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Exploring detergent insolubility in bovine hippocampal membranes: a critical assessment of the requirement for cholesterol

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Abstract

The phenomenon of detergent insolubility of bovine hippocampal membranes in Triton X-100 was monitored by estimating the presence of phospholipids in the insoluble pellet. This represents a convenient and unambiguous assay and reports the dependence of the *extent* of phospholipid solubilization on detergent concentration. The advantage of this approach is its ability to accurately determine the extent of detergent insolubility in natural membranes. Importantly, our results show that when suboptimal concentrations of Triton X-100 are used for solubilization, interpretations of the mechanism and extent of detergent insolubility should be made with adequate caution. At concentrations of Triton X-100 that leads to no further solubilization, ~ 44% of phospholipids are left insoluble at 4 °C in bovine hippocampal membranes. Cholesterol depletion using methyl-β-cyclodextrin enhanced phospholipid solubilization at low detergent concentrations but produced no significant change in the amount of insoluble phospholipids at saturating detergent concentration. Progressive solubilization by the detergent resulted in insoluble membranes that contained lipids with higher fatty acyl chain order as reported by fluorescence polarization studies using 1,6-diphenyl-1,3,5-hexatriene (DPH). These results suggest that it is the presence of such lipids rather than their association with cholesterol that determines detergent insolubility in membranes.

Keywords: Detergent insolubility; Hippocampal membrane; Cholesterol; Phosphate assay; Fluorescence polarization

1. Introduction

It now appears that constituent lipids of eukaryotic cell membranes are not randomly distributed but rather interact preferentially to form domains in the plane of cell membranes. These domains are thought to be laterally segregated entities with lipid-protein composition and physical properties distinct from that of the bulk membrane. Early observations that a fraction of cell membranes were resistant to solubilization by certain non-ionic detergents at low temperatures [1,2] and evidence gathered from model membrane systems with compositions mimicking these domains [3–6] have suggested that these fractions represent a liquid-ordered like phase in an otherwise fluid membrane. In the liquid-ordered phase, the lipids are packed tightly, as in an ordered gel phase [7,8] but exhibit rapid lateral diffusion [9] as in a liquid crystalline phase. Such detergent-resistant fractions are enriched in sphingolipids, cholesterol and lipids that have saturated fatty acyl chains. The phenomenon of detergent insolubility has been explained on the basis of physicochemical principles like the presence of differentially miscible lipids with a high melting temperature such as sphingolipids and lipids with saturated acyl chains [10] and/or their preferential interactions with cholesterol [11,12]. The idea of such specialized membrane regions (domains) assumes importance in cell biology since physiologically important functions such as membrane sorting and trafficking [13] and signal transduction processes [14] have been attributed to these domains on account of their unique lipid–protein composition and their potential to sequester signaling molecules in the plane of the membrane. However, most work in this area is concentrated on cellular systems such as polarized epithelial cells, fibroblasts and cells of the immune system. Neuronal cells represent a challenging system for such

Abbreviations: BCA, bicinchoninic acid; CMC, critical micellar concentration; DMPC, dimyristoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; G_{M1}, monosialosyl-*N*-tetraglycosyl ceramide; GPI, glycosylphosphatidylinositol; MLV, multilamellar vesicles; T_m, melting temperature

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studies since their lipid composition is rather unique and has been correlated with the increased complexity in the organization of the nervous system during evolution [15]. Yet, there have been relatively few reports on the isolation and characterization of such membrane fractions from neuronal systems.

Work from our laboratory has established native membranes prepared from the bovine hippocampus as a natural source for the seven-transmembrane domain, G-protein coupled, serotonin$_{1A}$ (5-HT$_{1A}$) receptor. We have successfully solubilized and characterized the 5-HT$_{1A}$ receptor from bovine hippocampal membrane in a functionally active form and have shown modulation of ligand binding by membrane cholesterol content (Pucadyil and Chattopadhyay, unpublished observations), guanine nucleotides, alcohols, metal ions and covalent modifications [16–23]. In light of the proposed significance of detergent-resistant membrane domains in signal transduction, the question of whether detergent resistance can be observed in bovine hippocampal membranes assumes relevance. In this paper, we have explored detergent insolubility in bovine hippocampal membranes and have provided quantitative estimates of the extent of insolubility in Triton X-100 by assaying for insoluble phosphate derived from phospholipids. Our results show that this method can be applied to characterize the nature and mechanism of detergent insolubility in natural membranes. In addition, steady-state fluorescence polarization studies using 1,6-diphenyl-1,3,5-hexatriene (DPH) provide novel insights into the possible mechanisms that contribute to the phenomenon of Triton X-100 insolubility.

