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Enhanced cholesterol accessibility for PFO interaction in membranes containing POPE and POPS phospholipids.

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Abstract of the Thesis

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Cholesterol biosynthesis regulation via the SREBP (sterol regulatory element binding protein) pathway has previously been elucidated to exhibit a switch-like response in the ER at ~ 5 mol% cholesterol. It has been proposed that this threshold value is mainly due to the control of the concentration at which cholesterol becomes accessible by the phospholipid composition of the ER membrane. We have conducted experiments to investigate this hypothesis. *Perfringolysin O* (PFO) was applied as a sterol sensor that binds membranes and oligomerizes upon cholesterol exposure in artificial membranes. Using this system, we detected a progressive enhancement of cholesterol accessibility as more PE (phosphatidylethanolamine) and PS (phosphatidylserine) phospholipids were incorporated into SUVs composed of PC (phosphatidylcholine). Further studies involving the uptake of various biologically relevant fatty intercalants (triglycerides, diglycerides, fatty alcohols) into PE/PS-containing vesicles reduced the threshold to a value as low as 2 mol% cholesterol. We discuss the hypothetical contributions of PE, PS, and various fatty intercalants in lowering the threshold concentration at which the SREBP pathway decides to transcriptionally activate or deactivate cholesterol biosynthesis.

Table of Contents

List of Figures

Page

List of Tables

Page

Introduction

Cholesterol, being one of the central lipids in mammalian cells, is known to be metabolically and structurally involved in a wide range of biological processes. Not only does this primarily synthesized sterol affect the cellular membrane physiology and participate in signaling pathways (*1*, *2*), but defects in the tightly regulated homeostatic control of cholesterol levels in a cell can also render the organism to be susceptible to the development of a number of diseases. In terms of pathophysiology, cholesterol has most prominently and notoriously exerted its influence in atherosclerosis, via an excess accumulation of cholesterol (*3*), but there are other diseases in which cholesterol and its regulation are involved. Examples include Alzheimer disease (*4*), cholesterol gallstone disease (*5*), Tangier Disease (*6*), conditions associated with dyslipidaemia (*7*), and potentially cancer (*8*). Thus, given the abundance of cholesterol-mediated physiological processes, cells have evolved pivotal mechanisms that engage in the strict regulation of cholesterol homeostasis.

Dietary cholesterol is continuously processed and circulated – from the intestinal cells, cholesterol is assembled into chylomicrons (lipoproteins) and subsequently assimilated into very low density lipoprotein (VLDL) particles by liver cells (*9*). VLDLs become precursors to low density lipoproteins (LDL), which develop into a key source of cholesterol for peripheral cells. Full circulation of the cholesterol molecules is then completed when reverse transport back to the liver in the form of high density lipoproteins (HDLs) occurs (*9*).

While this represents one form of regulation for dietary cholesterol, the sterol regulatory element binding protein (SREBP) pathway exhibits a different, yet important mode of cholesterol regulation. The SREBP pathway is an essential transcriptionally controlled, cholesterol feedback system that is centered in the endoplasmic reticulum (ER) of mammalian cells. SREBPs belong to a family of transcription factors, whose inactive precursors are embedded within the ER membrane. With the responsibility to regulate genes involved in cholesterol biosynthesis and uptake, three major SREBP isoforms have been identified in mammalian cells – SREBP-1a, SREBP-1c, and SREBP-2. Each exerts a distinct response to activate genes related to cholesterol, fatty acid and triglyceride biosynthesis (*10*). Brown and Goldstein have elucidated the mechanism by which the SREBPs turn on cholesterol synthesis in

sterol-depleted cells, as well as deactivate SREBP-responsive genes in adverse conditions (*11*). In cholesterol-deprived conditions, signals send the SREBP precursor to the Golgi, where it undergoes two proteolytic events. The cleavages liberate the mature SREBP transcription factor that is now capable of activating a wide variety of SREBP-responsive genes in the nucleus. In the case of cholesterol biosynthesis, only the SREBP-1a and -2 isoforms are concerned. (*10*) Other SREBP-mediated products include fatty acids and triglycerides, and it is the isoform in action that differentiates the genes to be stimulated. One crucial product of SREBP-2 activation is 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase). This enzyme catalyzes the rate-limiting step of producing mevalonate – the precursor that gives rise to the production of cholesterol and a variety of isoprenoid compounds (*12*). Other SREBP-2 responsive genes include those that encode for HMG CoA synthase, farnesyl diphosphate synthase, squalene synthase, and LDL receptors that endocytose cholesterol delivered by LDLs (*10*). Thus, with the wide range of effectors expressed upon SREBP activation, the significance of these transcription factors is clearly evident for the synthesis of critical membrane lipid constituents.

