strips to 1.0 Lo, the length at which agonist stimulation results in maximal isometric force. Muscle strips, held at 1.0 Lo, were then pinned to Sylgard blocks and transferred to a tube that contained 37°C Krebs solution. After an incubation period of at least 15 min, muscles attached to Sylgard blocks were rapidly frozen by immersion in liquid N_2. The frozen tissue was easily separated from the block with a spatula, ground to a fine powder under liquid N_2, and stored at −70°C.

Fractionation of tissue: preparation of the particulate and microsomal fractions

Sixty to one hundred milligrams of frozen powdered tissue was homogenized at 4°C in 1 ml of 250 mM sucrose, 2 mM EGTA, 20 mM HEPESNa (pH 7.3), and 0.5 mM $MgCl_2$ containing aprotinin, leupeptin and pepstatin A, each at a concentration of 5 μg/ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 μM calpain inhibitor peptide, 0.5 mM sodium orthovanidate, and 0.5 mM sodium pyrophosphate. Because fine powder was our starting material, only gentle homogenization was required, using a glass Tenbroeck homogenizer at 300 rpm, 10 strokes. The homogenate was centrifuged at 15,000 *g* for 2 min. Pellets, which we call "the particulate fraction," and supernatants were quick frozen in liquid N_2 in the presence of 10% glycerol and stored at −70°C. We have emphasized studies of the particulate fraction, because this fraction contains plasma membrane (42), where most liquid-ordered membranes might exist. The particulate fraction also contains 45.1 ± 4.8% of total cellular glucose 6-phosphatase activity, an endoplasmic reticulum marker (43). Galactosyl transferase activity [a golgi marker (44)] in the particulate fraction was <1% of that measured in an equivalent weight of isolated liver golgi.

We studied microsomes so that the phospholipid and cholesterol contents in this fraction could be compared with these contents in the particulate fraction. We also wanted to measure plasma membrane marker proteins in microsomes to determine whether plasma membranes were lost from the particulate fraction during homogenization. Microsomes were collected by centrifuging the low-speed supernatant at 424,000 *g* for 20 min.

Detergent extractions

Our initial experiments used nondetergent extraction of membranes (45). This technique did not extract sufficient membranes to study, in that we could barely detect any lipids in OptiPrep gradient fractions; we therefore have used only detergent extraction approaches in this study.

It has been critiqued (5) that many of the published studies of detergent-resistant membranes have not adequately described extraction protocols and that much of the data are nonquantitative. Our detergent extractions are described in terms of detergent-lipid ratios and relationship of detergent concentrations to critical micelle concentration (CMC), extraction time duration, and other characteristics. Our phospholipid and cholesterol data are given in terms of percent of totals in the particulate fraction, and we always quantitated extractable and nonextractable phospholipids and cholesterol.

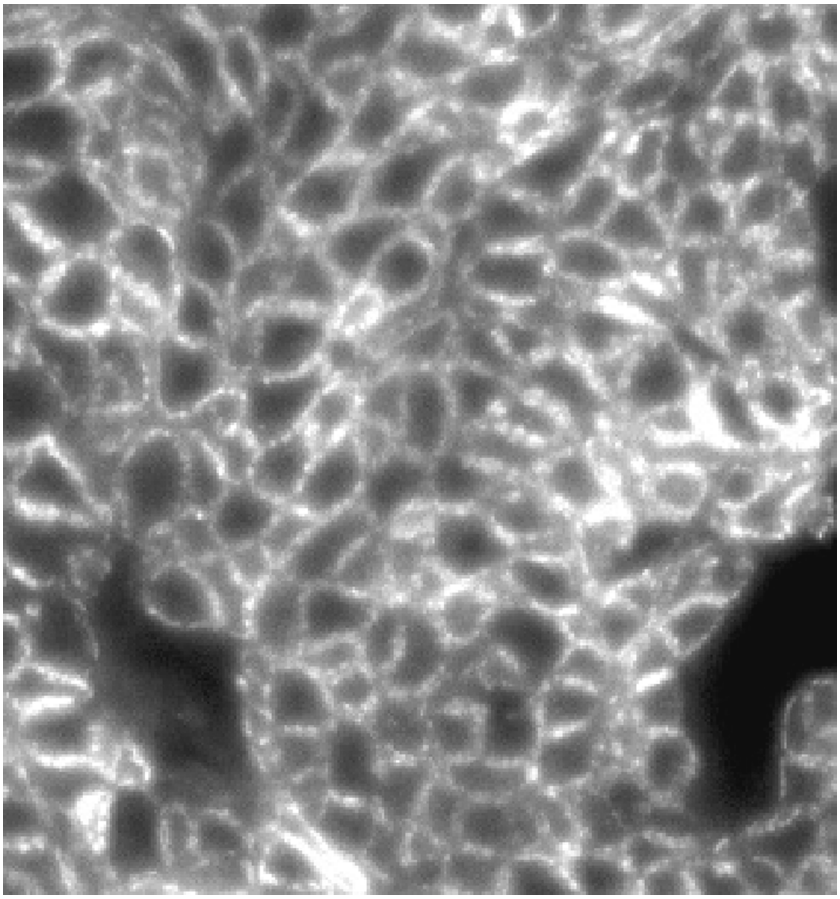

Fig. 1. Porcine tracheal smooth muscle (PTSM) histology. Packing of smooth muscle cells in intact smooth muscle cut in cross-section. Resting muscle held at 1.0 Lo was fixed in formaldehyde, carried through 10%, 20%, and 30% sucrose solutions, frozen with liquid N_2, cut with a microtome into 6 μm thick cross-sections, and incubated with monoclonal antibodies to caveolin-1 (Cav-1) followed by incubation with TRITS secondary antibody. The image was photographed using a fluorescent microscope.

Triton X-100. Extractions and treatments were performed at 4°C. The washed particulate fraction was resuspended and homogenized in 0.5 ml of extraction buffer that contained 0.5% Triton X-100 (giving 7.5 mM micelles in excess of the CMC = 0.2 mM; detergent-lipid ~11:1 (mol/mol; PC + phosphatidylethanolamine (PE) + phosphatidylinositol (PI) + sphingomyelin [SM] + cholesterol), aprotinin, leupeptin, and pepstatin A, each at a concentration of 5 μg/ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 μM calpain inhibitor peptide, 0.5 mM sodium orthovanadate, and 0.5 mM sodium pyrophosphate. We kept the homogenization procedure and time of exposure to detergents approximately constant in different experiments. The lipids found in the Triton X-100 extract were not sensitive to changes in salt concentrations in the range 0–600 mM NaCl, and we kept NaCl constant at 150 mM. The homogenate was centrifuged at 15,000 *g* for 2 min. Following centrifugation, the nonsedimentable fraction containing extracted lipid (supernatant) and a pellet were collected. The pellet was frozen and stored for lipid analysis. Two hundred thirty milligrams of solid sucrose was added to the supernatant and dissolved by slow mixing for 1 h. This material was then placed on isopycnic centrifugation gradients. Two experiments were performed. In an additional experiment, we vigorously sonicated the extract prior to isopycnic centrifugation. The extracts were split into two 0.5 ml portions. One portion was subjected to sonication for 5 min at 1 watt keeping temperature at 0°–10°C. Half-second bursts were given every second for 10 s, followed by 10 s with no sonication. This protocol was repeated 15 times, giving a total of 150 bursts. The second portion served as a control. Two-hundred thirty milligrams of sucrose was added to each extract, as above.