2. Materials and methods

2.1. Materials

Triton X-100, bicinchoninic acid (BCA), DPH, methyl-β-cyclodextrin, Tris, sucrose, EDTA, sodium azide, phenylmethylsulfonyl fluoride (PMSF), iodoacetamide and Na_2HPO_4 were obtained from Sigma Chemical (St. Louis, MO). BCA reagent kit for the estimation of protein was from Pierce (Rockford, IL). Amplex Red cholesterol assay kit was from Molecular Probes (Eugene, OR). All solvents used were of analytical grade. Fresh bovine brains were obtained from a local slaughterhouse within 10 min of death and the hippocampal region was carefully dissected out. The hippocampi were immediately flash frozen in liquid nitrogen and stored at − 70 °C till further use.

2.2. Methods

2.2.1. Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described previously [17]. Briefly, bovine hippocampal tissue (~ 100 g) was homogenized as 10% (w/v) in a polytron homogenizer in buffer A (2.5 mM Tris, 0.32 M sucrose, 5 mM EDTA, 5 mM EGTA, 0.02% sodium azide, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4). The homogenate was centrifuged at 900 × *g* for 10 min at 4 °C. The supernatant was filtered through four layers of cheesecloth and the pellet was discarded. The supernatant was further centrifuged at 50,000 × *g* for 20 min at 4 °C. The resulting pellet was suspended in 10 vol. of buffer B (50 mM Tris, 1 mM EDTA, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4) using a hand-held Dounce homogenizer and centrifuged at 50,000 × *g* for 20 min at 4 °C. This procedure was repeated until the supernatant was clear. The final pellet (native membranes) was suspended in a minimum volume of buffer C (50 mM Tris, pH 7.4), homogenized using a hand-held Dounce homogenizer, flash frozen in liquid nitrogen and stored at − 70 °C. Protein concentration was assayed using the BCA assay kit [24].

2.2.2. Treatment of native membranes with Triton X-100

Bovine hippocampal membranes and detergent stocks were incubated at the desired temperature for 5 min to reach thermal equilibrium before solubilization. Membranes were incubated with varying concentrations of Triton X-100 which was aliquoted from a stock solution (146 mM or 10% w/v, assuming mol. wt. = 625 g/mol) in buffer C. Aliquots ranging from 10–200 μl volume, corresponding to a final concentration range from 0–29.2 mM (0–2% w/v), were added from the detergent stock. The final volume of the membrane and detergent mixture was made up to 1 ml with buffer C. All solubilization experiments were carried out with 1 mg protein (protein concentration of the membranes was 10–13 mg/ml) which was equivalent to ~ 1 μmol total lipid phosphate, in 1 ml of buffer C for 30 min at the desired temperature (4 ° or 37 °C) with intermittent shaking. Membranes were spun at 100,000 × *g* for 45 min at 4 °C. The supernatant was decanted and the pellet was resuspended in 1 ml of buffer C. The pellet was briefly sonicated to achieve a homogeneous suspension.

2.2.3. Cholesterol depletion of native membranes

Bovine hippocampal membranes were depleted of cholesterol using methyl-β-cyclodextrin as described earlier [25]. Membranes with a typical protein concentration of 2 mg/ml were treated with 40 mM methyl-β-cyclodextrin in buffer C at room temperature with constant shaking for 1 h. Membranes were spun down at 50,000 × *g* for 10 min and resuspended in buffer C. Cholesterol was estimated using the Amplex Red cholesterol assay kit as described earlier [26].

2.2.4. Estimation of inorganic phosphate

Concentration of phosphate was determined subsequent to total digestion by perchloric acid [27] using Na_2HPO_4 as standard. Dimyristoylphosphatidylcholine (DMPC) was used as an internal standard to assess lipid digestion.

