Comparison of phospholipid composition of lipid rafts in rat heart, isolated with two different methods, using detergent or detergent-free high salt solution.

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Raunvísindadeild
Háskóli Íslands
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15 eininga ritgerð sem er hluti af
Baccalaureus Scientiarum gráðu í lífefnafræði

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Háskóli Íslands
Reykjavík, febrúar 2014

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Útdráttur

Frumuhimna heilkjarna lífvera er gerð úr fosfólípíðum, sfingólípíðum, kólesteróli og himnupróteinum. Himnuflekar eru afmörkuð svæði innan frumuhimnunnar sem innihalda hátt hlutfall kólesteróls og sfingólípíða. Þessi svæði hafa fjölbreytilega líffræðilega virkni, m. a. sem vettvangur fyrir boðflutninga og við innfrumun efna með caveolum, sem eru sérstakt form himnufleka. Himnuflekar voru einangraðir úr rottuhjörtum með notkun annaðhvort sterkrar Na_2CO_3 lausnar án sápuefna eða með Triton X-100 sápuefnalausn til að leysa himnur utan fleka og spuna á súkrósastigul þar sem himnuflekar settust í léttari hluta hans. Próteinmæling ásamt þerriblettun á flekapróteinum voru notuð til að sýna viðurvist himnufleka. Lípið voru dregin úr himnuflekum með aðferð Folch og fosfólípíð greind með ^{31}P-NMR. Fosfólípíðasamsetning himnuflekanna var mismunandi eftir einangrunaraðferðum: Toppar fosfatidýletanólamín-plasmalógens og fosfatidýletanólamíns runnu saman og toppur fosfatidýlseríns greindist ekki í himnuflekum einangruðum með sápuefnalausn, en aðgreining þeirra var góð í himnuflekum einangruðum án sápuefna. Hlutfall cardiolípins og fosfatidýlglýceróls, sem eru fosfólípíð úr hvatberum, var hærra í himnuflekum einangruðum með sápuefnalausn, sem bendir til meiri mengunar frá hvatberahimnum í himnuflekum einangruðum með sápuefnalausn. Aðgreining fosfólípíða-toppa var töluvert betri í himnuflekum einangruðum með sápuefnalausri lausn en þegar sápuefni var notað. Ennfremur bentu mótefnalitun og próteinmæling til þess að einangrun himnuflekanna hafi tekist betur þegar þeir voru einangraðir án sápuefna.

Abstract

The plasma membrane of eukaryotes is made of phospholipids, sphingolipids, cholesterol and membrane proteins. Lipid rafts are subdomains of the plasma membrane which are rich in cholesterol and sphingolipids. These domains have diverse biological functions, such as serving as platforms for signal transduction pathways and endocytosis by means of caveolae, a class of lipid rafts. Lipid rafts were isolated from rat hearts using a detergent-free, Na_2CO_3 buffer or 1% Triton X-100 detergent solution to solubilize non-raft membranes, and then samples were centrifuged in a discontinuous sucrose gradient to achieve floating of the lipid rafts into the lighter fractions. Total protein measurements and immunoblots were used to verify the presence of lipid rafts. Lipids were then extracted by the method of Folch and the phospholipids were analyzed by ^{31}P-NMR spectroscopy. It was not possible to distinguish between phosphatidylethanolamine, phosphatidyl-ethanolamine plasmalogen and phosphatidylserine in the sample isolated with the detergent method. The mitochondrial phospholipids cardiolipin and phosphatidylglycerol were found in higher percent amounts in the detergent isolated samples than detergent-free, which could indicate more contamination from mitochondrial membranes in these samples. The phospholipid peak separation was considerably better in the spectra from rafts isolated with the detergent-free method. Furthermore, immunoblots of raft markers and total protein measurements indicated a better isolation of lipid rafts with the detergent-free method.

Hereby I declare that this essay was written by me and has not been used by part or whole to a higher degree.

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Adam Erik Bauer, kt. 091089-2709

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Abbreviations

ER – endoplasmic reticulum

PE- phosphatidylethanolamine

PS – phosphatidylserine

PC – phosphatidylcholine

PI – phosphatidylinositol

GPI – glycosylphosphatidylinositol

SPH – sphingomyelin

PE plas – phosphatidylethanolamine plasmalogen

CL – Cardiolipin

PG – phosphatidylglycerol

GM3 – monosialodihexosylganglioside

DRM – detergent-resistant membrane

EDTA - Ethylenediaminetetraacetic acid

PBS – phosphate buffered saline

MES - 2-morpholinoethanesulfonic acid

GM1 – monosialotetrahexosylganglioside

HRP – horse radish peroxidase

PVDF – polyvinyldifluoride

IgG – Immunoglobulin G

BHT – butylated hydroxytoluene

$Cr(acac)_3$ - Chromium (III) acetylacetonate

Acknowledgment

I am first and foremost grateful to Valgerður Edda Benediktsdóttir for giving me the opportunity to do this research project and for the knowledge and experience I have gained from it. I also want to thank Bjarni Ásgeirsson for all assistance, accessibility of appliances and taking the time to review this paper. Lastly, I want to thank my parents for their tremendous support during my studies.

1 Introduction

1.1 The Plasma Membrane

Biomembranes made from lipids are found both inside and around the Eukaryotic cell. The plasma membrane encloses the cell separating the interior from its environment, and internal membranes separate and characterize different organelles, such as the nucleus and Golgi apparatus (1). Despite their differing functions, all biological membranes have a common general structure consisting of a thin film of a lipid bilayer about 5 nm thick and integral proteins, held together by noncovalent interactions. A bilayer of phospholipids forms in aqueous solutions because of the amphiphatic characteristics of the phospholipid molecule. It has a polar hydrophilic head facing into the hydrophilic aqueous phase and two non-polar aliphatic, hydrophobic tails forming an inner hydrophobic environment. Since these molecules are cylindrical the closed bilayer is their most thermodynamically favorable formation. It is energetically unfavorable for the bilayer to have edges so consequentially it forms a sealed compartment. The bilayer provides the cell with a semi-permeable barrier not permitting the passage of most water-soluble molecules which are instead regulated by membrane proteins. These proteins also control nearly all other functions of the membrane (2).

Roughly about 50% of the mass of the plasma membrane in animal cells comes from lipids and 50% from proteins. Glycolipids and cholesterol consist of about 40% of the total lipids and the rest of phospholipids.

Both lipids and proteins are free to move laterally and rotate in the membrane to a certain extent which is a consequence of the property of lipid bilayers as two-dimensional fluids, which is a critical trait for membranes. The fluidity of biomembranes is dependent on temperature and lipid composition. Short fatty acid chains in the phospholipids give the membrane more fluidity than longer chains at the same temperature, since the short chains are less rigid. The fatty acids in phospholipids can range from 14 to 24 carbon atoms. Unsaturated fatty acid chains with one or more cis-double creating kinks in the fatty acids are harder to pack together than unsaturated fatty acid chains, every double bond giving a more fluid membrane at lower temperatures (1,2).

When phospholipids and cholesterol are mixed the permeability barrier properties of the membrane bilayer is enhanced. The amount of cholesterol in eukaryotic cell membranes can be up to one molecule per phospholipid. The hydroxyl group of the cholesterol positions itself close to the polar head groups of the phospholipids and its rigid hydrophobic steroid rings interact, and partly immobilize, the hydrocarbons closest to the polar head groups. This makes the lipid bilayer less deformable without altering the membrane fluidity, since the cholesterol molecule prevents hydrocarbon chains from crystallizing (2).

Membrane proteins have been described as floating icebergs in a sea of lipids in the fluid mosaic model of Singer and Nicolson, as shown in figure 1,(3) but later studies show that the plasma membrane structure is in fact not random, but contains structures that organize the distribution of proteins in the membrane. These structures are called lipid rafts (4).

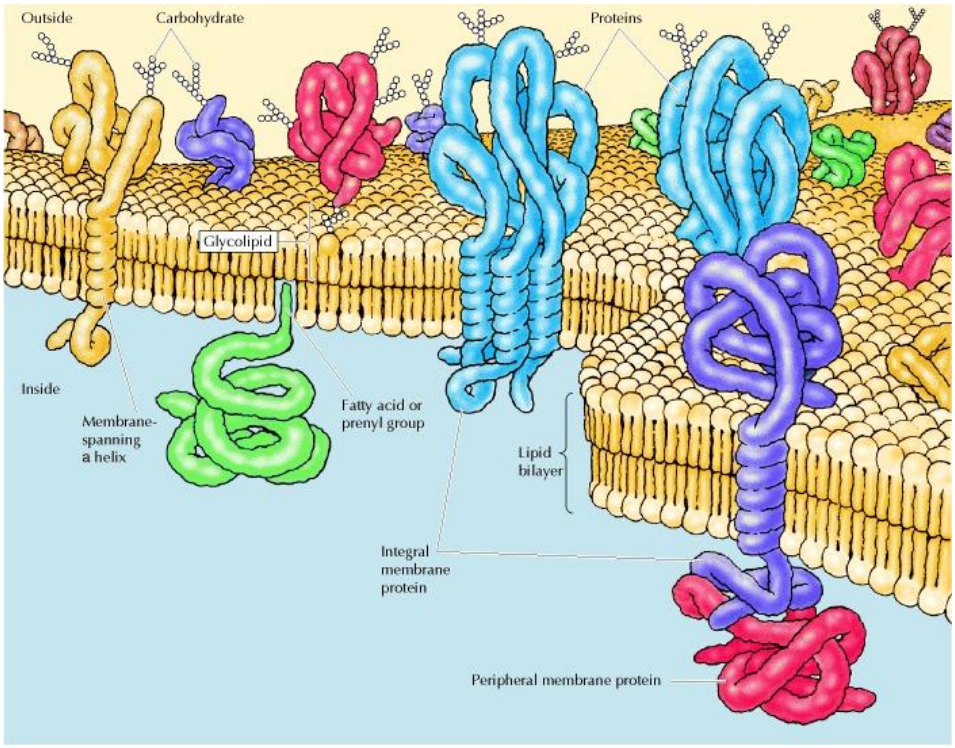

Figure 1 Fluid mosaic model of membrane structure (1).