OG. We performed OG extractions in two types of experiments: *a*) using Triton X-100-extracted pellets to attempt to release Triton X-100-resistant membranes; and *b*) using homogenized particulate fractions to elucidate characteristics of OG extractions; i.e., why this detergent could extract membranes from Triton X-100-resistant pellets. Extractions were performed using 35 mM OG, which is 10 mM in excess of the CMC. The detergent-lipid ratio was ~48:1 (mol/mol; PC + PE + PI + SM + cholesterol). The washed particulate fraction was resuspended in 0.5 ml of 20 mM HEPESNa (pH 7.3), 0.5 mM $MgCl_2$, 600 mM NaCl, 35 mM OG, and peptide protease and phosphatase inhibitors, as above. Six hundred millimolar NaCl was included, because initial experiments showed that maximal extraction required high salt to obviate ionic interactions. The sample was homogenized on ice (six strokes with the Tenbroeck homogenizer over 2 min). Centrifugation, collection of the pellet and supernatant, and preparation for isopycnic centrifugation were identical to techniques described above.

Continuous OptiPrep isopycnic density gradients

OptiPrep was used for isopycnic centrifugations, because it forms its own gradient. We first prepared a discontinuous Opti-

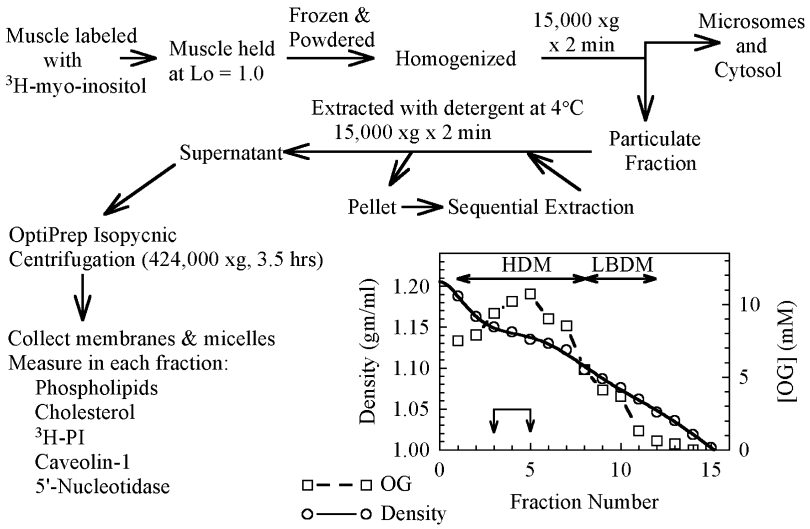

Fig. 2. Approaches and methodology. This is described in the text. The inset illustrates the typical relationship of different fractions collected using isopycnic centrifugation to their densities (obtained in "dummy runs"). Densities (circle) were measured by weighing 100 μl aliquots of each gradient fraction. Data were fit to an 8th-order polynomial. This plot also shows data obtained where octyl glucoside (OG) was injected into the 35–20% OptiPrep interface (vertical, connected arrows) prior to centrifugation to illustrate [OG] profile in the gradient after isopycnic centrifugation. [OG] in each fraction (square; mean of two gradients) was measured by drying 2 μl aliquots of each fraction from two separate OG extracts run on isopycnic density gradients, dissolving in 30 μl of CH_3OH, spotting the equivalent of 0.8 μl of the original 2 μl on a TLC plate, developing in $CHCl_3$-CH_3OH-H_2O (65:25:4; v/v/v), and staining with 0.2% $KMnO_4$ in 0.4 N NaOH, a general stain for hydroxyl groups (41).

Prep gradient in extraction buffer: 0.4 ml 35%, 0.6 ml 20%, 0.6 ml 15%, 0.6 ml 10%, and 0.2 ml 5%. (The use of a discontinuous gradient speeds up the formation of the continuous gradient during centrifugation.) Half milliliter samples of material, collected and treated as described above, were injected and centrifuged for 1.5×10^6 *g* h (424,000 *g* for 3.5 h at 4°C) in a TLA 100.3 angled rotor in a Beckman Optima TLX ultracentrifuge. This centrifugation protocol is equivalent to 29.6 h in a SW41 rotor at 39,000 rpm, a common rotor and speed used by other investigators. We usually injected samples at the 35–20% OptiPrep interface above a bottom cushion (to trap highly dense material). In experiments designed to lengthen the gradient, we injected the sample at the bottom of the gradient. Fractions of 0.2 ml were collected from the bottom of the gradient, and 10% glycerol added. Fractions were quick frozen in liquid N_2 and stored at −70°C. The density profile was determined with a dummy gradient. The inset of Fig. 2 illustrates density data obtained in a typical dummy gradient. This figure also illustrates diffusion of OG during the gradient and that the concentration of this detergent in collected low buoyant density fractions was below the CMC.

Lipid extraction, TLC, and quantitation

Aliquots of 25–100 μl for each fraction (including each gradient fraction, each wash and detergent extract not placed on a gradient, final pellets, and the microsomes) were extracted by first generating a single solution by addition of 0.5 ml of $CHCl_3$-CH_3OH (1:1; v/v, containing 0.5% butylated hydroxytoluene) and then causing phase separation by addition of 0.2 ml 0.88% KCl. The upper phase was removed, and the lower phase was washed with 0.2 ml of CH_3OH-H_2O (1:1; v/v). The upper phase was again removed, and the lower phase was dried under vacuum and dissolved in 30 μl of $CHCl_3$-CH_3OH (2:1; v/v). The entire volume and a 20 μl wash of each sample were spotted on a 1.2% boric acid-impregnated TLC plate (Whatman LK5), dried under vacuum for 20 min (readmitting N_2), and developed to 4 cm below the top with $CHCl_3$-C_2H_5OH-$(C_2H_5)_3N$-H_2O (30:34:30:8; v/v/v/v) (46). The plate was then dried under vacuum for 20 min at 37°C, cooled to room temperature, and developed to the top with n-C_6H_{14}-$(C_2H_5)_2O$-$(C_2H_5)_3N$ (20:40:0.6, v/v/v). One or two tracks on the plate contained quantitative mass standards (for SM, PC, PE, and cholesterol) as well as a sample of [^{3}H]PI. Phosphatidylserine (PS) could not be determined, because it comigrated with OG, some of which was always present in the lipid extract. Plates were first counted for ^{3}H in a Berthold Automatic TLC-Linear Analyzer and then charred for lipid and quantitated by densitometry (41). Polyphosphoinositides were extracted with $CHCl_3$-CH_3OH-conc HCl (267:133:1; v/v/v) (41) and separated using oxalate-impregnated plates (47).