Samples without perchloric acid digestion produced negligible readings.

2.2.5. Lipid extraction of detergent-insoluble pellets

Lipid extraction was carried out according to Bligh and Dyer [28]. The extracts were dried under a stream of nitrogen at 45 °C. The dried extracts were resuspended in a mixture of chloroform–methanol (1:1, v/v).

2.2.6. Sample preparation for fluorescence polarization measurements

DPH in methanol was added to membranes at a final probe concentration of 1 mol% with respect to total phospholipid content. Membranes containing 50 nmol of total phospholipids were suspended in 1.5 ml of buffer C and used for fluorescence polarization experiments. Membranes were vortexed for 1 min after addition of the probe and kept in the dark for 1 h before measurements. Background samples were prepared the same way except that the probe was omitted. Lipid extracts containing 20 nmol of total phospholipids in chloroform–methanol (1:1, v/v) was mixed with 0.2 nmol of DPH in methanol and dried under a stream of nitrogen at 45 °C. The samples were kept under vacuum for at least 3 h to remove all traces of solvent. Samples were hydrated at 80 °C by adding 1.5 ml of buffer C that was maintained at the same temperature, and vortexed for 3 min to disperse the lipids and form homogeneous multilamellar vesicles (MLVs). The MLVs were kept at 80 °C for an additional 1 h. Samples were kept in the dark till they attained room temperature before fluorescence was measured. Such high temperatures for hydrating the samples were necessary due to the presence of lipids with high melting temperature in neuronal tissues [29]. Fluorescence was measured in Hitachi F-4010 spectrofluorimeter using 1-cm-path length quartz cuvettes at room temperature (23 °C). Excitation and emission wavelengths were set at 358 and 430 nm with bandpasses of 1.5 and 20 nm, respectively. Fluorescence was measured with a 30-s interval between successive openings of the excitation shutter to reverse any photoisomerization of DPH [30]. The optical density of samples measured at 358 nm was ~ 0.16. Fluorescence polarization was performed using a Hitachi polarization accessory. Polarization values were calculated from the equation [31]:

$$P = (I_{VV} - GI_{VH})/(I_{VV} + GI_{VH})$$

where I_{VV} and I_{VH} are the measured fluorescence intensities with excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is equal to I_{HV}/I_{HH}.

Membranes and lipid extracts used for fluorescence polarization studies were not processed any further for removal of any possible residual Triton X-100. This does not affect interpretation of data from these experiments (see Results).

3. Results

The general view of membrane solubilization by detergents is described as a three-stage process (reviewed in Refs. [32,33]). Detergent monomers partition into the membrane without solubilization (stage I) until a saturation point is reached which coincides with the effective critical micellar concentration (CMC) of the detergent. At this stage, it is believed that membranes co-exist with lipid–protein–detergent mixed micelles (stage II). Any further increase in detergent concentration results in progressive delipidation of the lipid–protein–detergent mixed micelles, forcing the lipids to distribute among the increasing concentrations of detergent micelles (stage III) leaving behind, in some cases, a detergent-insoluble residue.

We estimated the extent of solubilization of hippocampal membranes by assaying the amount of inorganic phosphate derived from phospholipids present in the insoluble pellet. We refer to phospholipids to imply lipids that have a phosphate head group which would qualify all classes of glycerophospholipids and sphingolipids. We find this approach to be simple yet unambiguous in its interpretation since it represents the solubilization of a fairly 'bulk-marker', namely phospholipids, as they are the predominant class of lipids present ubiquitously in cell membranes. The phosphate content measured in the insoluble pellet after detergent solubilization must have originated mainly from phospholipids since ~90% of the phosphate content in native membranes could be recovered when membranes were extracted of lipids. The phosphate content of native membranes was found to be 1069 ± 14 nmol/mg protein while that of the lipid extract of native membranes was 936 ± 10 nmol/mg protein. The phosphate content in the insoluble pellet was therefore measured as it is without extraction.