1.2 Major lipids in Cell Membranes

The main phospholipids in most eukaryotes are phosphoglycerides, which have two long-chain fatty acids linked to glycerol molecule by ester bonds. The third alcohol of the glycerol is esterified to phosphoric acid and the phosphate group is linked to different types of head groups. These phospholipids are synthesized in the endoplasmic reticulum (ER) by special enzymes. The main phosphoglycerides are phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylinositol (PI). PI and PS are usually found in the cytosolic monolayer. Since PS has a net charge of -1, a significant charge difference between the layers is created. When PS appears on the extracellular leaflet of the membrane, it is a marker for phagocytic cells to ingest and degrade the cell. PI is concentrated in the cytosolic monolayer of the cell membranes, where it serves as a precursor for signaling molecules of the phosphatidylinositol messenger pathway. Lipid kinases first activate PI by adding one or more phosphate groups to the inositol ring. Upon agonist activation of the receptor, the lipid is cleaved into inositolphophates and diglycerides, both serving as 2^{nd} messenger molecules. Glycosilated PI is also an important protein anchor for membrane proteins in the extracellular leaflet, called a glycosylphosphatidylinositol (GPI) anchor (2) PC typically accounts for >50% of all cell membrane phospholipids and therefore is the main bilayer-forming lipid (5).

Sphingomyelin (SPH) is built from a sphingosine backbone instead of glycerol. Sphingosine contains one amino group and two hydroxyl groups at one end of the compound. A fatty acid tail is attached to the amino group and a phosphocholine group to the terminal hydroxyl group. This leaves one hydroxyl group free, which in turn can form hydrogen bonds. Glycolipids are made from sphingosine and sugars, but contain no phosphate group and are not detected by ^{31}P NMR. They are only found in the extracellular monolayer of the lipid bilayer (2).

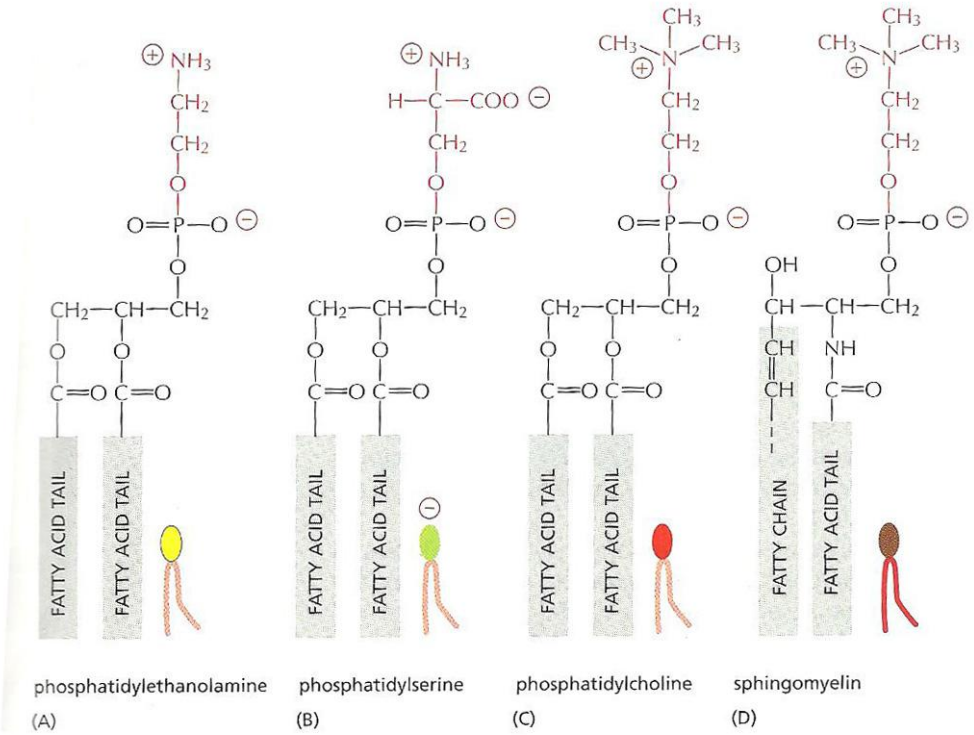

Figure 2 The common types of phospholipids found in cell membranes (2).

Plasmalogens such as phosphatidylethanolamine plasmalogen (PE plas) differs from PE by containing a fatty alcohol attached to a glycerol backbone with an ether linkage. These phospholipids make up 80-90 % of myelin membrane phospholipids and the first reaction in their formation is catalyzed by animal peroxisomes (2).

Cardiolipin (CL) is a phospholipid exclusively found in the mitochondria. It consists of two phosphatidylglycerols (PG) whith four fatty acyl chains. Its main function is regulating various kinds of mitochondrial proteins such as the electron transport complexes, carrier proteins and phosphate kinases (6). CL amounts to about 20% of the phospholipids of the inner mitochondrial membrane (2).

1.3 Lipid rafts

Lipid rafts are sub-domains of the plasma membrane, consensually defined at the 2006 Keystone Symposium of Lipid Rafts and Cell Function as follows: "Lipid rafts are small (10-200 nm), heterogeneous, highly dynamic, sterol-and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions" (7). These domains are split into two types, flat lipid rafts and invaginated, caveolin-rich lipid rafts called caveolae, seen in figure 3 (4).

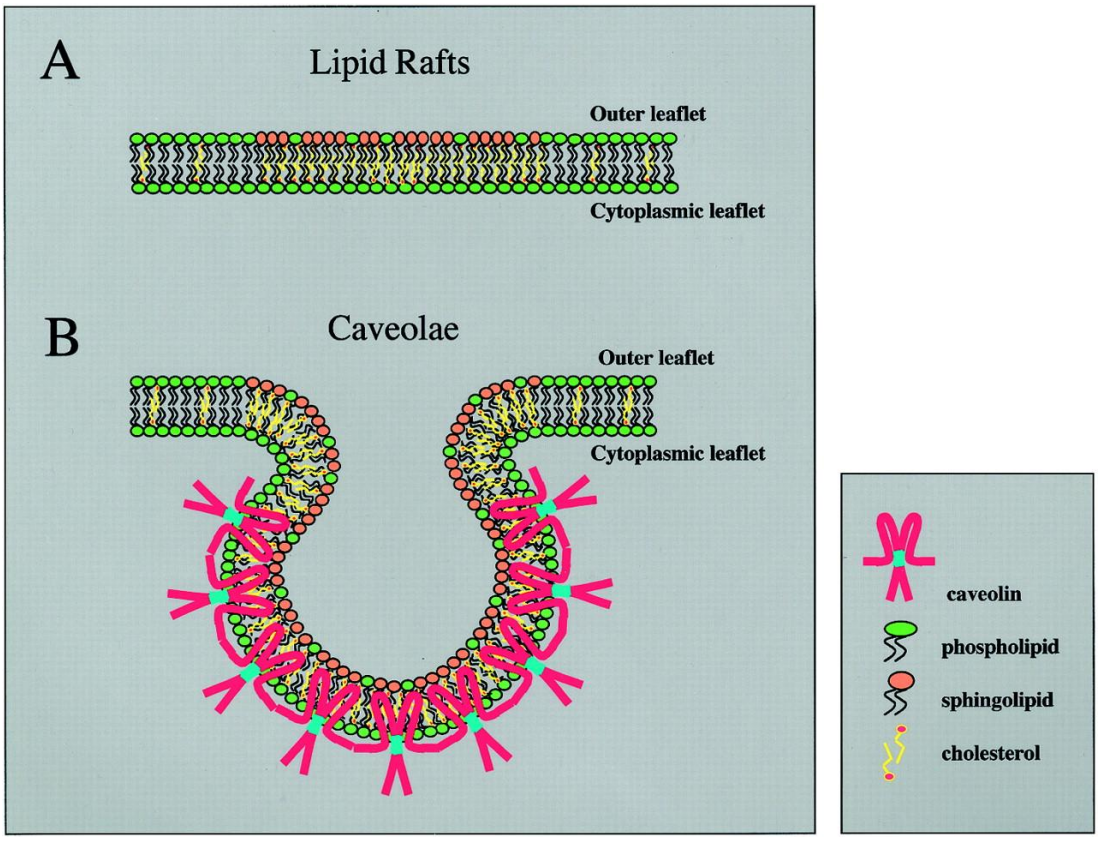

Figure 3 Detailed organization of lipid rafts and caveolae membranes (8).

The lipid composition of lipid rafts differs from that of the plasma membrane. Sphingomyelin levels are 50% higher in lipid rafts than in the plasma membrane and since they contain more saturated fatty acids the rafts are thicker and more rigid than the surrounding plasma membrane. The higher levels of sphingomyelin in contrast to decreased levels of phosphatidylcholine give a similar net amount of choline-containing lipids in the lipid rafts and the plasma membrane (4).

Lipid rafts are heterogenic and have many diverse biological functions, depending on cells and location. The main role of lipid rafts is considered to accumulate proteins involved in certain signal transduction mechanisms. These proteins include receptor tyrosine kinases, GPI-anchored receptors, cell adhesion molecules such as integrins and Notch1, ion channel proteins and others (5). Thus the rafts can create a physical separation of proteins that

would otherwise interact, creating a regulated control mechanism of a signal transduction pathway (4). It has been demonstrated that lipid rafts in nervous system cells have been implicated in neurotrophic factor signaling, cell adhesion and migration, axon guidance and neurite outgrowth, synaptic transmission, neuron-glia interactions and myelin genesis (5).

Caveolae (Figure 3) are distinguished from flat lipid rafts mainly by the invaginated structure, caused by caveolin which inserts hydrophobic loops into the membrane from the cytosolic side (2,4). Due to the high levels of cholesterol in lipid rafts compared to the plasma membrane caveolin has been linked to regulation of cholesterol (4). Like most other lipids, cholesterol is formed in the ER where it binds to caveolin at a 1:1 ratio. Caveolin is involved in the transport of cholesterol to the plasma membrane but the cholesterol is not evenly distributed when it arrives to the plasma membrane, instead most of it adheres to the lipid rafts (9).

Studies have shown that insulin receptors are partially localized in caveolae in intact cells since they can form complexes with caveolin-1 and monosialodihexosylganglioside (GM3) (5). Caveolae are also known to partake in endocytosis and to collect cargo proteins regulated by its lipid composition, rather than its protein composition like other clathrin and COPI or COPII coated vesicles. The caveolae are pinched of the plasma membrane using dynamin and the cargo is delivered to a compartment called caveosome, or to the plasma membrane on the opposite side (2).

Several proteomic analyses have identified proteins from the ER and mitochondria in lipid raft fractions but since both these membranes types are known to be low in cholesterol, further studying is necessary to confirm that lipid rafts truly exist in these membranes. If it is so, proteins and lipids found in the lipid raft fraction cannot be guaranteed to be derived only from the plasma membrane (10).