Two additional components are functionally needed for the establishment of cholesterol homeostasis in this pathway: the SREBP Cleavage Activating Protein (SCAP) and the Insulin induced gene (INSIG) protein. SCAP was first characterized in sterol-resistant mutants, as an ER membrane protein that contained five sterol-sensing domains (SSD) (*13*). Because the mutant strains did not exhibit the regular phenotype of inhibiting cholesterol biosynthesis in sterol-abundant situations, it was determined that SCAP is necessary for proper SREBP regulation (*13*). Structurally, C-terminal WD repeats in SCAP impart its ability to associate with the C-terminal regulatory domains of SREBP (*14*). Formation of this complex is important for two roles of SCAP – a SREBP escort protein and a sterol sensor. In times of cholesterol deprivation, SCAP leads the passage of SREBP from the ER to the Golgi (*15*) by means of vesicle budding (*16*). This initiates the first SREBP cleavage reaction (*17*), which is also dependent on the establishment of an SREBP-SCAP complex (*18*, *19*). In sterol-excessive conditions, cholesterol binds with high affinity and specificity to the SSD of SCAP (*20*). This triggers a conformational change that attracts INSIG to bind to the SREBP-SCAP complex (*21*). INSIG is an ER resident membrane protein that withholds the release of SCAP (and thus SREBP) from the ER, via its association with the SSD of SCAP (*22*). Formation of the ternary INSIG-SCAP-SREBP complex

in the ER prevents the transport of SREBP to the Golgi for proteolytic activation. Consequentially, this results in a global reduction of cellular cholesterol levels.

Studies have been conducted to investigate the cholesterol concentration from which deviations would drive the SREBP-SCAP complex to diverge into two antagonistic paths – transport-dependent SREBP activation or ER retention. Radhakrishnan et al. have demonstrated that SREBP-2 transport to the Golgi is precisely and acutely disrupted once ER cholesterol levels exceed 4 – 5 mol% (*23*). This switch-like response in the ER is further reproduced in experiments that utilize *Perfringolysin O* (PFO) as a sterol sensor analogue of SCAP (*24*). PFO, is one of the many pore-forming toxins in the family of cholesterol-dependent cytolysins (CDCs) (*25*). It is secreted by the Gram-positive bacterium *Clostridium perfringens* (*26*). The soluble PFO monomers have four domains, in which domain 4 of the C-terminal region is indispensable for cholesterol recognition and interaction (*27*). Once in contact with cholesterol, a large pre-pore complex – an oligomer that comprises of 24 – 50 PFO monomers, is formed and poised above the membrane (*28*). A subsequent vertical collapse in domain 2 brings domain 3 into close proximity with the membrane (*29*), and as this occurs, the six α-helices within domain 3 are simultaneously rearranged to form a pair of amphipathic β-hairpins. With the change in its structural properties, domain 3 inserts into the membrane in conjunction with other monomeric counterparts to form a large oligomeric β-barrel pore (*29*).

One sequence of PFO that has been commonly exploited experimentally is its six tryptophan residues located in domain 4 (*24*, *27*, *30*). This allows for the detection of PFO-cholesterol binding through an increase in the Trp fluorescence intensities. With the use of PFO as a probe and mimic for SCAP, Sokolov et al. re-generated the switch-like response at 5 mol% cholesterol in ER liposomes (24). Specifically, purified ER membrane lipids were extracted to build the ER liposomes, which lacked many of the purified ER membrane proteins. These results suggest that the enhanced chemical activity of cholesterol is an effect of the ER membrane phospholipid composition (*24*). In vitro studies have shown that PFO binding, oligomerization and pore formation are enhanced in lower pH (5.1) conditions (*30*). However, even at pH 5.1, the lowest cholesterol concentration required to induce PFO binding to vesicles was observed to be 10 – 15 mol% in DiPhyPC (1,2-diphytanoyl-*sn*-glycero-3-phosphocholine)/cholesterol mixtures (*30*). As a result, the 5 mol% cholesterol concentration at which PFO binds to cholesterol

(reflecting cholesterol-SCAP binding) has never been observed in model membranes of defined lipid composition, and the lipid composition that produces this effect remains unresolved.