The effect of increasing concentrations of Triton X-100 on the extent of solubilization of bovine hippocampal membrane phospholipids at 4 °C is shown in Fig. 1. The data indicate a progressive loss of membrane phospholipids with increasing concentrations of the detergent. The results show efficient solubilization occurring at low concentrations, typically below 7.3 mM (0.5% w/v), beyond which increasing the concentration of detergent causes only a marginal increase in the extent of solubilization. Fig. 1 shows that a large proportion (~ 44%) of membrane phospholipids remain insoluble in Triton X-100 even at the highest concentration of detergent used. Since we have used concentrations of Triton X-100 well above its CMC (0.3 mM or 0.019% w/v [30]), these results reflect an inherent property of Triton X-100 to render a significant portion of the hippocampal membrane insoluble rather than an insufficiency in the concentration of detergent used. In addition, the degree of detergent insolubility remained unaffected even if the treatment was carried out for longer time periods (up to 3 h, data not shown). Since detergents such as Triton X-100 are considered quick membrane solubilizers on

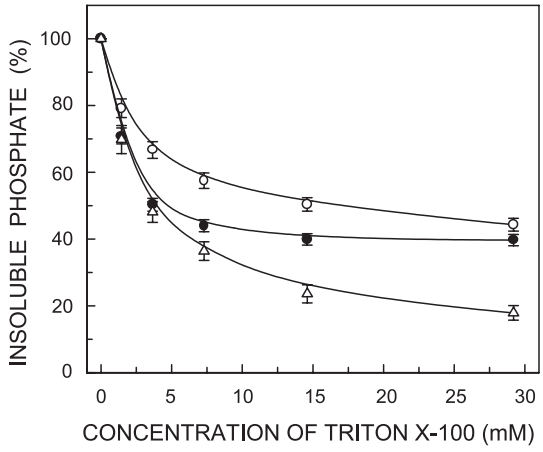

Fig. 1. Detergent insolubility in bovine hippocampal membranes treated with increasing concentrations of Triton X-100. Detergent insolubility was measured by assaying the content of total inorganic phosphate present in pellets after solubilization with different concentrations of Triton X-100. Solubilization was carried out for native (○) and cholesterol-depleted (●) membranes at 4 °C, and native membranes (△) at 37 °C. The ordinate represents percentage of insoluble phosphate normalized with respect to the appropriate control for each treatment. The data points are the means ± S.E. of duplicate points from three independent experiments. See Materials and methods for other details.

account of their lipophilicity [34,35], these observations suggest that the process of solubilization has reached equilibrium.

Isolation of detergent-insoluble membrane fractions are usually carried out at low temperature (4 °C) in order to enrich the fraction of such detergent-resistant, liquid-ordered phases in cell membranes. Similar experiments carried out at physiological temperature (37 °C) have been earlier shown to reduce the detergent-resistant fraction and have been employed as a control [36]. Fig. 1 shows that when solubilization of bovine hippocampal membranes was carried out at 37 °C, the solubilization ability of Triton X-100 was found to be enhanced at all detergent concentrations used. The increased solubility of phospholipids at higher temperatures can be attributed to the phase state of membrane lipids (we consider any change in CMC due to the change in temperature insignificant here since the detergent concentrations used are much above the CMC). An earlier study has alluded to a relationship between melting temperature (T_m) of phospholipids and their relative resistance to solubilization [37]. In addition, another study reported the insolubility at 4 °C of phospholipids in the gel state with 16–18-carbon chain fatty acids which are the predominant class of lipids in natural membranes [38]. These studies suggest that resistance to solubilization mediated by nonionic detergents is greater in bilayers composed of saturated lipids which exhibit higher T_m than other constituent lipids in membranes. Our observation of increased solubilization at higher temperature (37 °C) can be explained on this basis since at the higher temperature, some of the phospholipids could be in a disordered state.

Cholesterol is often found enriched in detergent-insoluble fractions isolated from many natural sources [2]. It is considered an essential constituent of such fractions and studies carried out in model membrane systems have addressed the specific molecular requirements for sterols in general to facilitate the formation of liquid-ordered states that display detergent resistance [11,39]. To address the role of cholesterol in conferring detergent insolubility to hippocampal membranes, cholesterol depletion was carried out using methyl-β-cyclodextrin and detergent insolubility was tested. The treatment with methyl-β-cyclodextrin resulted in depletion of ~ 90% of membrane cholesterol without any significant effect on phospholipid content (see Table 1).