No published studies, which we know of, have been done to determine the phospholipids in lipid rafts isolated from rat hearts.

1.4 Detergent and Detergent-free Isolation

Lipid rafts were first defined by their low density and insolubility in cold 1% Triton X-100 and were considered to remain intact in the detergent solution while non-raft lipids were dissolved, as seen in figure 4. Because of this they received the acronym DRM (detergent-resistant membrane) (4).

The detergent-free isolations first used by Song *et al*. (11) rely on high pH or hypertonic sodium carbonate solutions which generate membrane fragments of lipid rafts which can be separated by density gradient centrifugation similarly to the detergent treated membranes. Both these methods do alter the lateral order of the membranes to some extent (5).

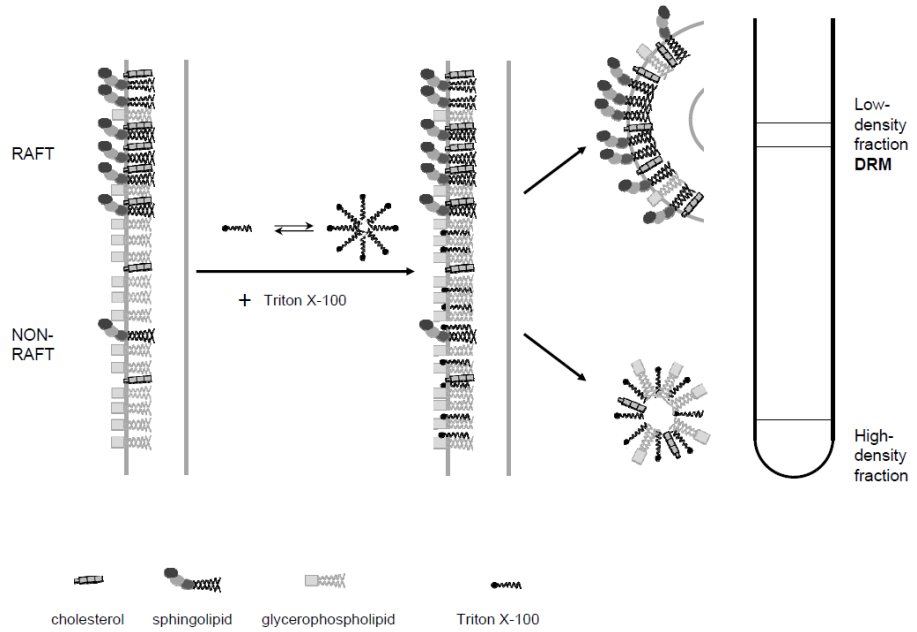

Figure 4 Insolubility of lipid raft components in the presence of Triton X-100 and preparation of DRMs (5).

The use of non-detergent buffers to isolate rafts gives lipid rafts similarities to the DRM isolated with 1% Triton X-100 method, but also some differences. The main difference is that DRM rafts, contained double amount of cholesterol compared to the non-detergent rafts as can be seen in figure 5 (4).

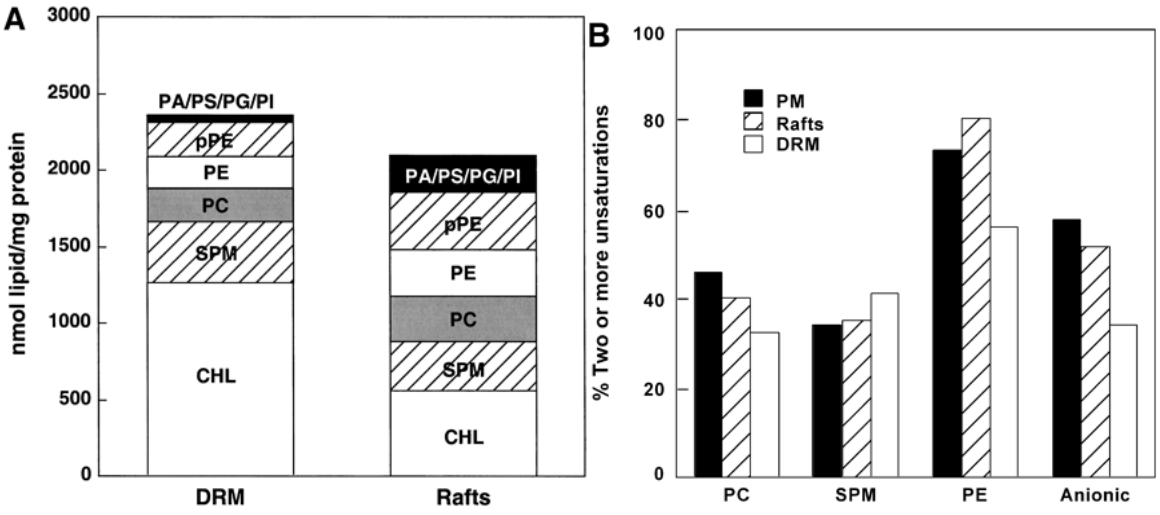

Figure 5 The different composition of phospholipids in DRMs and lipid rafts from KB cells. A) Lipid composition of DRMs and non-detergent rafts. B) Distribution of double bonds in acyl side chains by class of lipids (4). CHL= cholesterol, SPM = sphingomyelin, pPE = phosphatidylethanolamine plasmalogen, PM = plasma membrane.

According to the same study the DRM contain higher concentrations of lipids found primarily in the outer membrane and less of the lipids found in the inner membrane, that is the anionic lipids and PE. From these results it was concluded that using the 1% Triton X-100 method the inner leaflet lipids of the rafts is partly dissolved and therefore not isolated as part of the DRM (4). Other studies show that tiny modifications of various conditions during the isolation procedure, such as pH, the ratio of detergent to sample, mechanical procedures used to promote membrane solubilization and temperature, could affect the results considerably. Maintaining sample temperature at 0-4°C is crucial since temperature fluctuation can promote lipid aggregation (5).

Membranes prepared with the detergent method provide a cleaner starting material for proteomic analysis and has a higher ratio of true positives to false positives with respect to raft proteins (10).

García-Marcos *et al*.(12) have studied the different lipid composition of lipid rafts from rat submandibular glands isolated by these two methods. They found significant differences in protein profiles. Their target receptor $P2X_7$, which is supposed to be located in lipid rafts, was solubilized when Triton X-100 was used but was found in lipid raft fractions when the detergent-free method was used.

1.5 ^{31}P–NMR

Many methods are available for phospholipid analysis such as high-performance liquid chromatography, thin-layer chromatography, soft ionization mass spectrometry, gas chromatography, $^{1}H/^{13}C$ NMR and ^{31}P NMR. All these methods have their advantages and drawbacks, for example the recording of a ^{31}P NMR might take some time but the method and work is considered rather user-friendly. The ^{31}P nucleus has high natural abundance (100%) and a high magnetogyric ratio of nuclei used in NMR which leads to good detectability. These features make it possible for individual phospholipid classes and even fatty acid compositions to be determined if the samples are prepared carefully. The main problem is the relaxation time of phosphorus which is in the range of about 1.5-2.0 seconds, so a wait of nearly 10 seconds must be administered before each pulse. This results in time consuming measurements or poor signal-to-noise ratios.

During sample extraction, inorganic phosphate (P_i) can be extracted to some extent. Also, when samples are stored, some P_i is formed by phospholipid decomposition. If the resonance of P_i becomes high in the sample, some other resonances might be lost. This can be avoided by administering several steps of washing the organic extract with distilled water.

Since the phospholipids are amphipathic, a single solvent cannot be used since that would promote the formation of either vesicles or inverse micelles, depending on the polarity of the solvent. The most common solvent used is mixtures of chloroform, methanol, and a very small quantity of an aqueous, saturated EDTA solution. The most common references used for the spectra are external 85% H_3PO_4 and Bu_3PO_4. By using these methods all major phospholipid classes can be detected in a single recording, and since the slight overlap of SM and PE can occur, simple adjustments such as shifting pH or changing temperature of the measurement can improve the isolation. Different length of the fatty acids of phospholipids result in different chemical shifts (13), which could result in peak overlapping, as can be seen in figure 6.

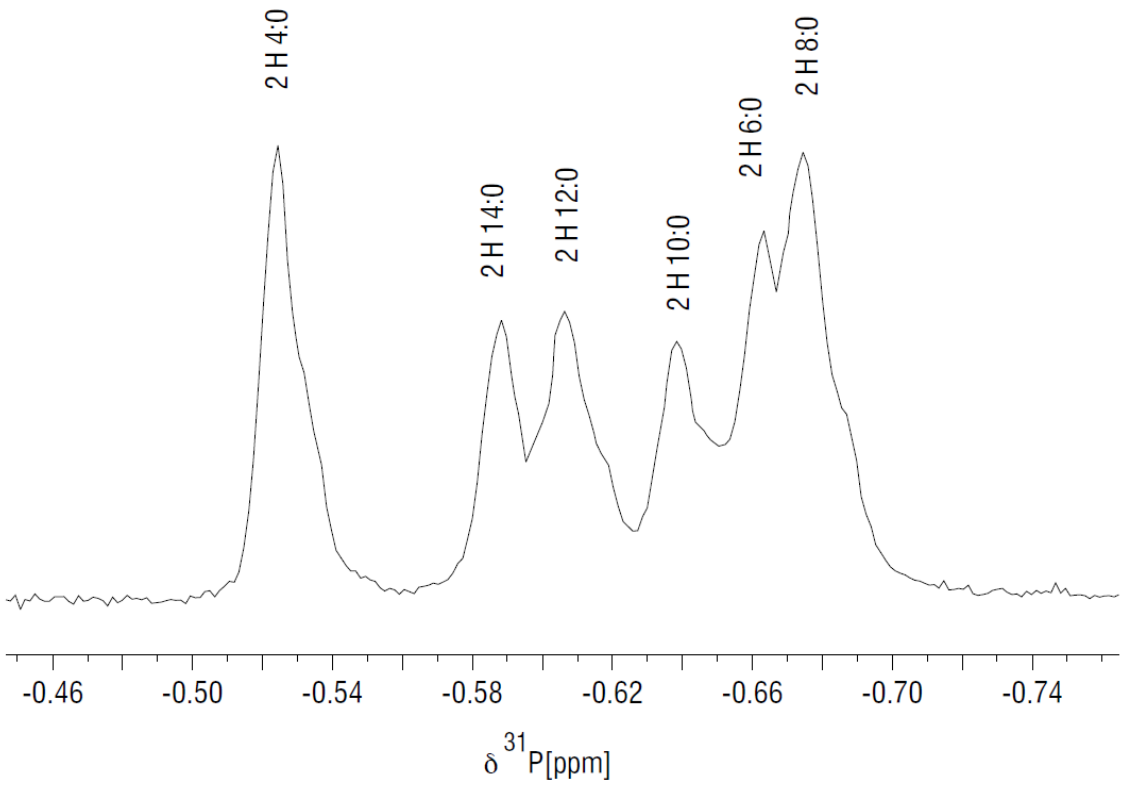

Figure 6 ^{31}P NMR spectrum of phosphatidylcholines with varying fatty acid compositions. The number of carbon atoms is given at the top of each peak, as well as the number of double bonds of each PC species. Spectra referenced to external 85% H_3PO_4 (13).