5′-Nucleotidase and Cav-1 measurements

Liquid-ordered membranes contain glycosylphosphatidylinositol (GPI)-anchored proteins, and Cav-1. 5′-nucleotidase (5′-N) (a GPI-anchored protein) activity was assayed as described in Baron, Pring, and Coburn (41). We quantified Cav-1 content using dot blots (SDS-PAGE proved that the polyclonal Cav-1 antibody recognized one protein at 21 kDa). One microliter aliquots were spotted on 0.45 μ nitrocellulose membranes. The membranes were washed three times with 20 mM TrisCl (pH 7.5) containing 0.2 M NaCl and 0.1% Tween, blocked with 5% dry milk in the same solution and incubated with primary antibody then with secondary antibody conjugated to HRP, and developed using enhanced chemiluminescence. The densities of the spots on the X-ray film were quantified using densitometry at 448 nm in a Shimadzu CS-390 scanner. X-ray films of lower intensity were used so that the spot intensities were below film saturation.

Curve fitting

Mean densities were determined by using single or double gaussian fits of the data (Easyplot, version 4.0.1, Spiral Software).

Single gaussian: $y = (a)e^{([-[(b-x)/c]2])^2} + f$ *(Eq. 1)*

Double gaussian: $y = (a)e^{([-[(b-x)/c]2])^2} + (f)e^{([-[(g-x)/h]2])^2} + i$ *(Eq. 2)*

RESULTS

Recoveries of the various phospholipids and cholesterol

We compared lipids extracted from frozen ground tissue with $CHCl_3$-CH_3OH (2:1; v/v) and $CHCl_3$-CH_3OH-conc HCl (267:133:1; v/v/v) (41) versus the sum of each lipid determined in microsomes + particulate fraction after homogenization and washing to test the effects of homogenization and separation into microsomal and particulate fractions on lipid recoveries. Data are given in **Fig. 3**. A surprising finding was the loss of polyphosphoinositides, while recoveries of other phospholipids (mean = 99%) and cholesterol (82%) were satisfactory. Loss of polyphosphoinositides was not reversed by Ca^{2+} and Mg^{2+}chelation using EGTA or EDTA during homogenization at 4°C. The phospholipase C inhibitor 2-nitro-4-carboxyphenyl N,N′-diphenylcarbamate did not reverse the loss of polyphosphoinositides; however, this loss was shown to be due to hydrolysis to Ins(1,4)P_2 and Ins(1,4,5)P_3 (determined by HPLC analysis; data not given). To date, we have not been able to develop a method to preserve polyphosphoinositides. Previous studies showing enrichment of polyphosphoinositides in detergent-resistant membranes (48, 49) usually reported data as percent of recovered lipid species.

Lipid contents in the particulate and microsomal fractions

Table 1 lists the lipid contents of the particulate and microsomal fractions. The major phospholipids (PI, PS, SM, PC, and PE) were almost evenly divided between the particulate and microsomal fractions. Higher contents of cholesterol were found in the particulate fraction (36.9 ± 1.8%) than in the microsomes (24.0 ± 3.8%). One hundred percent of total membrane-associated 5′-N activity and 88.1 ± 1.9% of the total cellular Cav-1 content were recovered in the particulate fraction, indicating that the particulate fraction contained most of the plasma membrane in this tissue. The finding that 5′-N activity was absent in microsomes indicates that this fraction did not contain plasma membrane that was disrupted from the particulate fraction during preparation. The finding of Cav-1 in microsomes was expected, because this protein has been identified in golgi (50).

TABLE 1. Lipid, caveolin-1, and 5′-nucleotidase distribution between the particulate and microsomal fractions

Component	Particulate Fraction	Microsomes
	Lipid Composition	
	mol% of total tissue lipid Pi ± SEM (n)	
PI	2.96 ± 0.14 (7)	2.79 ± 0.22 (7)
PS	4.96 ± 1.03 (5)	3.85 ± 0.77 (5)
SM	10.1 ± 0.5 (5)	8.83 ± 0.70 (5)
PC	20.5 ± 1.1 (5)	19.8 ± 1.3 (5)
PE	13.8 ± 0.4 (5)	12.3 ± 0.7 (5)
Cholesterol	36.9 ± 1.8 (3)	24.0 ± 3.8 (2)
	Protein Distribution	
	% of content in total tissue	
Cav-1	88.1 ± 1.9 (9)	11.0 ± 1.9 (9)
5′-N[a]	67.8 ± 1.5 (6)	0

Cav-1, caveolin-1; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; 5′-N, 5′-nucleotidase.

[a] The remainder was found in the cytosol.

Triton X-100 extractions

Table 2 shows the distribution between extracted lipids [micelles + high-density membranes (HDMs) and LBDMs] and the pellet. As expected, because Triton X-100 has been extensively used to separate detergent-resistant membranes

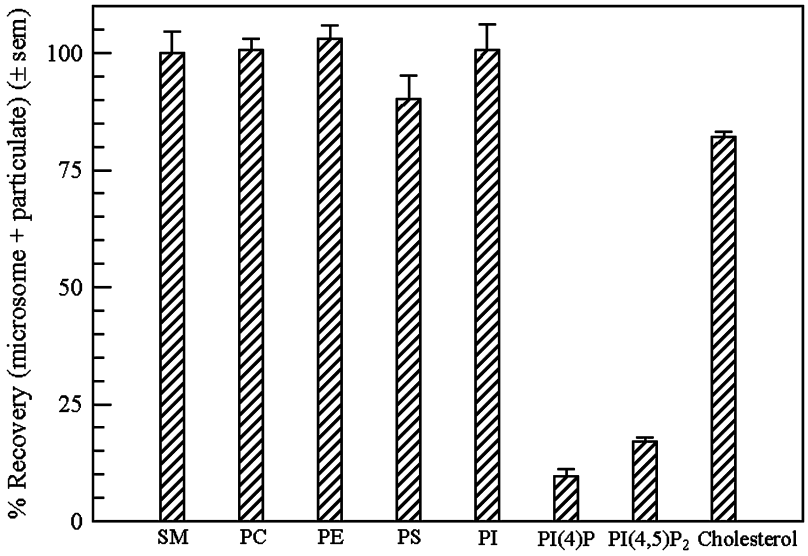

Fig. 3. Recovery of lipids following homogenization of PTSM. Lipids were extracted and quantified as described in Baron, Pring, and Coburn (41). Values are ±SEM (n = 6 except for cholesterol, where n = 3).