As shown in Fig. 1, the extent of phospholipids solubilized in cholesterol-depleted membranes appears to be greater than that in native membranes at lower detergent concentrations. Interestingly, this difference in the extent of solubilization gradually reduces as the detergent concentration is increased. At higher concentrations, the process of solubilization reaches saturation and the amount of phospholipids left behind (~ 40%) is found to be similar to that observed with native membranes. These results indicate that the ability of membrane cholesterol to confer resistance to Triton X-100-mediated solubilization is limited to only lower concentrations of the detergent. The extent of detergent insolubility at higher concentrations of detergent therefore appears to be insensitive to the actual membrane cholesterol content. The significance of this result in light of the proposed role of cholesterol to maintain a detergent-insoluble liquid-ordered state of membranes will be discussed later.

The composition of detergent-resistant membranes obtained from various sources indicates an enrichment in

Table 1
Estimation of cholesterol and phospholipid contents in native and methyl-β-cyclodextrin-treated membranes

Membranes	Cholesterol content (nmol/mg protein)[a]	Phospholipid content (nmol/mg protein)[b]	Cholesterol: phospholipid ratio[c]
Native	446.7 ± 9.7 (100%)[d]	964 ± 43 (100%)[d]	0.46
Treated with 40 mM MβCD	45.3 ± 0.4 (10%)	905 ± 26 (94%)	0.05

[a] Cholesterol content was assayed using the Amplex Red cholesterol oxidase-based assay and was normalized with respect to total protein. The data represents the means ± S.E. of duplicate points from three independent experiments. See Materials and methods for more details.

[b] Phospholipid content of normal and cholesterol-depleted membranes was assayed as described in Materials and methods. The data represent the means ± S.E. of nine independent experiments.

[c] Cholesterol to phospholipid ratio was calculated for native and cholesterol-depleted membranes from data given in columns 2 and 3.

[d] Numbers in parentheses indicate percentage of cholesterol and phospholipid contents of membranes normalized with respect to control.

cholesterol, sphingomyelin and phospholipids bearing long saturated acyl chains, and have been characterized biophysically to represent membranes in a liquid-ordered state [40]. Such phases are characterized by an intermediate degree of motional restriction or order in fatty acyl chains between that of lipids in the gel and fluid phases. We characterized the detergent-insoluble fractions obtained with increasing concentrations of Triton X-100 in terms of the fatty acyl chain order and packing by measuring steady-state polarization of the fluorescent probe DPH in these membranes. Fluorescence polarization monitors the rotational diffusion of the membrane embedded probe which is sensitive to the packing of fatty acyl chains [41]. Fluorescence quenching experiments in model membranes containing co-existing gel and fluid phases have earlier shown that DPH partitions homogeneously between these phases [42]. The partitioning property of DPH is particularly advantageous since it reports the phase-averaged rotational properties of fatty acyl chains without bias toward any particular region of the membrane. Importantly, the polarization values determined remained identical even after dilution of membrane samples, indicating the absence of any scattering artifact.

Fig. 2 shows that the fluorescence polarization values of DPH in detergent-resistant membranes obtained by treating native hippocampal membranes with increasing concentrations of detergent at 4 °C increases sharply (from 0.352 to 0.379) up to a detergent concentration of 7.3 mM (0.5% w/v). The detergent-insoluble membrane fractions (and lipid extracts of these fractions, see later) have not been specifically treated to remove any residual detergent. In any event, presence of residual detergent would increase the fluidity of these fractions and result in a decrease in fluorescence polarization. Since we observe an increase in polarization upon increasing the detergent concentration (see Fig. 2 and Table 2), this would not be a complicating factor in our interpretation of the polarization data. Interestingly, this increase parallels the increased solubility of phospholipids in the same range of detergent concentrations (0–7.3 mM or 0–0.5% w/v) as shown in Fig. 1. In addition, similar to the trend in phospholipid solubilization, further increase in detergent concentration does not significantly change the polarization value, which remains around 0.382. The remarkable similarity between the extent of phospholipid solubilization and the increase in polarization values of DPH in the detergent-resistant membranes implies that with increasing detergent concentrations, phospholipids in the fluid state get solubilized preferentially. This results in an enrichment of ordered phospholipids in the detergent-resistant membranes, which gives rise to higher polarization.