Comparing ^{31}P NMR chemical shifts of phospholipids is precarious if different solvents, standards or extractions methods were used. Different methods can cause considerable differences in chemical shifts, even if the same sample is used (13). Most scientist isolating lipid rafts use distinct accredited methods so results can be comparable.

2 Materials and methods

2.1 Animals and diets

All animal procedures were approved by the Experimental Animal Committee, Ministry for the Environment in Iceland (License number: 0113-04). Sixteen two months old Sprague-Dawley rats (Tacoma, Lille Skensved, Denmark) were used. All rats were provided fresh food daily and consumed water and food *ad libitum.* The rats were injected with heparin (1000-2000 units) in the abdomen 5 minutes before slaughter and then anesthetized. The heart was excised and rinsed by retrograde perfusion with phosphate

buffered saline (PBS) at 4°C, frozen in liquid nitrogen (N_2) and stored at -80°C until isolation of lipid rafts.

2.2 Materials

All salts and buffer materials were >99% pure from Sigma-Aldrich unless stated otherwise.

2.3 Lipid raft isolation

Lipid rafts were isolated either by using a detergent-free, Na_2CO_3 buffer (25mM 2-morpholinoethanesulfonic acid (MES), 150 mM NaCl, 250 mM Na_2CO_3, pH 11) or detergent solubilization with Triton X-100 solution (25 mM MES, 150 mM NaCl and 1% Triton X-100 (pro analyse, Merck) at pH 7,4) as described by Cavalli *et al* (14). All procedures were performed at ≤ 4°C. One rat heart, which ranged from 0,96g-1,36g, was added to 7 mL of either Na_2CO_3 buffer or the Triton X-100 solution, with 70 µL of protease inhibitor cocktail. The heart was cut into small pieces with scissors and homogenized in three steps as follows: 1) By Polytron (Brinkman Instrument PCU-2-110), setting 5, repeated three times for 20 seconds each, with 30 second cooling intervals with ice-water. 2) With a chilled dounce homogenizer, 10 strokes, done with hand, on ice. 3) By sonication (Fisher 60 Dismemberator Sonicator), amplitude 60, repeated three times for 20 seconds with 30 second cooling intervals in ice-water. The homogenate in Na_2CO_3 buffer was centrifuged at 1000g for 10 minutes at 4°C (2530 rpm, rotor 1619, Hettich Universal 320 R centrifuge). Homogenates in Triton X100 were centrifuged at 425g for 5 minutes at 4°C (1590 rpm, rotor 1619, Hettich Universal 320 R centrifuge) as demonstrated by Hattersley *et al* (15). 6 mL of supernatant were collected and distributed to three 12 mL centrifuge tubes (Beckmann 344059, Ultra-Clear ultracentrifuge tubes) along with 2 mL of 80% sucrose solution (w/v),containing a buffer with 25 mM Tris, 2 mM EGTA and 150 mM NaCl at pH 7.4, and mixed thoroughly making a 40% sucrose solution. Four mL of 30% sucrose solution were layered slowly on top of the supernatant, followed by addition of 4 mL 5% sucrose solution layered on the top, establishing a discontinuous sucrose gradient. The sucrose gradient was centrifuged at 38,000 rpm (280,000g) for 18.5 hours in a Beckmann SW41.Ti rotor, at 4°C. Twelve 1 mL fractions were collected and numbered from the top. Lipid rafts were assumed to be in fractions 4-6. All samples except the small amount used for protein measurements and dot blots were frozen in liquid nitrogen and stored at -85°C until further use for lipid extraction.

2.4 Quantitative determination of proteins

The total protein concentration in the 12 sucrose gradient fractions was measured in triplicate by the methods described by Zaman & Verwilghen (16). Bovine serum albumin standard from Sigma-Aldrich was used. The fractions from each rat heart with the same number were combined and diluted, if needed, to fit within the standard plot. 15 µL portions of the samples, blank or standard and by 165 µL of Coomassie brilliant blue dye (0.05% Coomassie G-250, 0.5 M perchloric acid) were placed in a 96 well plate. The solution was shaken, allowed to stabilize for 10 minutes and absorbance was measured at 650 nm.

2.5 Immunoblots

The sample volume for immunoblots (dot blots) was 1-30 µL, calculated from protein concentration in each fraction. Each dot blot sample was made to contain an equal protein amount of about 0.3 µg. Two heart samples prepared at the same time were blotted in parallel. All 12 fractions were blotted in one of the samples but only fractions 1-6 of the other sample. Fractions that did not contain any measureable amount of protein were omitted from the dot blots.

Primary antibodies, produced in rabbits, to the lipid raft proteins Caveolin-3 (Thermo Scientific, PA1-066) and Flotilin-1 (Sigma-Aldrich F1180), and antibody to the raft lipid monosialotetrahexosylganglioside (GM1), (horse radish peroxidase (HRP) conjugated cholera toxin B, Sigma-Aldrich C-3741) were used for the dot blots to indicate presence of lipid rafts. Whatman polyvinyldifluoride (PVDF) membrane sheets were cut to appropriate size strips and incubated in methanol for 1 minute, followed by incubation in a buffer containing 39 mM glycine, 48 mM Tris-HCl, 0,047% SDS, 20% methanol, at pH 8.3 (transfer buffer) for 15 minutes. The membranes were placed on blotting paper, which was soaked in the transfer buffer to prevent drying. Samples were applied to the membrane as small dots with 1 cm between them. Each drop of the same sample was allowed to absorb completely into the membrane before the next one was administered on the same dot. After addition of samples, membranes were blocked for three hours in 5% non-fat milk in 20 mL of block buffer (12.5 mM HEPES, 70 mM NaCl, 1% Tween 20, at pH 7.1) at 4°C on a platform shaker. Membranes were washed 2 times with 20x diluted block buffer (blot buffer) after blocking. The three different primary antibody solutions were prepared in the blot buffer containing 1.5% non-fat milk and diluted as follows: cholera toxin B, 1:5000, anti-caveoilin-3, 1:7500 and anti-flotillin-1, 1:7500. Membranes were incubated in these solutions over-night at 4°C on a platform shaker. The morning after, the strips were washed with the blot buffer: 2x short washes, 1x 15 minutes on a platform shaker and finally 3x5 minute washes on a platform shaker. A HRP conjugated secondary antibody (anti-rabbit IgG, Santa Cruz sc-2004) was diluted 1:5000 in 1,5% non-fat milk in blot buffer and the washed caveolin-3 and flotillin-1 membranes were added and incubated for 2 hours. After incubation, the membranes were washed as before.

Development of the membranes was done with carbazole staining. 10 mg 3-amino-9'-ethylcarbazole was dissolved in 5 mL dimethylformamide and mixed with 45 mL 50 mM acetic acid at pH 5.0 and 25 µL hydrogen peroxide. The solution was poured over the membranes and incubated for 10-60 minutes, or until sufficient coloring was observed. The membranes were then scanned to achieve digital pictures.

2.6 Lipid extraction

The lipid extraction was performed on fractions 3-6, which were assumed to contain lipid rafts, as described by Folch (17). Methanol and chloroform (Sigma 98-99%) with 5 mg Butylated hydroxytoluene (BHT)/100 mL was used to prevent oxidation of lipids. Each fraction was split in to three tubes containing 1 mL. If volume was less than 1 mL in any tube 0,73% NaCl solution was added up to 1 mL. The samples were dropped slowly into 1.25 mL of methanol. 2.5 mL of chloroform were added, mixed well, and shaken periodically for 1 hour. Then the samples were spun at 3000 rpm (1000g) for 10 minutes so that a thin white layer separated the upper and lower phase. The upper phase was carefully

withdrawn and put into a clean tube and the lower phase was also withdrawn and combined with the upper phase leaving only the white protein layer which was discarded. 1.25 mL methanol and 1.0 mL 0.73% NaCl solution were added giving a final mixture of chloroform/methanol/water 1:1:0.8 W/W, respectively. Samples were centrifuged again at same settings and the lower phase was collected. Samples were stored at -20°C until ready for ^{31}P-NMR.

2.7 ^{31}P-NMR measurement

Preparation of samples for ^{31}P-NMR was performed as described by K.R. Metz and L.K. Dunphy (18). After drying the sample with N_2 gas stream, 1.0 mL of tri-n-butylphosphate (about 0.3 mg/mL) in $CDCl_3$ was added as a standard and then 0.504 mL of methanol - 0.2 M Cs-EDTA solution (0.2 M EDTA, 0.46 M CsOH at pH 6.0) 1:4 (v/v) was added to the tube followed by addition of 0.2 mL $CHCl_3$ with 87 mM $Cr(acac)_3$. The final ratios of solvents were chloroform/methanol/water 100:36:9 and $Cr(acac)_3$ concentration was about 10 mM. The sample was spun for 10 minutes at 2500 rpm. The colorless aqueous upper phase was discarded and the dark red chloroform-methanol lower phase was put into a NMR tube. ^{31}P-NMR measurements were done at 307 K and also 298 K and 312 K in NMR sample 3.

3 Results

3.1 Preparation

The average sample mass of the hearts isolated with the detergent-free method (usually 1 heart) was 1.10 g ± 0.10 (average ± SD, n=8) and the mass of the samples isolated with 1% Triton X-100 was 1.20 ± 0.11 (average ± SD, n= 4)

3.2 Total protein in sucrose gradient fractions.

Figure 7A shows a representative sample of total protein concentration in 12 sucrose gradient fractions isolated with the detergent-free method, numbered from the top. The protein concentration rises after fraction 7 in a parabolic-like way. A peak in protein concentration in fraction 3-6 can be seen in Figure 7B, which could indicate the isolation of lipid rafts in those fractions, as was expected.