The enhanced cholesterol accessibility to PFO or SCAP in ER membranes can be clarified by the active cholesterol hypothesis. Within membranes, cholesterol molecules choose the phospholipids they interact with, based on their structural features. The strength of cholesterol-phospholipid interactions was outlined to weaken in the following order: sphingomyelin (SM) > phosphatidylcholine (PC) > phosphatidylserine (PS) > phosphatidylethanolamine (PE) (*31*). The structural reasoning for this order may be partly explained by a decrease in head group size (from PC to PE). However, because SM shares the same head group size as PC, other effective variables, such as phospholipid acyl chain length and degree of unsaturation, are required to account for this difference (*32*). Thus, the combined chemistry of different head groups and phospholipid acyl chains confer different levels of van der Waals, hydrophobic and hydrogen bonding stabilizations (*33*). Two models have been proposed to explain for the preference indicated above. Cholesterol may prefer to shelter under the umbrella provided by the large head groups, as specified by the umbrella model (*34*). Alternatively, cholesterol can form condensed complexes with the selected phospholipid structures (*35*), and remain locked in place until a threshold complexing capacity is reached. When cholesterol levels exceed the complexing or umbrella capacity, free and uncomplexed cholesterol units emerge to physically signal downstream regulatory pathways (*36*).

Furthermore, this enhanced chemical activity of cholesterol outlines its importance in activating the SREBP pathway. There is a dramatic difference in the amount of total cellular cholesterol that is held in the ER (0.5%) (*37*) as compared to that observed for the plasma membrane (25 – 40%) (*38*). This disparity is further emphasized by studies that show a large 10-fold increase in ER cholesterol can result from a small 1.5 fold increase in the plasma membrane (*37*). Specifically, this illustrates how small fluctuations in plasma membrane cholesterol levels can easily activate a much larger portion of cholesterol molecules in the ER. With the release of free ER cholesterol, cholesterol becomes furnished with additional exposure for SCAP binding. Aiming for cholesterol homeostasis, sterol-dependent conformational changes in SCAP will thus undertake the initial responsibility of restoring the optimal cholesterol-fatty acid ratio in membranes (*39*, *40*).

Another essential function that intersects at the ER is the synthesis of triacylglycerol (TAG) (*41*), and its subsequent transport into lipid droplets and nascent VLDLs (*42*). The key enzyme for TAG biosynthesis - acyl-CoA:diacylglycerol acyltransferase (DGAT), resides in the ER membrane and catalyzes the esterification of diacylglycerol with acyl-CoA (*43*). Mainly functioning as an energy storage molecule, triglycerides are also regulated by the SREBP pathway, exclusively by the SREBP-1a and 1c isoforms (*10*, *44*). In a hypothetical model, triglycerides generated from the DGAT esterification reaction are temporarily released into the ER membrane before subsequent relocation to lipid droplets or lipoproteins (*45*). Grasping onto the concept that triglycerides are highly hydrophobic molecules, we hypothesized that their brief existence in the ER membrane may induce cholesterol displacement from their phospholipid complexes. In terms of the umbrella model (*34*), triglycerides may deprive the small polar head groups of cholesterol from protection by the large phospholipid head groups. Hence, this disruption induces an increase in cholesterol exposure and accessibility to external activity.