The pattern of change in fluorescence polarization with increasing concentrations of detergent is similar when membranes are treated at 37 °C with Triton X-100 (see Fig. 2). This is somewhat surprising since the extent of phospholipid solubilization observed under these conditions (4° and 37 °C) was found to be different (see Fig. 1). We speculate that the presence of membrane lipids such as cerebrosides, ceramides and gangliosides, which do not

Table 2
Fluorescence polarization of DPH in MLVs prepared from lipid extracts and in natural membranes

Treatment	Triton X-100, mM (% w/v)	Fluorescence polarization	
		Lipid vesicles[a]	Natural membranes
Membranes	0	0.339 (1.00)[b]	0.352[c] (1.00)[b]
treated	3.65 (0.25)	0.363 (1.07)	0.375 (1.07)
at 4 °C	14.60 (1)	0.373 (1.10)	0.382 (1.09)
Cholesterol-depleted	0	0.296 (1.00)	0.322 (1.00)
membranes treated	1.46 (0.1)	0.331 (1.12)	0.360 (1.12)
at 4 °C	14.60 (1)	0.394 (1.33)	0.403 (1.25)
Membranes	0	0.347 (1.00)	0.348 (1.00)
treated	1.46 (0.1)	0.349 (1.01)	0.367 (1.05)
at 37 °C	3.65 (0.25)	0.367 (1.06)	0.379 (1.09)

[a] Lipids were extracted according to Bligh and Dyer [28] from detergent-resistant membrane pellets obtained after treating hippocampal membranes at the above conditions of detergent concentrations and temperature. MLVs were prepared from these extracts and were used for DPH fluorescence polarization experiments. Fluorescence was measured at room temperature (23 °C) for polarization experiments. The ratio of probe/total phospholipid was maintained at 1:100 (mol/mol) in all cases. Representative values are indicated here. See Materials and methods for other details.

[b] The numbers in parentheses indicate the fractional increase in fluorescence polarization over controls (no detergent added) for the respective treatments.

[c] Polarization values for natural membranes are from Fig. 2.

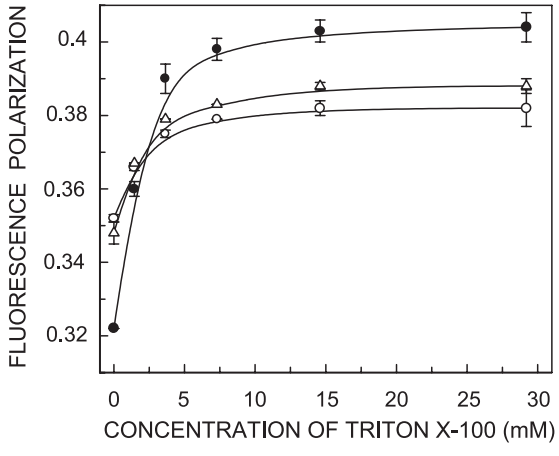

Fig. 2. Fluorescence polarization of DPH in detergent-insoluble fractions from bovine hippocampal membranes treated with increasing concentrations of Triton X-100. The ratio of probe/total phospholipid was maintained at 1:100 (mol/mol) in all cases. Solubilization was carried out for native (○) and cholesterol-depleted (●) membranes at 4 °C, and native membranes (△) at 37 °C. The resultant detergent-insoluble membrane fractions were used for fluorescence polarization experiments. Fluorescence polarization measurements were carried out at room temperature (23 °C). The data points are the means ± S.E. of duplicate points from three independent experiments. See Materials and methods for other details.