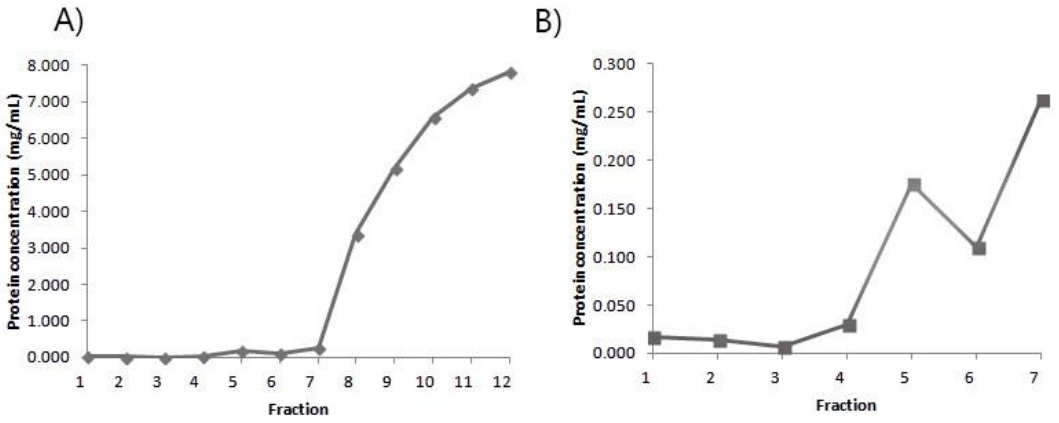

Figure 7 Protein concentration in each sucrose gradient fraction from a sample isolated with the detergent-free method (see Materials and Methods). A) Protein concentration in fractions 1-12, B) Protein concentration in fractions 1-6 of the same sample. 15 µL sample of each fraction was mixed with 165 µL Coomassie brilliant blue dye and absorbance was measured at 650 nm

Figure 8A shows a representative sample of total protein concentration in 12 sucrose gradient fractions isolated with the Triton X-100 method, numbered from the top. The bulk of protein is in the lower fractions 6-12 similarly to the results in figure 7A. Figure 8B shows no significant protein concentration in fractions 1-4, but a rise in 5-6.

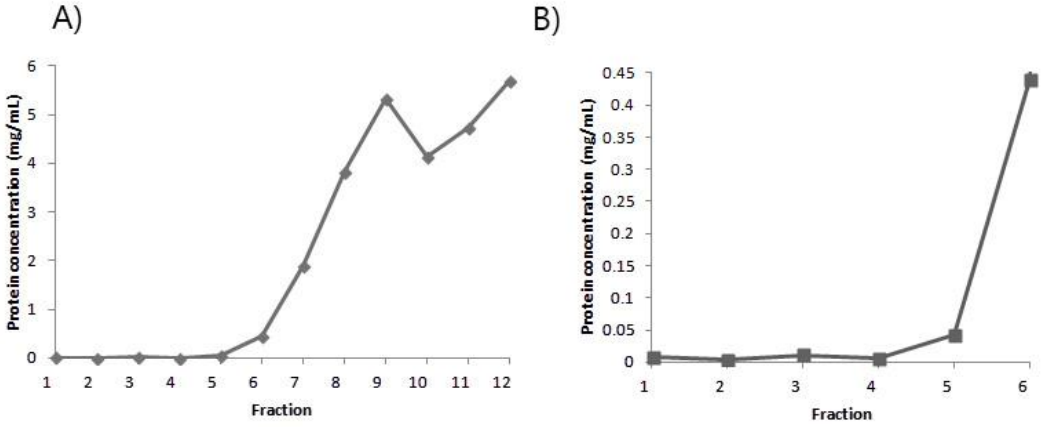

Figure 8 Protein concentration in each sucrose gradient fraction from a sample isolated with the Triton X-100 method (see Materials and Methods). A) Protein concentration in fractions 1-12. B) Protein concentration in fractions 1-6 in the same sample. 15 µL sample of each fraction was mixed with 165 µL Coomassie brilliant blue dye and absorbance was measured at 650 nm.

3.3 Immunoblots (dot blots)

Dot-blots were performed on all samples with antibodies to Flotillin-1, Caveolin-3 and GM1. The presence of these lipid raft markers in fractions indicates where lipid rafts are most likely to be found and the intensity of the color represents their concentration. Figure 9 shows representative dot blots of the sucrose gradient fractions 1-12 from a sample isolated with the detergent-free method.

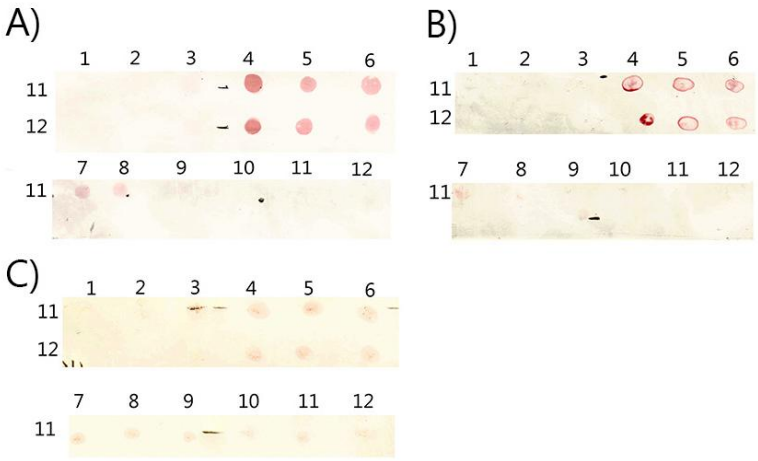

Figure 9 Presence of Caveolin-3(A), Flotillin-1(B), and GM1(C) in sucrose gradient fractions from two samples isolated with the detergent-free method. All dots contained the same amount of protein, about 0.3 µg (see Materials and Methods).

When the detergent-free method is used, the highest concentration of the lipid raft markers appears in sucrose gradient fractions 4-6 and relatively little or no indication in high-density fractions 7-12, which was expected. For GM1 some staining is seen also in the high-density fractions. Dot blots for other samples prepared with the same method showed similar results.

Figure 10 shows dot blots of sucrose gradient fractions 1-12 for samples isolated with the method using the detergent Triton X-100.

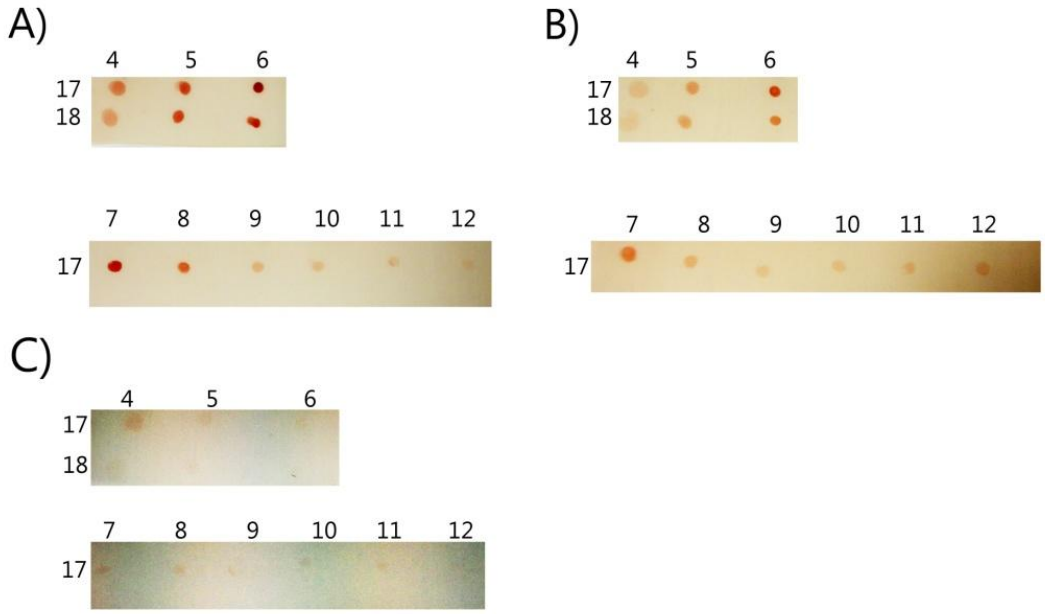

Figure 10 Presence of Caveolin-3(A), Flotillin-1(B), and GM1(C) in sucrose gradient fractions from samples isolated with the Triton X-100 method. All dots contained the same amount of protein, about 0.3 μg (see Materials and Methods).

The lipid raft markers in the Triton X-100 preparation were seen in all the sucrose gradient fractions. The blots were darker in fractions 5-7 than in the high-density fractions 8-12 for Caveolin-3 and Flotillin-1 which indicates that fractions 5-7 contain more of the lipid rafts. This does not apply for GM1 since it seems that the intensity of the dots were similar in all fractions.

3.4 Phospholipid composition with ^{31}P-NMR

Lipid rafts were assumed to be in fractions 3-6 and each ^{31}P NMR sample, except sample 2, contained lipid rafts from four hearts. Position of each phospholipid was found by using chemical shift measurements with comparison with the positions published by Metz and Dunphy (18). NMR sample 1 (figure 11) and sample 2 (figure 12), were isolated by the detergent-free, Na_2CO_3 buffer. Both measurements were performed at 307 K.