TABLE 2. Distribution of particulate fraction lipids and Cav-1 between micelles + HDM, LBDM, and the pellet

	Triton X-100 (0.5%)			OG (35 mM) + NaCl (600 mM)		
	Micelles + HDM[a]	LBDM[b]	Pellet[c]	Micelles + HDM[a]	LBDM[b]	Pellet[c]
	% of total recovered from the particulate fraction ± SEM					
SM	19.9 ± 1.6	33.1 ± 1.6	45.2 ± 3.7	18.3 ± 0.7	56.2 ± 0.8	21.7 ± 1.3
PC	59.2 ± 1.3	31.9 ± 1.4	7.3 ± 2.2	15.3 ± 1.6	58.1 ± 1.6	24.0 ± 1.6
PI	65.0 ± 1.3	25.7 ± 1.1	9.0 ± 2.5	30.0 ± 0.8	56.0 ± 0.7	11.4 ± 0.9
Cholesterol	35.8 ± 0.2	23.9 ± 0.1	38.6 ± 3.0	16.7 ± 0.8	57.7 ± 0.7	21.8 ± 1.3
Cav-1	30.9 ± 1.1	20.6 ± 1.0	48.3 ± 6.0	27.9 ± 4.3	60.6 ± 5.4	2.7 ± 0.1

HDM, high-density membrane; LBDM, low buoyant density membrane; OG, octyl glucoside. n = 6–12 except for Triton X-100: Micelle + HDM and LBDM, where n = 2.

[a] Micelles + HDMs: collected in fractions 1–7 (1.12–1.20 g/ml).

[b] LBDM: collected in fractions 8–12 (1.05–1.11 g/ml).

[c] Pellet: 15,000 *g* × 2 min pellet remaining after detergent extraction.

from solubilized membranes, this detergent failed to extract a high percentage of total particulate fraction SM, cholesterol, and Cav-1 from the pellet. Membranes remaining in the pellet contained 45.2% SM, 7.3% PC, 9.0% PI, and 38.6% cholesterol of these lipids present in the original particulate fraction. The percent composition of membranes remaining in the pellet were: 20 ± 1.7% SM, 6.5 ± 2.0% PC, 10.0 ± 1.9% PE, 1.2 ± 0.3% PI, and 62.2 ± 4.8% cholesterol. The percent composition of each lipid was computed as a percent of the sum of PC + PE + SM + PI + cholesterol. PS was not used in calculating percent composition because this lipid comigrated with OG on TLC and was not measured.

Extracted material in the 15,000 *g* for 2 min supernatant contained a little over half of the total particulate fraction SM, cholesterol, and Cav-1, and over 90% of total particulate fraction PC and PI. **Figure 4** shows results of isopycnic centrifugation separation of Triton X-100-extracted lipid. In evaluating these data, we assigned LBDM as those collected over a range of 1.05–1.11 g/ml, which corresponds to the mean density (1.08 g/ml) of LBDM reported by Brown and Rose (33) obtained using a continuous sucrose gradient. This range of densities also is within the range of LBDM collected using discontinuous sucrose gradient centrifugation in the interface between 5% (1.02 g/ml) and 30% (1.13 g/ml). We cannot distinguish between nonfloating or poorly floating micelles from HDMs collected at densities >1.11 g/ml. In general, except for SM, most of the extracted lipids in the gradient were found in the high-density region. However, 25% to 33% of total particulate fraction phospholipids and 24% of total particulate fraction cholesterol were collected in the low-density region. Cav-1 appears to partially separate into two density groupings centered around 1.13 and 1.085 g/ml. Table 2 gives mean values for the partition between micelles + HDM and LBDM. Because of the buoyant density profile shown in Fig. 4, it is unlikely that the presence of PC at buoyant densities of 1.05–1.11 g/ml was due to diffusion into this region of the gradient. Vigorous sonication had no effect on the lipid density profile (Fig. 4, inset).

OG extraction of the Triton X-100-resistant pellet

Because a major goal in this study was to determine the buoyant densities of membranes that remained in the Triton X-100-resistant pellet, our challenge was to obtain a method of extracting these membranes in a form in which they could be subjected to isopycnic centrifugation. In most published studies in which cultured cells were lysed and homogenized in the presence of Triton X-100, this has not been a problem, because membrane particles were apparently adequately separated and the homogenized lysate could be directly injected into the isopycnic gradient. Using the particulate fraction obtained from PTSM, in which plasma membrane is tightly bound to the extracellular matrix (42), homogenized Triton X-100-resistant pellet could not be injected directly into our gradient. To our knowledge, no method for extraction of membranes from Triton X-100-resistant pellets applicable to tightly bound membranes has been previously described. We therefore explored the use of another detergent, OG, to extract membrane particles. It was found that 35 mM OG in 600 mM NaCl effectively extracted membranes from the Triton X-100-resistant pellet. Isopycnic centrifugation profiles (**Fig. 5A**) show that most of the extracted phospholipids and cholesterol were collected as LBDM and that <10% had densities >1.11 g/ml. Gradient profiles shown in Fig. 5A also suggest that SM, cholesterol, and PC coisolated in LBDM. This is analyzed below. These membranes contained (percent composition) 21.8% SM, 59.6% cholesterol, 10.5% PE, <0.4% PI, and 7.7% PC. In this manuscript, we call these membranes "high SM/cholesterol LBDM". High-SM/cholesterol LBDM accounted for 28.3% of total phospholipid + cholesterol found in the original particulate fraction. Particulate fraction 5′-N activity (15.3 ± 0.7%), and 31.0 ± 1.8% of particulate fraction Cav-1 content were recovered in these membranes. **Table 3** lists the percent composition of high SM/cholesterol LBDM and compares these values with the percent composition of the particulate fraction. SM and cholesterol percent compositions were greater and PC, PE, and PI percent compositions were smaller than values in the original particulate fraction.