have a phosphate group, exhibit high melting temperature ($T_m \sim 70-80$ °C) [29] and typically display detergent insolubility in membranes [36,43,51], could account for this observation. Fig. 2 also shows that cholesterol-depleted membranes show a marked increase in DPH polarization upon treatment with increasing concentrations of Triton X-100. Since it is well known that cholesterol induces order in biological membranes, cholesterol-depleted membranes display a lower polarization value (0.322) than native membranes (0.352) in the absence of any added detergent. Interestingly, treatment with increasing concentrations of the detergent results in a remarkable increase in fluorescence polarization of DPH. The fluorescence polarization reaches a value of 0.404, implying an extremely rigid environment around the probe at the highest concentration of detergent used. This shows that even though the detergent insolubility of phospholipids in cholesterol-depleted membranes is comparable to that observed in native membranes at higher Triton X-100 concentrations (see Fig. 1), the insoluble fraction consists of lipids that exhibit greater order packing in their fatty acyl chains approaching that observed in pure gel states in model membranes. The higher DPH polarization value observed in cholesterol-depleted membranes compared to native membranes (see Fig. 2), at concentrations of detergent that leads to no further phospholipid solubilization (7.3–29.2 mM or 0.5–2%, see Fig. 1), in fact further supports our earlier interpretation on the preferential enrichment of lipids that exhibit greater order packing in their fatty acyl chains for the following reasons. Cholesterol is known to induce disorder in membranes composed of lipids in the ordered phase (those exhibiting high acyl chain order and hence a high DPH polarization value) [44]. If one considers that cholesterol depletion does not affect the types/classes of phospholipids left eventually insoluble in the pellet, a valid assumption based on data from previous literature with model membranes [37,43], but rather lowers the effective concentration of detergent required to reach saturation in solubilization (see Fig. 1), then our observation that the detergent-insoluble fractions isolated from cholesterol-depleted membranes exhibit higher polarization values indicates the presence of lipids that exhibit tight packing at their fatty acyl chains. These fractions would, however, have shown a lower fluorescence polarization value in the presence of cholesterol as is seen with control detergent-insoluble membrane fractions (see Fig. 2).

Taken together, our results indicate that detergent-insoluble fractions obtained after treatment of bovine hippocampal membranes with increasing concentrations of Triton X-100 exhibit greater fatty acyl chain packing. However, a concern arises due to the presence of native proteins in hippocampal membranes and its effect, if any, on the fluorescence polarization values. To rule out any possible influence of membrane proteins on the earlier reported fluorescence polarization values (Fig. 2), we measured fluorescence polarization of DPH incorporated in MLVs formed from lipids extracted from the detergent-insoluble fractions (see Table 2).

The results show that the overall increase in polarization upon increasing detergent concentration is also present in experiments carried out with vesicles formed from extracted lipids. In addition, the fractional increase observed when increasing concentrations of detergent are used is found to be more or less constant in both the cases (see Table 2). These results reinforce our earlier interpretation that the fluorescence polarization data obtained for natural membranes are due to an enrichment of certain classes of lipids exhibiting a greater fatty acyl chain order.

4. Discussion

In this paper, we have examined the phenomenon of Triton X-100 insolubility in bovine hippocampal membranes by measuring the amount of insoluble phospholipids. This represents a convenient and unambiguous method to accurately analyze the extent of detergent insolubility in natural membranes owing to the ease with which they sediment and the ubiquitous nature of phospholipids in membranes. More importantly, our results indicate that the extent of detergent insolubility depends on the concentration of detergent used, a point to be remembered when isolation and quantitation of such fractions are carried out. The use of suboptimal detergent concentrations would therefore overestimate the amount of detergent-insoluble fraction on account of incomplete solubilization. Utilizing this approach, we show here that a significant portion of hippocampal membranes ($\sim 44\%$) remains insoluble at the highest concentration of Triton X-100 used.