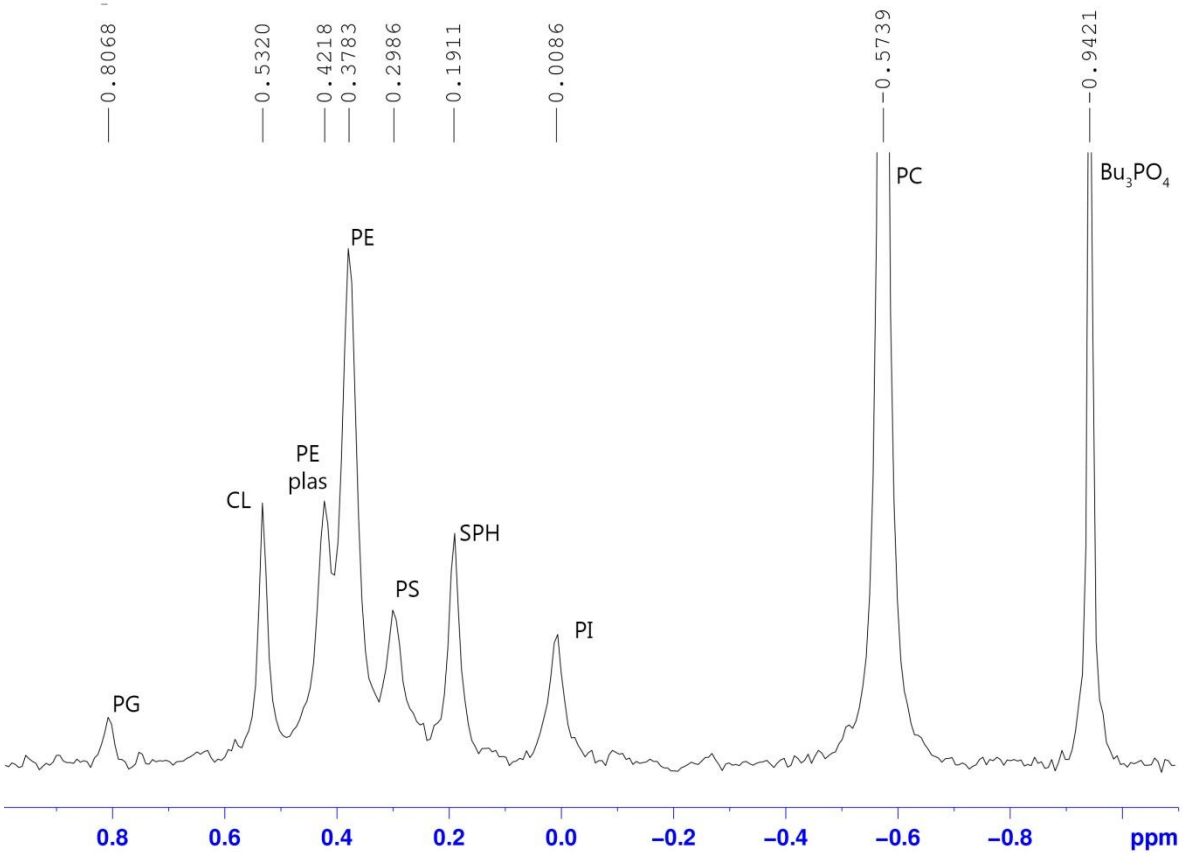

Figure 11 ^{31}P-NMR spectra (161.98 MHz), acquired at 307 K, of phospholipids from NMR sample 1, isolated by using a detergent-free, Na_2CO_3 buffer. One mL of 0.356 mg Bu_3PO_4 /mL standard was used. The positions of each phospholipid and corresponding integration of the peak can be seen in table1.

Table 1 Results from ^{31}P-NMR spectra shown in figure 11.

Peak	Chemical shift (ppm)	Integration
Bu_3PO_4	-0.9421	100.000
PC	-0.5739	319.368
PI	0.0068	41.913
SPH	0.1911	56.844
PS	0.2986	59.135
PE	0.3783	164.009
PE plas	0.4218	72.368
CL	0.5320	60.088
PG	0.8068	9.800

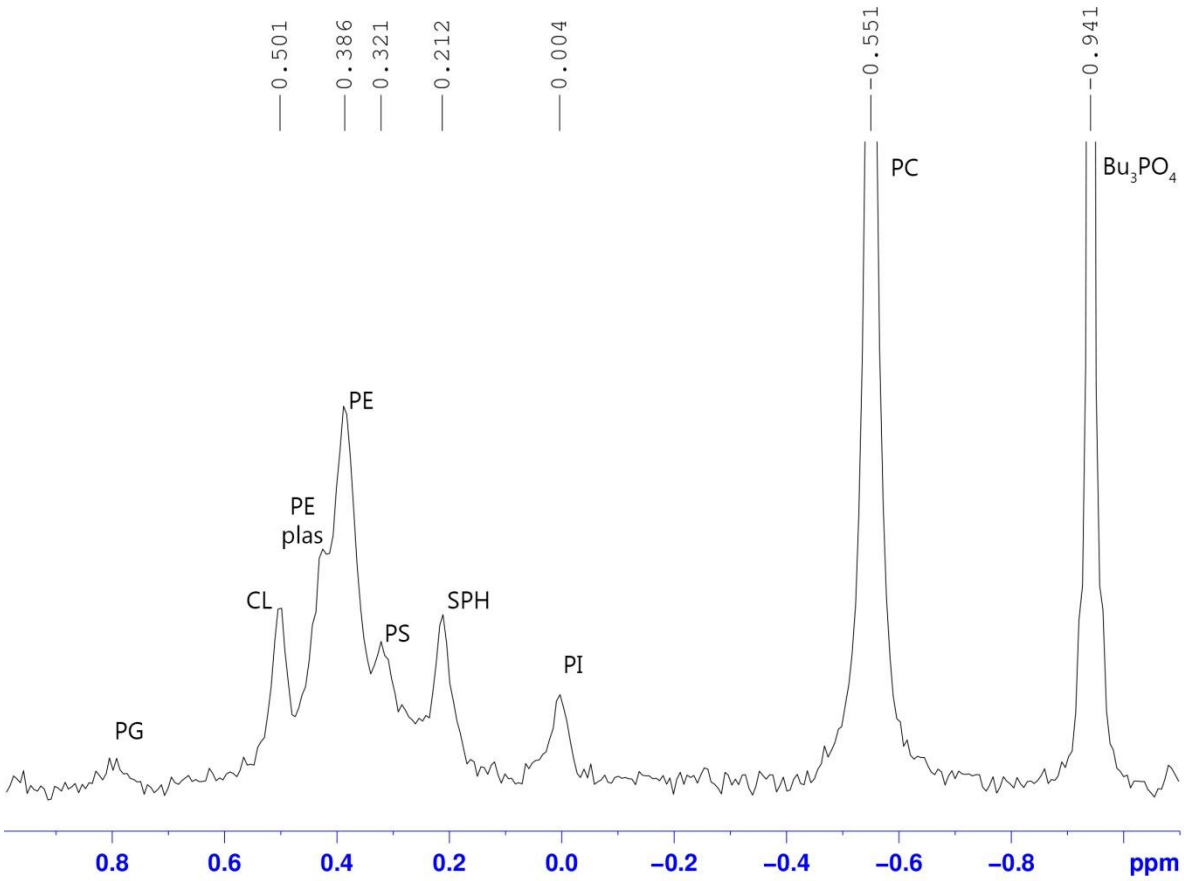

Figure 12 ^{31}P-NMR spectra (161.98 MHz), acquired at 307 K, of phospholipids from NMR sample 2, isolated by using a detergent-free, Na_2CO_3 buffer. One mL of 0.356 mg Bu_3PO_4 /mL standard was used. The positions of each phospholipid and corresponding integration of the peak can be seen in table 2.

Table 2 Results from ^{31}P-NMR spectra shown in figure 12.

Peak	Chemical shift (ppm)	Integration
Bu_3PO_4	-0.941	100.000
PC	-0.551	116.875
PI	0.004	12.452
SPH	0.212	24.421
PS	0.321	25.112
PE	0.386	51.869
PE plas	-	30.876
CL	0.501	20.831
PG	0.80	3.336

NMR sample 3 (Figures 13-15) was isolated by the Triton X-100 method. The sample was measured at three different temperatures, 298 K, 307 K and 312K to enable estimation of mol % of PS and to give SPH and PE + PE plas peak separation.

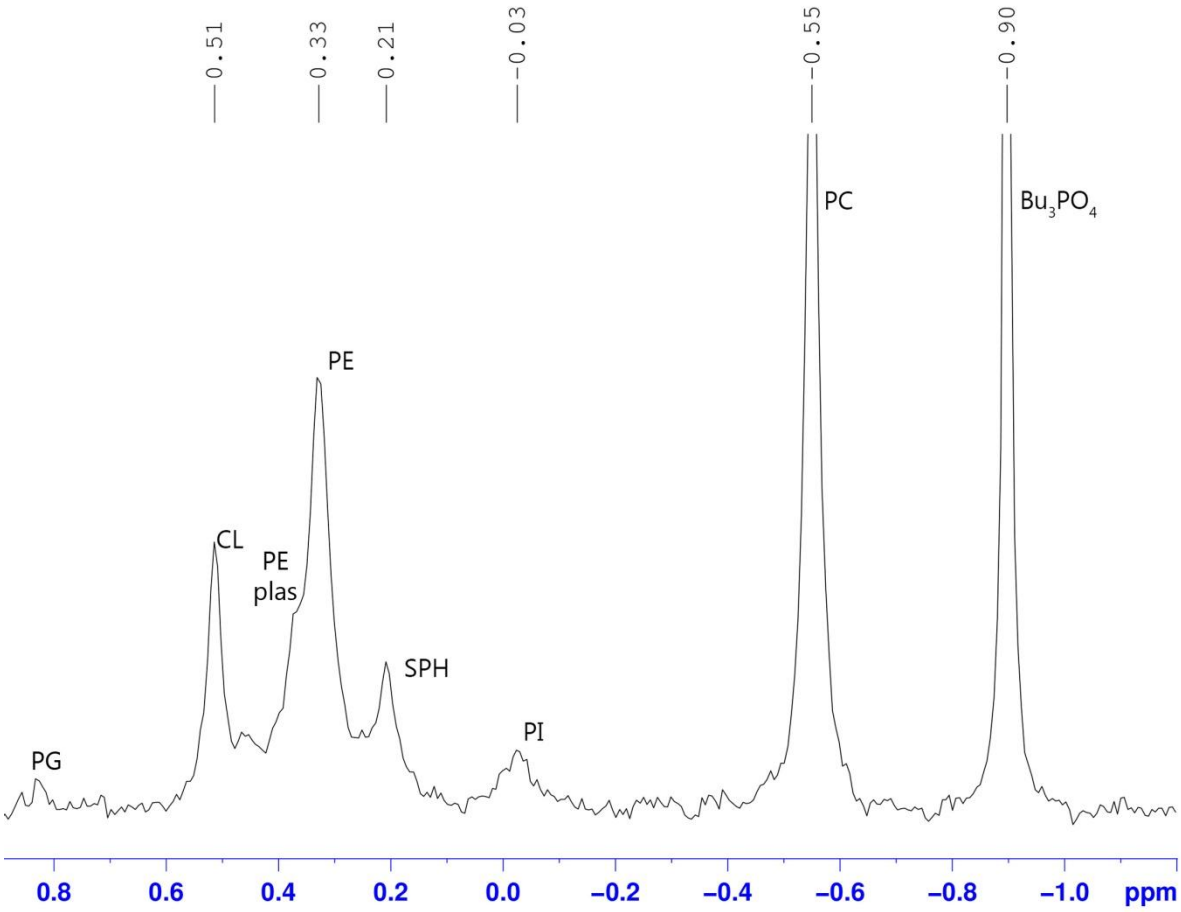

Figure 13 31P-NMR spectra (161.98 MHz), acquired at 298 K, of phospholipids from NMR sample 3, isolated by the detergent solubilization with Triton X-100 solution. One mL of 0.356 mg Bu_3PO_4 /mL standard was used. The positions of each phospholipid and corresponding integration of the peak can be seen in table 3.