A number of investigations have already identified molecules capable of displacing sterols from their phospholipid complexes to increase their cholesterol chemical activities. These molecules are termed as amphipaths or membrane intercalators that structurally resemble cholesterol in terms of having a small polar head group and a hydrophobic body. Examples include ceramides (*46*, *47*), alkanols (*39*, *48*), 1,2-dioctanoyl-*sn*-glycerol (*49*), saturated and unsaturated fatty acids, fatty acid methyl esters, terpenes, sphingosine derivatives, alkyl ethers, ketones, aromatics and cyclic alkyl derivatives (*50*, *51*). We have performed a series of in vitro experiments to test whether certain lipid membrane compositions would reconstitute the degree of ER cholesterol accessibility. PFO vesicle-binding experiments have been carried out in different phospholipid mixtures – DOPC, DOPC/POPE/POPS (1:1:1), and POPE/POPS (1:1)[1]. The lowest cholesterol concentration needed for PFO binding was 4.2 mol% in PE/PS mixtures. A cholesterol level of 7.9 and 13.2 mol% was required for PFO binding to PC/PE/PS and PC vesicles, respectively. In addition to these phospholipid mixtures, we have separately incorporated 5 mol% of triglycerides (triolein or tripalmitin), diglycerides (1-palmitoyl-2-oleoyl-*sn*-glycerol [PO glycerol] or dipalmitin) and fatty alcohols (tetradecanol, octadecanol, oleoyl alcohol) to each lipid composition.

Here, we further characterize the cholesterol-displacing characteristics of triglycerides,

[1] Abbreviations: DOPC – 1,2-dioleoyl-*sn*-glycerol-3-phosphocholine; POPE – 1-palmitoyl-2-oleoyl-*sn*-glycero-3-

diglycerides and alkanols by providing estimates for the concentration at which cholesterol transitions from a complexed to an active state. The lowest cholesterol concentration we have managed to achieve for PFO binding, was 1.9 mol% in PE/PS mixtures containing 5% triolein. We tested diglycerides based on the rationale that DGAT substrates are embedded in the ER membrane prior to esterification (*45*). The assimilation of 5% PO glycerol in PC/PE/PS reduced the cholesterol need of 7.9 mol% to 5.1 mol%. This 35% difference in the switch-point for PFO binding was the greatest we have attained with diglycerides. To summarize, in hopes of reproducing the 5 mol% cholesterol switch-point, we have used PFO as a mimic for SCAP to probe for the emergence of active cholesterol in various lipid mixtures. An approximate value of the concentration required for greater cholesterol exposure and accessibility has been quantitated.

These results may contribute new insights to the development of future cholesterol-lowering methods. A potential strategy for treating harmful sterol-excessive conditions may involve the manipulation of HMG CoA reductase (HMGR) levels in the cell. Among the diverse selection of amphipaths tested, many were found to lower HMGR activity (*50*). Given that HMGR regulates a critical checkpoint for the mevalonate pathway (*12*), a reduction in its activity would profoundly limit the synthesis of its downstream lipid products. Assuming that our experiments imitate the nature of cholesterol regulation of the SREBP pathway, fatty intercalants may similarly exert a minimizing effect on HMGR activity. Undesirable accumulation of cholesterol and certain lipid components may be restricted and reduced. At the level of the ER membrane, introduction of slightly higher concentrations of triglycerides, diglycerides or other fatty intercalants may possibly lower the productive output of the mevalonate pathway, and thus restore the homeostatic lipid concentrations.

Experimental Procedures

Materials

All phospholipids: 1,2-dioleoyl-*sn*-glycerol-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phosphor-L-serine] (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycerol (PO glycerol) and cholesterol, were purchased from Avanti Polar Lipids (Alabaster, AL). Triolein was acquired from Supelco Analytical (Bellefonte, PA), and the remaining membrane intercalators – tripalmitin, dipalmitin (mixture of ~ 50% 1,2- and 50% 1,3- isomers), tetradecanol, octadecanol and oleoyl alcohol were purchased from Sigma-Aldrich (St. Louis, MO). Lipids were diluted and stored in ethanol or chloroform at –20 °C and lipid concentrations were determined by dry weight. TALON bead resin was purchased from Clontech (Mountain View, CA). All other chemicals were reagent grade.