Previous studies have used marker proteins such as glycosylphosphatidylinositol (GPI)-anchored proteins, caveolin or specific lipids like G_{M1} to assess detergent insolubility [45,46]. A potential limitation of this approach is the implicit assumption of uniform presence of such marker molecules in regions of the membrane exhibiting detergent insolubility. Moreover, the presence of a specific marker may be limited to a particular cell type [47] making it difficult to quantitatively compare and estimate detergent insolubility among various cell types. The present method does not suffer from such limitations as phospholipids are ubiquitously present in cell membranes. Since the principal target for detergent action in most cases are bulk lipids, namely phospholipids, which induce the solubilization of membrane proteins, we feel the present method can be used to precisely estimate the onset and kinetics of detergent insolubility. In combination with experiments carried out under different conditions such as at different temperatures or using membranes with altered lipid composition, mechanistic aspects of detergent insolubility can be addressed in natural membranes by this approach. In

addition, this method to determine detergent insolubility would help avoid complication in interpretation that is often accompanied with some of the other methods used.

Insolubility in nonionic detergents such as Triton X-100 at 4 °C has been utilized to suggest the existence of membrane fractions that are enriched in cholesterol, sphingolipids and lipids with saturated fatty acyl chains [2]. These fractions are thought to be the equivalent of liquid-ordered phases shown to exist in model membrane systems of similar lipid composition [4]. It has also been shown that these lipids exist in a gel-like phase in the absence of cholesterol. The nature of insolubility at 4 °C has been suggested to reflect an inability of Triton X-100 to solubilize model membranes existing either in the liquid-ordered state or gel-like state [6,37]. Interestingly, these tenets have not been earlier tested in membranes isolated from a native source. As the presence of pure gel-like states in natural membranes is unlikely, especially with the abundance of cholesterol in membranes, detergent insolubility is thought to arise out of the existence of liquid-ordered states in the membranes. An important aspect of our results is that cholesterol does not appear to be crucial for detergent insolubility in hippocampal membranes (see Fig. 1). Experiments carried out using cholesterol-depleted membranes signify that the solubilization potential of Triton X-100 is indeed increased in the absence of cholesterol. However, our results clearly indicate that this is true only at lower concentrations of the detergent (Fig. 1). At higher detergent concentration, the extent of phospholipids solubilized reduces and reaches a value similar to that seen in native membranes. In view of DPH polarization results carried out in the resultant membrane fractions (see Fig. 2), we interpret these findings to suggest that detergent insolubility is not due to the presence of the putative liquid-ordered phase created by cholesterol in membranes. Instead, it is due to the gel state-preferring lipids in the membrane which eventually determine detergent insolubility though the gel-state lipids in the presence of cholesterol would have given rise to a liquid-ordered state in the membrane. Earlier reports have indicated that detergent treatment can lead to a selective enrichment of phospholipids exhibiting ordered packing of their fatty acyl chains in the insoluble fraction [48–51]. However, these studies do not provide any information on whether the ordered packing of phospholipids alone is sufficient to bring about detergent resistance independent of the presence of cholesterol. To the best of our knowledge, this is the first report highlighting the redundancy of cholesterol in conferring detergent resistance.

It should be noted here that whether detergent insolubility reflects the preexistence of isolated domains or is induced by detergents remains an open question although arguments for and against this have been provided [52,53]. Several studies utilizing detergent-free approaches have, however, yielded membrane fractions with similar lipid and protein composition as that of detergent-insoluble membranes [54,55].

Our results show enhanced phospholipid solubilization at 37 °C at all studied detergent concentrations. An increase in temperature could lead to a transition of membrane lipids from ordered gel-like to fluid-like phases bringing about an increase in solubilization. References to similar phenomena have been made earlier in model membranes and correlations drawn between the melting temperature T_m of the constituent lipids of the bilayer and its resistance to detergent solubilization [6,37]. In natural membranes, an increase in solubility of phospholipids might suggest local melting of lipids as the temperature is raised leading to progressive solubilization with increasing concentrations of detergent. This could be the experimental basis of solubilization carried out at high temperature (37 °C) being used as a control [36].

In summary, we report here a straightforward assay to address important issues related to the phenomenon of Triton X-100 detergent insolubility in the bovine hippocampal membranes. To the best of our knowledge, this is the first report in which the *extent* of detergent insolubility in Triton X-100 in neuronal membranes from a native source has been analyzed. In addition, by the use of native membranes with altered lipid composition in conjunction with fluorescence polarization measurements using the phase-insensitive probe DPH, we provide novel insights into the origin and mechanism of detergent insolubility in hippocampal membranes.

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