Table 3 Results from 31P-NMR spectra shown in figure 13.

Peak	Chemical shift (ppm)	Integration
Bu_3PO_4	-0.90	100.000
PC	-0.55	117.375
PI	-0.03	17.731
SPH	0.21	35.579
PE+PE plas	0.33	101.611
CL	0.51	35.721
PG	0.83	4.232

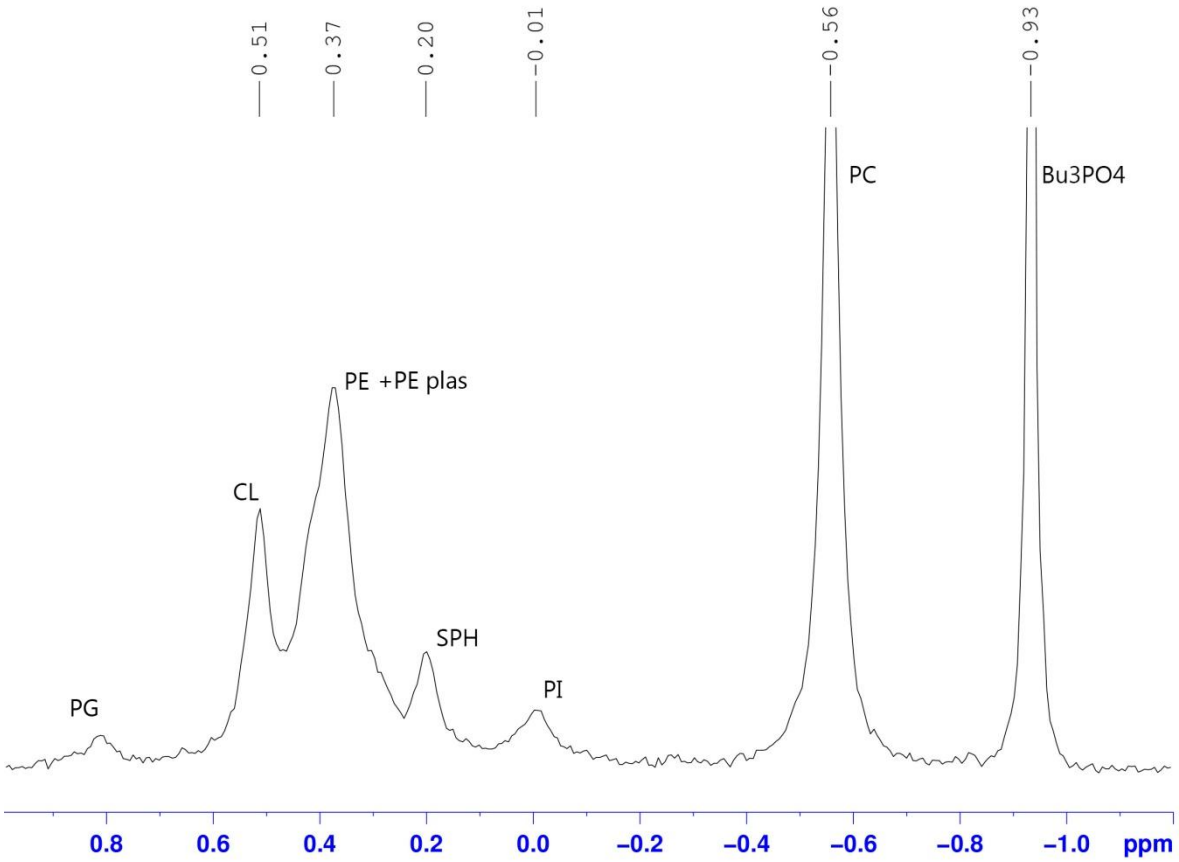

Figure 14 ^{31}P-NMR spectra (161.98 MHz), acquired at 307 K, of phospholipids from NMR sample 3, isolated by the detergent solubilization with Triton X-100 solution. One mL of 0.356 mg Bu_3PO_4 /mL standard was used. The positions of of each phospholipid and corresponding integration of the peak can be seen in table 4.

Table 4 Results from ^{31}P-NMR spectra shown in figure 14.

Peak	Chemical shift (ppm)	Integration
Bu_3PO_4	-0.93	100.000
PC	-0.56	120.612
PI	-0.01	15.605
SPH	0.20	22.758
PE+PE plas	0.37	117.456
CL	0.51	49.581
PG	0.82	6.896

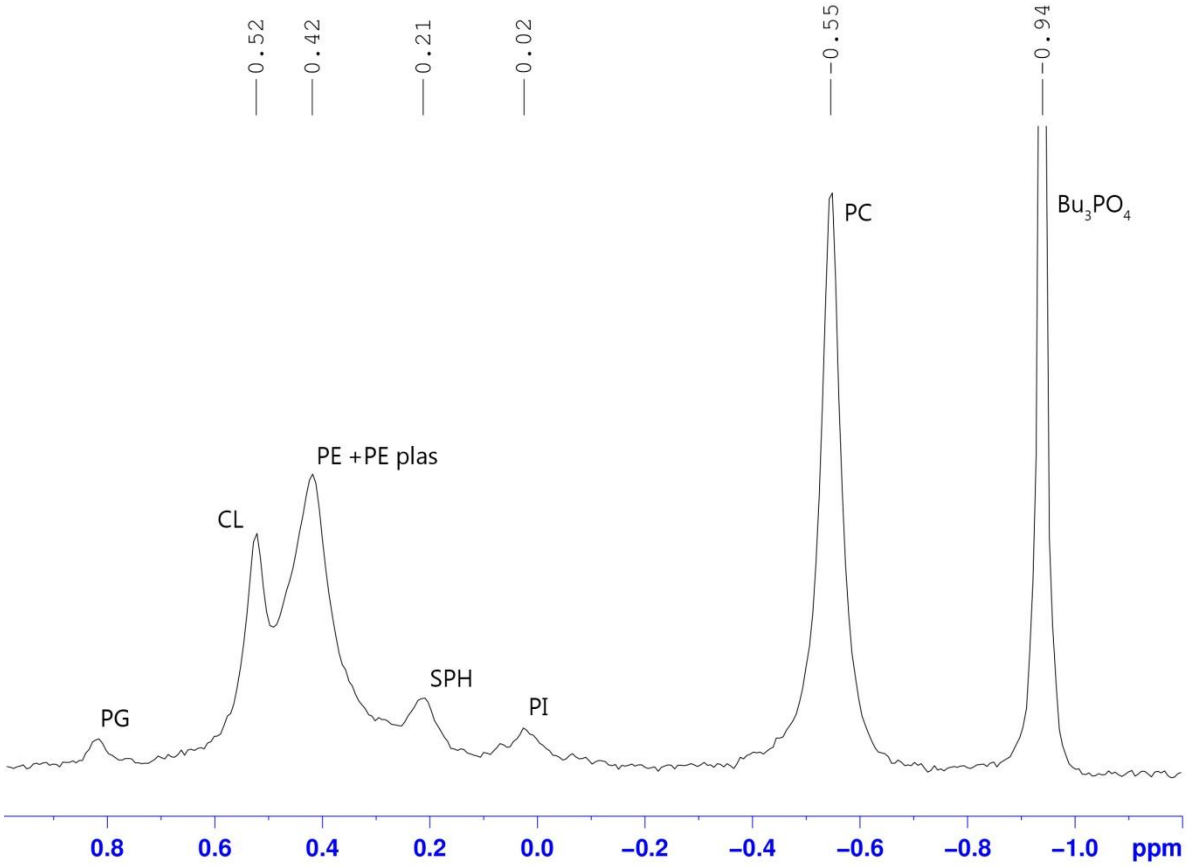

Figure 15 ^{31}P-NMR spectra (161.98 MHz), acquired at 312 K, of phospholipids from NMR sample 3, isolated by the detergent solubilization with Triton X-100 solution. One mL of 0.356 mg Bu_3PO_4 /mL standard was used. The positions of of each phospholipid and corresponding integration of the peak can be seen in table 5.

Table 5 Results from ^{31}P-NMR spectra shown in figure 15.

Peak	Chemical shift (ppm)	Integration
Bu_3PO_4	-0.94	100.000
PC	-0.55	119.926
PI	-0.02	13.498
SPH	0.21	22.913
PE+PE plas	0.42	111.533
CL	0.52	52.966
PG	0.82	7.617

3.5 Calculated mole percentage of phospholipids from ^{31}P-NMR results

The known concentration of phosphorus in the standard and its integration value was used to calculate the moles of each phospholipid measured in the ^{31}P-NMR spectra. One mL of 0.356 mg Bu_3PO_4 /mL standard was used. Since CL is the only phospholipid molecule that contains two P atoms its integration value is halved in all calculations of mol%.

Tables 6-7 show the calculated mol% for samples 1 and 2 which were prepared with the detergent-free Na_2CO_3 buffer. Total protein in sample 1 was 3.88 mg and total protein in sample 2 was 2.60 mg.

Table 6 Calculated mole percentage of each phospholipid from ^{31}P-NMR sample 1, acquired at 307 K, isolated by using a detergent-free, Na_2CO_3 buffer.

Peak	Integration value	µmole P	Mole percentage (%)
PC	319.37	4.25	42.39
PI	41.91	0.557	5.56
SPH	56.84	0.756	7.54
PS	59.14	0.786	7.85
PE	164.01	2.18	21.77
PE plas	72.37	0.962	9.60
Cl	60.09	0.399	3.99
PG	9.80	0.130	1.30
	Total :	**10.02**	

Table 7 Calculated mole percentage of each phospholipid from ^{31}P-NMR sample 2, acquired at 307 K, isolated by using a detergent-free, Na_2CO_3 buffer

Peak	Integration value	µmole P	Mole percentage (%)
PC	116.88	1.55	42.44
PI	12.45	0.166	4.52
SPH	24.42	0.325	8.87
PS	25.11	0.334	9.12
PE	51.87	0.689	18.84
PE plas	30.88	0.410	11.21
CL	20.83	0.138	3.78
PG	3.34	0.0443	1.21
	Total :	**3.66**	

The results from samples 1 and 2 show similar trends regarding phospholipid composition. Sample 2 shows lower value of total phospholipids which is because sucrose fraction 6 was excluded.