The percent compositions of lipids in the Triton X-100-resistant pellet after OG extraction were SM, 15.7 ± 3.5%; cholesterol, 68.6 ± 8.4%; PE, 9.1 ± 4.5%; PI, 3.0 ± 0.7; and PC, 3.6 ± 1.4%. These values are similar to the percent composition of lipids in the high SM/cholesterol

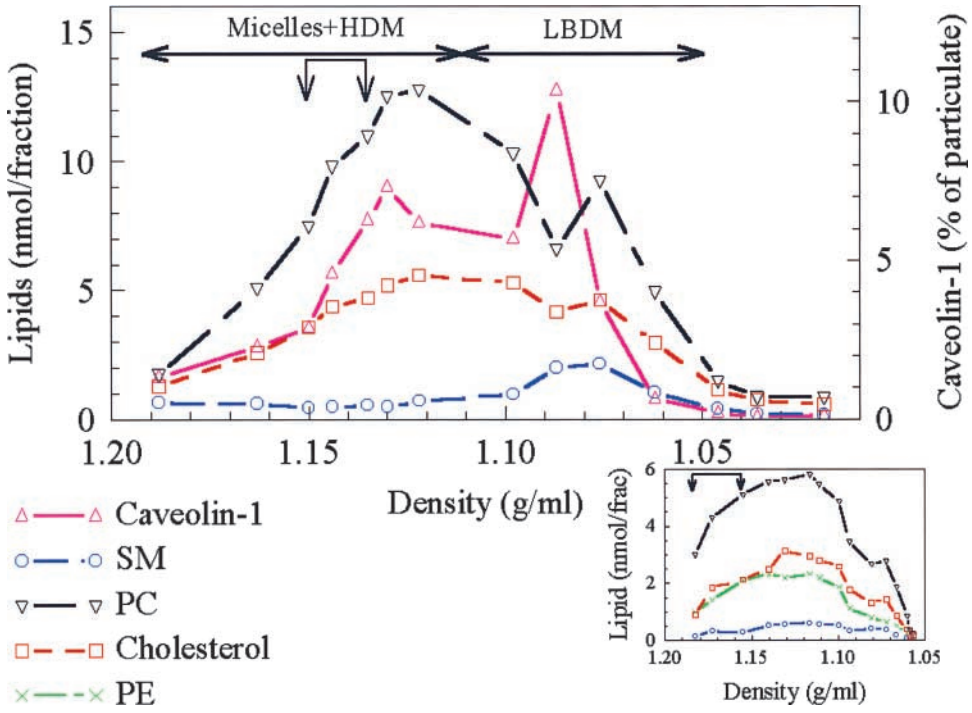

Fig. 4. Triton X-100 extract-isopycnic buoyant density gradients. Low-speed, nonsedimentable material was obtained after extracting the particulate fraction with 0.5% Triton X-100, 150 mM NaCl, 20 mM HEPESNa (pH 7.5), and 0.5 mM $MgCl_2$. As in Fig. 2 inset, vertical connected arrows indicate fractions where the extract was loaded prior to centrifugation. Data are means from two experiments: square, cholesterol; inverted triangle, phosphatidylcholine (PC); circle, sphingomyelin (SM); triangle, Cav-1. The inset shows that in a third independent experiment, there was no effect of vigorous sonication on density profiles obtained using isopycnic centrifugation. In this experiment, the sample was injected at the bottom of the gradient. Fractions were assayed and densities determined as described in the text. Square, cholesterol; X, phosphatidylethanolamine (PE); inverted triangle, PC; circle, SM.

LBDM extracted with OG, except that a small quantity of membranes that contained high PI contents were not extracted. Unextracted lipids accounted for 9.0% of SM, 10.8% of cholesterol, 2.2% of PE, 5.9% of PI, and 2.2% of PC present in the original particulate fraction.

We quantified enrichment in high SM/cholesterol LBDM by computing ratios of SM, cholesterol, and Cav-1 to PC and compared these ratios to those found in the original particulate fraction (Fig. 5B). SM/PC, cholesterol/PC, and Cav-1/PC were 2.83, 7.75, and 14.6, respectively, in high SM/cholesterol LBDM and 0.50, 1.80, and 2.6, respectively, in the original particulate fraction. 5′-N activity was not enriched relative to PC in these membranes. There were marked decreases in PI/PC in collected high SM/cholesterol LBDM compared with PI/PC in the particulate fraction.

Are high SM/cholesterol LDBMs homogenous regarding phospholipids and cholesterol, and are 5′-N and Cav-1 homogeneously distributed in these membranes?

We constructed gaussian fits and determined peak densities for SM, cholesterol, and PC. Gradient profiles were found to be symmetrical and had identical peak densities (SM 1.085 ± 0.001 g/ml and cholesterol 1.086 ± 0.001 g/ml). Because buoyant density is determined by protein-lipid ratios, results indicate that these fractions had homogeneous protein-lipid ratios. We also calculated gaussian fits addressing whether 5′-N and Cav-1 coisolated with SM and cholesterol. The mean density of the 5′-N peak was 1.084 ± 0.004, and this enzyme was shown to track with lipids in the LBDM region (Fig. 5C). Although most of the Cav-1 was associated with the major lipid peak, the Cav-1 gradient profile consistently had higher values on the dense side of the peak and a mean density for a single gaussian fit of 1.091 ± 0.001 g/ml, indicating that some of the Cav-1 is located in a higher density species (the double gaussian fit gave peaks with mean densities of 1.127 ± 0.002 and 1.088 ± 0.001 g/ml).

Properties of lipids extracted by OG from the particulate fraction

As indicated above, OG extracts of the Triton X-100-resistant pellet contained LBDM that had lipid compositions expected in liquid-ordered membranes. However, it was not determined whether this was due to OG selectively extracting liquid-ordered membranes or to the pellet containing only liquid-ordered membranes. To our knowledge, the lipid characteristics of membranes extracted by OG have not been studied previously using isopycnic centrifugation.