PFO C459A E167C is a fully functional and active, cysteine-less derivative of wild-type PFO in which C459 is substituted with an alanine residue and E167 is mutated to C for labeling purposes (*52*, *53*). This wild-type PFO derivative was expressed as reported previously (*28*), and a modified purification was performed (*30*). An overnight culture of BL21 DE3 pLysS competent cells (Stratagene, Cedar Creek, TX) transformed with the WT PFO C459A E167C plasmid (*53*), was grown in 100 ml of Luria-Bertani (LB) medium containing 100 μg/ml of ampicillin and 35 μg/ml of chloramphenicol. The culture was used to inoculate 2 L of LB medium containing 50 μg/ml of ampicillin, and the *E. coli* cells were grown until an A_{600} of 0.5 – 0.6 was reached. Upon induction of PFO expression by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3 hours, the *E. coli* cells expressing PFO were pelleted at 4 °C. Following overnight storage in – 80 °C, cell pellets were resuspended in 30 ml of NiA buffer (10 mM MES, 150 mM NaCl, pH 6.5) containing 150 μg/ml phenylmethylsulfonyl fluoride and 100 μg/ml chicken egg white lysozyme (Sigma-Aldrich). The bacterial suspension was incubated at room temperature for 30 min on a shaker and subsequently lysed by tip sonication with a cell disruptor (Heat Systems, Ultasonics, Inc, Plainview, NY). 15 s of sonication was followed by 15 s of cooling on ice, and this was repeated for two more times. After centrifugation (Dupont RC-5 centrifuge) at 15,000 rpm in a SS-34 rotor at 4 °C, the supernatant was mixed with 3 ml of

TALON metal affinity resin for 20 min at room temperature. Next, the TALON beads were pelleted with a tabletop centrifuge and added to a 0.8 × 4 cm poly-prep plastic column (BioRad, Hercules, CA). The beads were washed with ~ 5 – 10 mls of NiA buffer and then 1-ml aliquots of NiA buffer containing the following order of imidazole concentrations: 50 mM, 100 mM, 400 mM (3 aliquots), and 1M. PFO was usually obtained from the aliquots eluted with 400 mM imidazole. The 400 mM imidazole fractions were pooled and dialyzed overnight against 4 liters of HP Seph A buffer (10 mM MES, 1 mM EDTA, pH 6.5) containing 0.5 mM DTT (1,4-dithiothreitol; Roche Diagnostics, Indianapolis, IN). On the next day, gravity anion-exchange chromatography was performed. The dialyzed fractions were added to a 0.8 × 4 cm poly-prep plastic column loaded with SP-Sephadex resin (GE Healthcare, Piscataway, NJ). 1-ml aliquots of HP Seph A buffer containing increasing concentrations of NaCl were added to the column, increasing NaCl in 100 mM steps from 0 to 1 M with duplicate aliquots at 300 and 400 mM NaCl. PFO was eluted in the 300 and 400 mM fractions and the collected fractions were dialyzed overnight against 4 liters of PBS (10 mM sodium phosphate, 1 mM potassium phosphate, 137 mM sodium chloride, 13 mM potassium chloride) containing 0.5 mM DTT. Protein concentrations were derived from Beer's law after the A_{280} of the dialyzed fractions was measured (molar extinction coefficient = 74,260 $M^{-1}cm^{-1}$, molecular weight = 54,000 g/mol). Finally, the purified protein aliquots were stored at –20 °C.

Vesicle Preparation

Small unilamellar vesicles (SUV) were prepared at a concentration of 100 μM lipid in PBS (pH 5.1) by ethanol dilution. Similar to the protocol described previously (*47*), the desired concentration and combination of lipids dissolved in organic solvent were mixed and dried with 5 min of N_2 gas and 1 h of high vacuum. The dried lipids were briefly heated in a 70 °C water bath (10 min), and subsequently dissolved in 80 μl of ethanol. The ethanol-dissolved lipids were diluted by 50-fold upon addition of 4 ml of pre-heated PBS buffer (70 °C, pH 5.1), and were briefly vortexed. After the vesicles cooled to room temperature and were evenly distributed into 4 test tubes (1 ml each), the SUVs were ready for vesicle binding experiments.

Vesicle Binding Experiments

To probe for PFO-vesicle interactions, the intrinsic increase in Trp emission intensity was measured. This monitors an association between the domain 4 Trp residues and the cholesterol-containing membranes (*54*). The fluorescence experiments were performed by incubating 5 μg of PFO (from a stock solution containing ~ 1 – 2 mg/ml of protein) with 1 ml of SUVs prepared from the desired lipids. After 1.5 – 2 h of incubation at room temperature, fluorescence emission intensity was measured on a SPEX Fluorolog 2 spectrofluorimeter. To monitor tryptophan fluorescence, measurements were made with an excitation wavelength of 280 nm and an emission wavelength of 340 nm. Triplicate samples were prepared along with a single background control sample containing vesicles only (not incubated with PFO).