Tables 8-10 show the calculated mole percentage for sample 3 which was prepared with the 1% Triton X-100 detergent. Total protein in sample 3 was 4.33 mg.

Table 8 Calculated mole percentage of each phospholipid from ^{31}P-NMR sample 3 acquired at 298 K, isolated by the detergent solubilization with Triton X-100 solution.

Peak	Integration value	µmole P	Mole percentage (%)
PC	23.10	1.56	39.87
PI	3.49	0.236	6.02
SPH + PS	7.00	0.473	12.09
PE+PE plas	20.00	1.35	34.52
CL	7.03	0.237	6.07
PG	0.83	0.0563	1.44
	Total :	**3.91**	

Table 9 Calculated mole percentage of each phospholipid from ^{31}P-NMR sample 3 acquired at 307 K, isolated by the detergent solubilization with Triton X-100 solution.

Peak	Integration value	µmole P	Mole percentage (%)
PC	37.99	1.60	39.14
PI	4.92	0.207	5.06
SPH	7.17	0.303	7.39
PE+PE plas+PS	36.99	1.56	38.12
CL	15.62	0.330	8.05
PG	2.17	0.0917	2.24
	Total :	**4.10**	

Table 10 Calculated mole percentage of each phospholipid from ^{31}P-NMR sample 3 acquired at 312 K, isolated by the detergent solubilization with Triton X-100 solution.

Peak	Integration value	µmole P	Mole percentage (%)
PC	45.28	1.59	39.71
PI	5.10	0.179	4.47
SPH	8.65	0.305	7.59
PE+PE plas+PS	42.12	1.48	36.94
CL	20.00	0.352	8.77
PG	2.88	0.101	2.52
	Total :	**4.01**	

The results from the measurements at these three different temperatures are similar. The peak that show PE + PE plas have the highest variance in mol% which is explained by the fact that no separate PS peak is seen in any of the ^{31}P NMR spectra of the Triton X-100 prepared sample. Therefore PS is possibly overlapping this peak at 307K and 312K. The chemical shift of PS changes with temperature faster than the other phospholipids (18). At temperature 298 K its peak overlaps SPH, which explains the higher molar percentage for SPH at that temperature.

From these overlaps the mol% of PS can be roughly estimated to be 3-5% in sample 3.

The comparison of mol% of phospholipids isolated with both methods is shown in table 11. The values for PE and PE plas were combined for the detergent-free results for better comparison since the peaks overlap in the detergent isolated sample. For that sample, the average value from the three measurements at different temperatures was taken, when possible. Since PS overlaps SPH at 298 K, SPH is excluded and results from 307 K and 312 K were used. PS overlaps PE + PE plas at 307 K and 312 K so the value for PE + PE plas at 298 K is shown in table 11.

Table 11 Comparison of mol% of phospholipids in samples isolated by either method.

	Mol% of total phospholipid		
Phospholipid	**Detergent-free, Na_2CO_3 buffer**		**Detergent solution**
PC	42.39	42.44	39.57 ± 0.31
PI	5.56	4.52	5.18 ± 0.64
SPH	7.54	8.87	7.49 ± 0.10
PS	7.85	9.12	3-5
PE + PE plas	31.37	30.05	34.52
CL	3.99	3.78	7.63 ± 1.14
PG	1.30	1.21	2.07 ± 0.46

The greatest difference in phospholipid compositions between the two methods seems to be that the mol% of PC is lower and CL and PG is higher in the detergent isolated sample. It is hard to distinguish the difference in PE and PE plas since the peaks overlap in the sample isolated with the detergent method.

The total protein and total lipid in each NMR sample is summarized, as well as the lipid/protein ratio calculated, in table 12. Sample 3, which was isolated with the Triton X-100 method, is lower in lipid/protein ratio than the sample isolated with detergent-free method. Sample 2 in not comparable since it is missing sucrose gradient fraction 6.

Table 12 Comparison of lipid/protein ratio (w/w) from the three different NMR samples.

NMR sample	Protein (mg)	Lipid (µg)	µg lipid/mg protein
1	3.88	10.02	2.58
2	2.60	3.66	1.41
3	4.33	4.01	0.93

4 Discussions

The main goal of this study was to find whether if the two different isolation methods, using the detergent-free, Na_2CO_3 buffer or detergent solubilization with 1% Triton X-100 solution, would give different phospholipid compositions.

Proteins that are found in high-density sucrose gradient fractions 6-12 (Fig. 6A) are part of large cellular proteins and organelles and the lipid rafts should collect in the lighter sucrose gradient, between fraction 4-6. A protein peak around fraction 5 in the fractions from the detergent-free method indicated a good isolation. Most of the protein measurements for the detergent-free method gave this trend. If these results are compared to the results from the Triton X-100 solution isolation (fig. 7.A) it can be seen that the results differ. In fractions 1-6 (fig. 7.B) only a minute protein peak was seen around fraction 3, and the protein concentration was lower than in figure 6.B. This occurred in all the other detergent isolations. Protein concentrations in fractions 3-5 were around four to five times lower than of the detergent-free method. We decided to use the same fractions for lipid isolation nr. 3-6 although fraction 6 in the detergent isolate seems to be mixed with the bulk protein of the preparation in fractions 7-12.

The target molecules in the dot blots were the lipid raft proteins Flotilin-1 and Caveolin-3, and the raft lipid GM1. Prior lipid raft studies from our laboratory, using the same methods, showed with SDS-PAGE and Western blotting that these lipid raft markers located in fractions 3-6 (19). For this reason and to save time only dot blots were used to confirm the presence of the markers in the fractions. The dot blots from the detergent-free isolated samples showed dark red dots at fractions 4-6 for both the Flotilin-1 and the Caveolin-3 blots, and only faint dots in fractions 7-8 (fig. 9). On the GM1 blots, dots could be seen in fractions 4-12, but in fractions 4-6 the dots were darker than the ones in 7-12. The dot blots of lipid raft marker proteins in samples isolated with the detergent-free method indicated a good isolation of the lipid rafts, and their presence was mostly in fractions 4-6. This corresponded nicely with the protein concentration measurement (Fig. 6B). In the detergent isolated samples (fig. 10) the darkest dots for Caveolin-3 seem to be in fractions 5-8 and for Flotilin-1 in fractions 5-7 and dots were seen down to fraction 11. This trend was seen in all the detergent isolations. This indicates that the lipid rafts are not concentrating in fractions 4-6, like in the detergent-free isolation, but are somewhat distributed down the gradient. These results are not in unison with most other published results using the detergent method, where these marker molecules usually locate at fractions 4-7 (19, 20). Authors that have isolated lipid rafts from rat heart do not agree on their preference of isolation methods. Cavalli *et al.* (14) experienced better results with the detergent method but Head *et al.* (20) preferred the detergent-free method as they found that when using the detergent method the lipid raft marker proteins were not confined to fractions 3-6 in agreement with our results.

A total of three samples were investigated, thereof two samples prepared with the detergent-free method and one sample with the Triton X-100 detergent method. Spectra measured at 307 K were used for data interpretation and chemical shift measurements. The samples were prepared as described by Metz and Dunphy (18) and their published

chemical shifts of each phospholipid were used to identify the peaks. The locations of PS, SPH, PE, PE plas, CL and PG were all a bit shifted upwards compared to their publication but their samples were a mixture of rat liver and brain extracts (18) and we were working with heart samples which could cause some variation in chemical shifts.

Comparison of the spectra of samples 1 and 2 give similar results. The sample size for NMR sample 1 was greater than of Sample 2 so the separation of the peaks can be expected to be better in sample 1. All the common phospholipids were detected in samples 1 and 2 in distinct peaks. CL and PG, that are phospholipids found in mitochondria and not the plasma membrane, were also detected. Heart muscle cells have an unusually high number (hundreds to thousands) of mitochondria so contamination from the mitochondrial membrane can be expected.

When spectra for samples 1 and 2 are compared to sample 3 the biggest difference seen is in the resolution and separation of peaks. Although sample 3 contained the highest amount of protein of the three samples, it had only half of the lipid content of sample 1 (Table 12). Since the sample size affects quality of the spectra some peaks converge in sample 3, like PE and PE plas. In all the three different spectra for sample 3 no peak for PS is seen, but its presence can be assumed since at 298 K the mol% for SPH rises about 50% and at this temperature SPH and PS have similar chemical shifts. At higher temperatures PS shifts closer to PE, which is the result in higher mol% for PE+PE plas at 307 and 312 K. When mol% of the phospholipids in the three samples (Table 11) are compared the main differences are the lower values for PC and higher values for CL and PG when the sample is isolated with the detergent solution. The low mole% of PS estimated in the samples isolated using the Triton X-100 method is conclusive with results of García-Marcos(12) and L. Pike (4) and other studies that have shown that Triton X-100 is more likely to solubilise phospholipids present in the cytoplasmic leaflet of the cell membrane, which is where PS is located. Other groups have had problems with using detergent to isolate lipid rafts from heart. Head *et al.* (20) found that the Triton X-100 isolation method gave worse isolation of Caveolin-3 than the high salt, detergent-free method.

Our results (table 11) do not show as much difference in the inner leaflet phospholipids, such as PE plas and PI, between methods compared to the results of L. Pike (fig. 5) (4). However, the comparison of lipid/protein ratio from each ^{31}P NMR sample (table 12) shows that the Triton X-100 detergent method gives less than half the lipid/protein ratio of the sample isolated with the detergent-free method, which could indicate that the detergent method solubilizes more of the phospholipids in lipid rafts.

In conclusion, from these data it seems that the detergent-free isolation when working with rat hearts would be the optimal method even though lipid composition did not differ that much between methods. It results in lipid rafts confined at the upper fractions of the sucrose gradient and protein concentration in these fractions were up to five times higher than when the detergent method was used. Immunoblots of raft markers and total protein measurements indicated a better isolation of lipid rafts and the NMR spectra shows better peak separation and less signs of contamination from other organelle membranes.

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