OG was efficient in extracting lipid from the particulate fraction; i.e., ~80% of total lipid in the particulate frac-

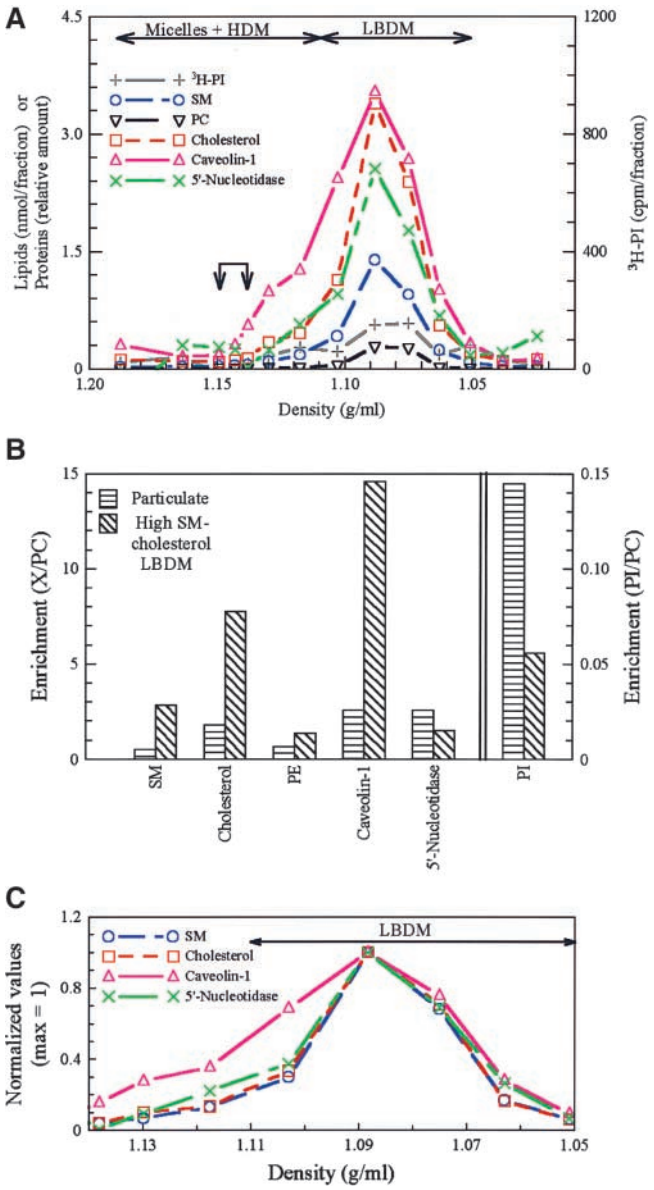

Fig. 5. SM cholesterol-enriched low buoyant density membranes (LBDMs), extent of enrichment, heterogeneity of Cav-1. A: OptiPrep gradient obtained using material obtained with sequential Triton X-100 and then OG extraction, as described in the text. Data are means of two gradients. As in Fig. 4, vertical connected arrows indicate fractions where the extract was loaded prior to centrifugation. X, 5′-nucleotidase (5′-N); triangle, Cav-1; square, cholesterol; inverted triangle, PC; circle, SM; +, [^{3}H]phosphatidylinositol (PI). B: Enrichment of different lipids, Cav-1, 5′-N, and PI in high SM/cholesterol membranes. LBDM components were compared with PC (ratios are mol/mol except for Cav-1 and 5′-N, where the amount in the original particulate fraction was set = 100). C: Recalculation of data in Fig. 5A, where the maximum height of each profile was set: X, 5′-N; triangle, Cav-1; square, cholesterol; circle, SM.

tion was extracted by OG with high salt. Table 2 lists lipid distributions between micelles + HDM, LBDM, and the pellet. Surprisingly, three-fourths of extracted phospholipids and cholesterol were collected as LBDM (**Fig. 6**), and a smaller percentage was recovered in the micelle + HDM fractions of the gradient. The micelle + HDM fractions obtained from the OG supernatant contained 15.3% and 30.0% of total particulate PC and PI, respectively, whereas these fractions obtained using Triton X-100 contained 59.2% and 65.0% of total particulate fraction PC and PI. Table 3 lists the percent composition of OG-generated LBDM that is similar to the percent composition of the original particulate fraction and markedly different from the percent composition of high SM/cholesterol LBDM. Buoyant density profiles were identical in OG-generated LBDM and high SM/cholesterol LBDM (1.05–1.11 g/ml) (Figs. 5, 6). The phospholipid and cholesterol contents of membranes in the OG-resistant pellet were consistent with a mixture of liquid-ordered and liquid-disordered membranes.

We vigorously sonicated OG extracts prior to isopycnic centrifugation to determine whether membrane particles containing different lipids could be separated. This had no effect on the gradient profile, indicating the stability of membrane particles (Fig. 6, inset). The same was true when we increased the length of the gradient or when we collected LBDM extracted using OG and then repeated the extraction and isopycnic centrifugation. In our usual experiments, [OG] was below the CMC in fractions containing LBDM (Fig. 2). We investigated whether the presence OG in fractions containing LBDM altered the gradient profile. Two different approaches were used. First, we refloated LBDM that had been isolated from a normal gradient through a layer of 35 mM OG + 600 mM NaCl; and second, we examined the floatation of normal OG-extracted material, but with maintenance of 30 mM OG throughout the gradient. Both procedures yielded particles of the same buoyant density and composition as found initially (data not shown).

DISCUSSION

High-SM/cholesterol LBDM

As described in Results, a major challenge was to extract Triton X-100-resistant membranes in the tightly assembled homogenized particulate fraction. Using 35 mM OG, we could extract 75% of the particulate fraction lipids that were resistant to Triton X-100 extraction that were then isolated as LBDM. This allowed us to study characteristics of these membranes. Extracted membranes had high SM and cholesterol contents and low PC contents and were nearly entirely collected in low buoyant density fractions. The strongest evidence that these membranes are liquid-ordered is their phospholipid and cholesterol compositions. SM and cholesterol molar concentrations in our high SM/cholesterol LBDM are similar to molar concentrations that evoked phase separation in model membranes (35, 51). That high SM/cholesterol LBDMs are liquid ordered is supported by other evidence cited in the introduction. In addition, in intact cells cholesterol and sphingolipid enhanced the Triton X-100 insolubility of GPI-anchored proteins by promoting the formation of detergent-insoluble-ordered membrane domains (52).

Because Cav-1 and 5′-N are known markers of liquid-ordered membranes, the finding that these proteins coiso-

TABLE 3. Percent composition of particulate fraction and isolated LBDM

	Particulate Fraction	OG-Generated LBDM[b]	High SM/Cholesterol LBDM[c]
		% of PC + PE + PI + SM + cholesterol[a]	
PC	24.3	24.5	7.7
PE	16.4	16.5	10.5
PI	3.5	3.4	<0.4
SM	12.0	11.7	21.8
Cholesterol	43.8	43.8	59.6
Lipid yield (nmol/100 nmol particulate fraction lipid)	100.0	73.2	28.3
Density range (g/ml)	~1.19	1.05–1.11	1.05 –1.11

[a] PS was not used in calculating the percent composition because this lipid comigrated with OG on TLC and was not measured. Errors ranged from 1.2% to 8.9% of the values given.
[b] Obtained by OG extraction of the particulate fraction.
[c] Obtained by OG extraction of the Triton X-100-resistant pellet.

lated with SM and cholesterol in high SM/cholesterol LBDM also supports a conclusion that these LBDMs were liquid-ordered membranes. However, the total 5′-N activity and Cav-1 content recovered in high SM/cholesterol LBDMs were only 15.3% and 31.0%, respectively, of total particulate fraction contents. The major portion of membrane-bound 5′-N (60.8 ± 6.2%) was found in the Triton X-100 extract. A significant portion of Cav-1 (30.9%) was also recovered in the micelle-HDM portion of the gradient obtained using the Triton X-100 extract. These findings suggest that large portions of these proteins were bound in liquid-disordered membranes, or lost from liquid-ordered membranes, but do not negate the significance of coisolation of these proteins with high SM/cholesterol LBDM. We are unable to find percent recovery data for detergent-resistant Cav-1 and 5′-N in previous reports for comparison with our data.