The fluorescence measurements of the control were subtracted from the corresponding sample readings, and the value for each sample was divided by the measurements obtained from cholesterol-free samples. This normalizes the Trp emission intensity to a value of 1 in the absence of cholesterol, and the values obtained for triplicate samples were averaged. Finally, the averaged sample points were fit to a sigmoidal curve, using the SlideWrite Plus program. For comparative purposes, the cholesterol concentration at which the increase in fluorescence emission intensity is half-maximal was identified. This is referred to as the threshold concentration for PFO binding and its value was defined by the equations (also determined with SlideWrite Plus) derived from the sigmoidal curves.

Results

A lower cholesterol concentration is required for PFO binding in vesicles containing POPE and POPS.

The cytoplasmic leaflet of the plasma membrane is preferentially enriched in phosphatidylethanolamine (PE) and phosphatidylserine (PS) (*55*), and the ER membrane is believed to have a similar composition. Therefore, PE and PS were incorporated into SUVs to prepare vesicles that are a rough imitation of the ER membrane. Binding of PFO to vesicles containing a DOPC/POPE/POPS (1:1:1) or a POPE/POPS (1:1) mixture was studied. Results were compared with those derived with vesicles containing DOPC only. Similar studies with vesicles comprised solely of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) were previously performed (*30*) and characterized to require a much higher cholesterol concentration for PFO binding. As more cholesterol was incorporated, we obtained a switch-like increase in Trp emission intensity for each composition, which corresponds to PFO binding. The main difference observed with different lipids was the cholesterol concentration at which PFO was bound. We refer to this cholesterol concentration as the threshold value for PFO binding. With greater PE and PS fractions within vesicles, lower concentrations of cholesterol were required for PFO binding.

As depicted in Fig. 1, an increase of PE and PS content in SUVs resulted in a reduction of the cholesterol threshold concentration. Individual curves and error bars generated for each lipid composition are shown in the Appendix section (Fig. S1), and Table 1 describes the threshold-lowering effects of incorporating PE and PS into PC-containing vesicles. In contrast to the requirement for ~ 13 mol% cholesterol for PFO binding to DOPC vesicles, a low threshold observed was ~ 4 mol% in vesicles comprised purely of PE/PS (1:1). Showing intermediate behavior, a mixture of PC/PE/PS (1:1:1) exhibited PFO binding at ~ 8 mol% cholesterol. Therefore, the addition of PE and PS into PC-containing vesicles profoundly reduces the cholesterol threshold concentration by 40%, and another 46% decrease results from the complete removal of PC from PC/PE/PS vesicles.