Because of the quantitative measurements made in this study, we determined that high SM/cholesterol LBDM accounts for 28% of total particulate membrane lipid. Because it is likely that these membranes originated in plasma membrane, these data are consistent with a postulate that high SM/cholesterol membranes (53) and raft-like membranes (17, 54) may constitute a large portion of

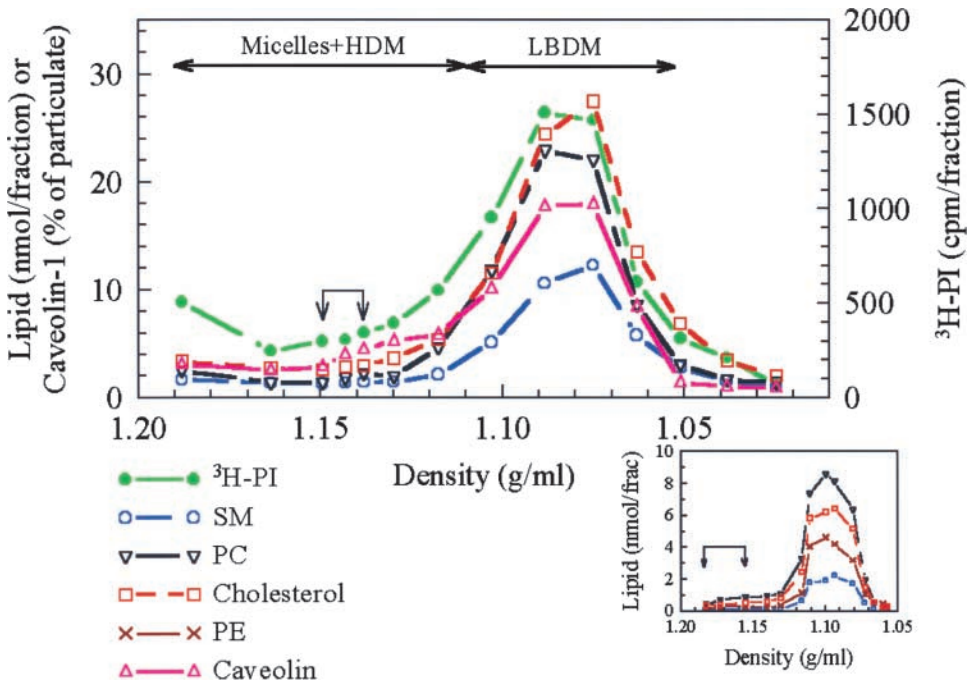

Fig. 6. OG extracts; isopycnic buoyant density gradients. Material in low-speed, nonsedimentable OG extracts, 35 mM OG, 600 mM NaCl, 20 HEPESNa (pH 7.5) were floated in OptiPrep gradients. Data are the average of three gradients. Density values used were obtained from the 8th-order polynomial fit in the Figure. Square, cholesterol; inverted triangle, PC; open circle, SM; closed circle, [^{3}H]PI; triangle, Cav-1. The inset shows isopycnic buoyant density gradients after sonication. The sonication technique is described in the text. Fractions were assayed and densities determined as described in the text. Square, cholesterol; X, PE; inverted triangle, PC; open circle, SM.

the plasma membrane. Because SM is concentrated in the outer leaflet of the plasma membrane (55), the percent of PTSM outer leaflet membrane made up of liquid-ordered membranes may be considerably greater than 30%. Our value for total high SM/cholesterol LBDM in the particulate fraction is probably underestimated, because the 25% of lipids that were not extracted from the Triton X-100-resistant pellet by OG had compositions expected in liquid-ordered membranes. In addition, LBDM extracted with Triton X-100 may have contained some liquid-ordered membranes, as well as high PC membranes.

Most previous investigators who separated liquid-ordered and liquid-disordered membranes using isopycnic centrifugation of cold Triton X-100 extracts of lysed, homogenized cultured cells have compared high and low buoyant density material without performing an analysis of different density fractions (i.e., used material that floated to the interface of 5% and 30% sucrose). The detailed analysis performed here allowed further characterization of high-SM/cholesterol LBDM. Collected LBDM had a narrow density range (Fig. 5A) that was symmetrical, and there were no differences in the SM and cholesterol density profiles. This finding suggests that these membranes were homogenous with respect to protein-lipid ratios. The finding that 5′-N coisolated with SM and cholesterol suggests that this protein was homogenously distributed in these membranes. The Cav-1 density profile consistently extended further into higher buoyant density fractions than did the SM or cholesterol density profile (Fig. 5C). This implies that some caveolae may have higher protein-lipid ratios than does the major portion of isolated LBDM. Recovery of 5′-N and Cav-1 in the Triton X-100 extract implies that these proteins are not necessarily unique markers of liquid-ordered membranes.

An important characteristic of high SM/cholesterol LBDMs is that they restricted PI. This finding is consistent with the accepted concept that lipids with unsaturated acyl chains cannot insert into liquid-ordered domains (1, 21, 23). Because the acyl chains of PI may not be altered during sequential phosphorylations to phosphatidylinositol 4-phosphate [PI(4)P] and to phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2], it is expected that mechanisms for anchoring polyphosphoinositides in the inner leaflet are identical to mechanisms for anchoring PI, and that polyphosphoinositides also would be restricted from these membranes unless they are bound to proteins associated with liquid-ordered membranes. Because in our study both PI(4)P and PI(4,5)P_2 were lost during the homogenization (prior to detergent extraction), we could not test the hypothesis (48, 49) that PI(4,5)P_2 is concentrated in detergent-resistant membranes.

Previous studies have found considerable variation in phospholipid and cholesterol contents of detergent-resistant LBDM (9, 33, 56–60). These results are shown in **Table 4** and compared with data obtained in the current study. In most of these studies, membranes were extracted from lysed cultured cells with cold 0.5% to 1.0% Triton X-100 and LBDM fractions collected. Our SM and cholesterol contents are similar to those reported by Gousset et al. (9); however, the Gousset membranes had a very high PC content. Our cholesterol and PC contents are similar to those reported by Pike et al. (60), but SM was restricted from the Pike membranes. PC contents in previous studies varied between 10.5% and 27% and PI content between 0% and 21%. Although some PC moieties that contain saturated acyl chains are expected to be components of liquid-ordered membranes (21), the majority of PCs in most cell types have unsaturated acyl chains, like PI. As indicated above, both lipids would be expected to be restricted from liquid-ordered membranes. Variation in data in previous studies could be explained if low buoyant density is not unique to liquid-ordered membranes, or if there is large variation in SM, cholesterol, and PC content in liquid-ordered membranes that depends on the presence of proteins or other lipid moieties; i.e., other sphingolipids. The high and variable PC and PI contents reported in some of the previous studies provide the strongest evidence that some detergent-resistant membranes isolated as LBDM also contained some liquid-disordered membranes. The low PC and PI contents in our high SM/cholesterol LBDM argue against major contamination with liquid-disordered membranes.

TABLE 4. Detergent-resistant LBDMs

Literature Reference	Starting Material	SM	Cholesterol	PC	PE	PI
		% composition (enrichment)[a]				
Baron and Blough (56)[b]	Reconstituted erythrocyte influenza virus receptor	38.3 (2.62)	46.4 (1.15)	13.0 (0.67)	1.2 (0.07)	1.1 (0.12)[c]
Brown and Rose (33)[d]	MDCK cells; whole lysate	14.5 (9.24)	42.7 (2.53)	16.3 (0.49)	23.0 (0.55)	3.5 (0.52)
Fridriksson et al. (57)[d]	RBL-2H3 mast cells; whole lysate	16.4 (32.0)	NA	36.0 (0.83)	27.1 (1.35)	20.5 (0.67)
Prinetti et al. (58)[d]	Rat cerebellar granule cells; 5′ × 1,300 *g* supernatant	10.3 (3.10)	29.4 (1.84)	54.1 (0.99)	5.4 (0.24)	0.8 (0.19)
Sawamura et al. (59)[d]	Homogenated PS2 transgenic mouse brains; + sonication	2.9 (NA)	34.6 (NA)	33.6 (NA)	27.4 (NA)	1.5 (NA)
Gousset et al. (9)[d]	Human platelets	21.0 (1.38)	50.9 (6.52)	19.5 (0.54)	8.5 (0.32)	0.0 (0.00)
Pike et al. (60)[d]	KB cells plasma membranes	8.4 (0.64)	60.0 (4.64)	10.5 (0.40)	20.5 (0.58)	0.7 (0.06)
This study[e]	Porcine tracheal smooth muscle particulate fraction	21.8 (1.81)	59.6 (1.36)	7.7 (0.32)	10.5 (0.64)	0.4 (0.12)

NA, not available and/or starting material composition unknown.

[a] Published data were recalculated as percent of total SM + cholesterol + PC + PE + PI; values in parentheses are percent in LBDM divided by percent in the starting material and indicate enrichment (values >1) or exclusion (values <1).

[b] Eluate of 1.5 M NaCl from DEAE chromatography of cold OG extracted membranes.

[c] PI + PS.

[d] Cold Triton X-100 extractions.

[e] Sequential cold Triton X-100-OG extraction.

OG extracts both low buoyant density liquid-ordered and liquid-disordered membranes

Unlike the case for Triton X-100 extractions, OG treatment of the particulate fraction did not generate high PC micelles as a principal mechanism for solubilizing liquid-disordered membranes. Instead, OG generated nonsedimentable membrane particles that floated in low buoyant density fractions of the gradient (Fig. 6). These fractions had phospholipid and cholesterol contents identical to those in the original particulate fraction, indicating that they contained both liquid-ordered and liquid-disordered membranes. We cannot exclude a possibility that liquid-ordered membranes aggregated with liquid-disordered membranes in the presence of OG. However, the finding that density profiles were unchanged using sonicated extracts and after repeated isopycnic centrifugation argues against this. Aggregation of membrane particles could occur as [OG] fell in the gradient (Fig. 2). Evidence that this was not occurring was obtained in the experiments in which [OG] was kept above the CMC in low buoyant density fractions, which did not change the buoyant density profile.

Collection of LBDM in OG extracts allowed us to obtain an estimate of the buoyant densities of liquid-disordered membranes. Because the range of buoyant densities of OG-generated LBDMs containing both liquid-disordered and liquid-ordered membranes (as suggested by their lipid composition) was identical to the range of buoyant densities of the liquid-ordered, high SM/cholesterol LBDMs, we conclude that liquid-disordered membranes have buoyant densities similar to those of liquid-ordered membranes; i.e., 1.05–1.11 g/ml. We could not find a previous estimate in the literature of low buoyant densities of liquid-disordered membranes.

Lipid compositional data indicate that OG extraction is not useful for selectively isolating liquid-ordered from liquid-disordered membranes by buoyant techniques. This conclusion follows from the findings that there was coisolation of high PC/PI and high SM/cholesterol membranes as LBDM, and that the OG-resistant pellet did not exclude either type of membrane. We conclude that OG extraction of apparently noncontaminated high SM/cholesterol LBDM membranes from the Triton X-100-resistant pellet was due to this pellet containing only these membranes. The role of OG in extracting Triton X-100-resistant membranes is that this detergent can apparently disrupt lipid-lipid interactions of liquid-ordered membranes.

OG has been considered to be a weaker detergent than Triton X-100 (61); however, our data indicate OG is able to extract liquid-ordered membranes, whereas Triton X-100 lacks this property. The structure of Triton X-100 shows this detergent molecule contains a large hydrophilic group, which might inhibit its penetration into tightly packed membranes containing proteins occupying a considerable amount of the adjacent hydrophilic space.

The hypothesis that low buoyant density is a unique characteristic of liquid-ordered membranes

The finding that OG-extracted high PC/PI membranes (as well as high SM/cholesterol membranes) were collected as LBDM suggests that low buoyant density may not be a unique characteristic of liquid-ordered membranes. Results (Fig. 4) of Triton X-100 extractions also support this postulate. Although this detergent solubilized high PC membranes as micelles, there was a smaller fraction collected from the extract supernatant as LBDM that had PC, SM, and cholesterol contents not consistent with the presence of pure liquid-ordered membranes.

The postulate that Triton X-100-resistant membranes are liquid ordered

Although results obtained in this study do not support the collection of LBDM fractions to separate liquid-ordered and liquid-disordered membranes, our data do support the postulate that Triton X-100-resistant membranes are liquid-ordered. The phospholipid and cholesterol percent composition of the Triton X-100-resistant pellet is consistent with the presence of liquid-ordered membranes that were not contaminated by high PC membranes.

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