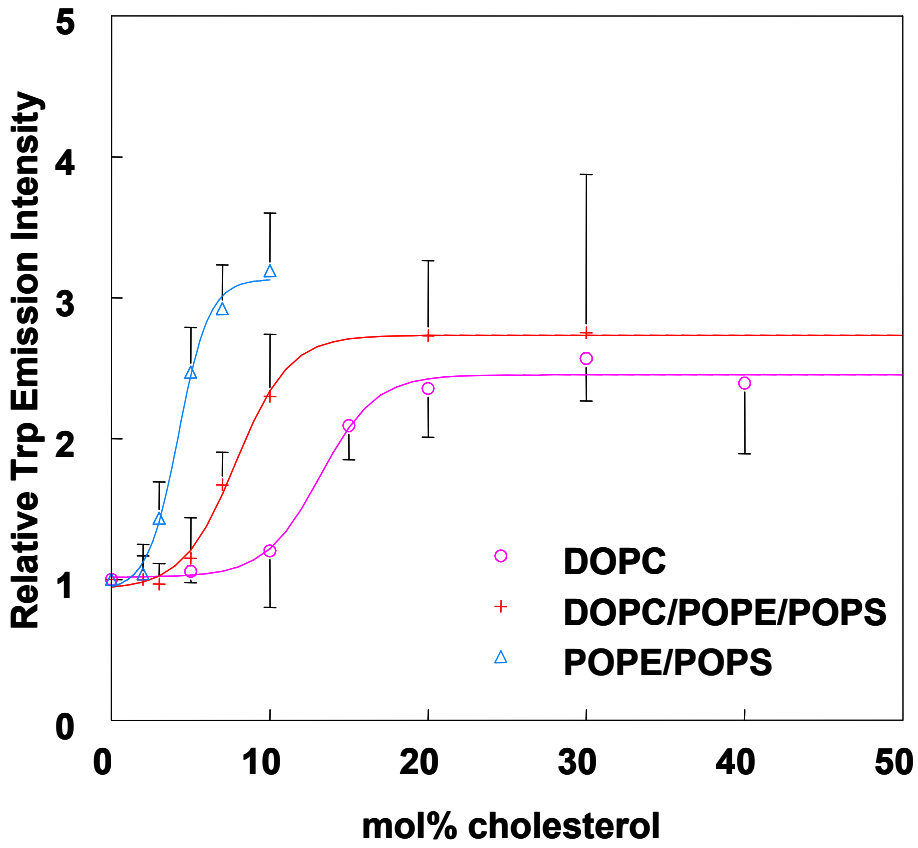

Figure 1 - Effect of different lipid compositions upon Trp emission intensity versus cholesterol concentration at pH 5.1. 100 μM SUVs were prepared from DOPC, DOPC/POPE/POPS (1:1:1) or POPE/POPS (1:1) lipid mixtures containing an increasing mol% of cholesterol, by ethanol dilution. After vesicle incubation with 5 μg of PFO for 2 h, Trp fluorescence was measured to assess PFO binding. Cholesterol-free sample readings were normalized to a value of 1, and the relative Trp emission intensities were plotted to fit a sigmoidal curve. Each sample point represents the average of multiple experiments (each consisting of triplicate samples) performed on separate days, and the cholesterol threshold concentrations were derived from the equations given by the curves.

Cholesterol-displacing effects of triglycerides, diglycerides, and fatty alcohols in DOPC/POPE/POPS membrane vesicles.

With the objective of reconstituting the 5 mol% threshold concentration observed in ER liposomes prepared from purified ER membrane lipid extracts (devoid of many ER membrane proteins), we have added a number of membrane-intercalating lipid molecules to vesicles. In SUVs containing an increasing mol% of cholesterol in DOPC/POPE/POPS, 5% of the aforementioned triglycerides (triolein, tripalmitin), diglycerides (PO glycerol, dipalmitin), and alkanols (tetradecanol, octadecanol, oleoyl alcohol) were included. Fig. 2 illustrates their threshold-lowering effects compared to the control vesicles (containing PC, PE, and PS only) in PFO binding experiments.

Fig. 2A shows the differential effects of the triglycerides that vary by the degree of acyl chain unsaturation. Tripalmitin, consisting of three saturated acyl chains, exhibited no significant effect in exposing cholesterol for PFO binding. However, the three mono-unsaturated fatty acyl chains of triolein seem to have lowered the threshold concentration by 25% (a difference of ~ 2 mol% cholesterol). An impact of acyl chain unsaturation was also observed for diglycerides as dipalmitin and PO glycerol enhance cholesterol accessibility by 20 and 35%, respectively. In other words, the substitution of one saturated acyl chain with one mono-unsaturated chain allowed for a threshold reduction of ~ 1 mol% (Fig. 2B). Similarly, a ~ 1 – 2 mol% decrease in cholesterol threshold concentration was witnessed for experiments involving the addition of fatty alcohols. This translates into a ~ 25% reduction in the threshold concentration when 5 mol% tetradecanol or octadecanol, respectively, was included. However, 5% oleoyl alcohol did not manifest as significant a degree of threshold-reduction, as it only induced a 10% decrease from the control (Fig. 2C). All cholesterol threshold concentrations and their percentage decrease from the relative control value (without fatty intercalants) for this and the following sets of experiments, are summarized and compared in Table 1. In summary, the lipid molecules presented above (with the exception of tripalmitin and oleoyl alcohol) increased cholesterol exposure and accessibility to PFO monomers.

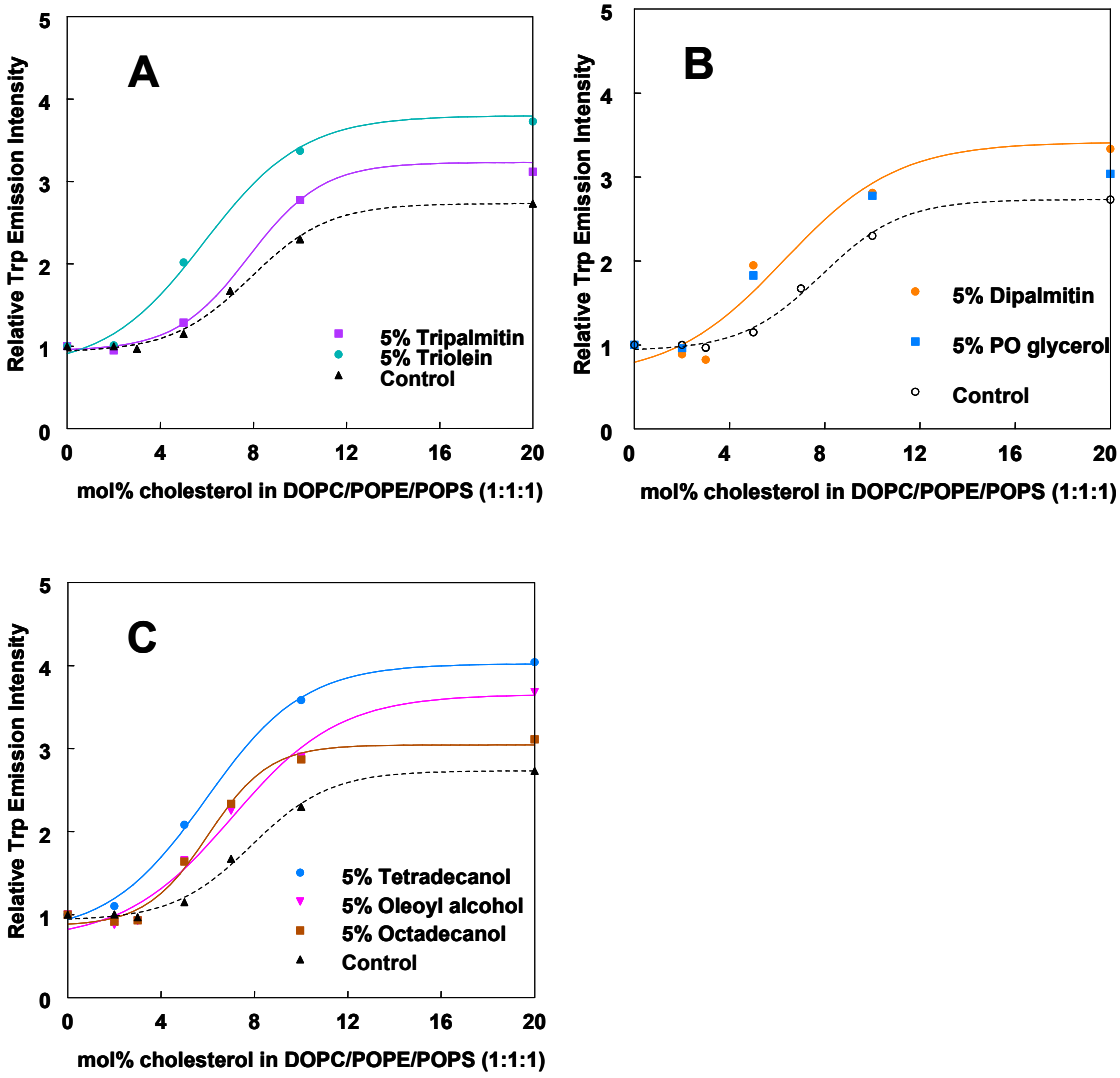

Figure 2 – PFO vesicle binding experiments with triglycerides, diglycerides, and fatty alcohols in DOPC/POPE/POPS (1:1:1) SUVs. Cholesterol displacement effects of 5 mol% (A) tripalmitin and triolein, (B) dipalmitin and PO glycerol, and (C) of tetradecanol, oleoyl alcohol and octadecanol. Experiments were performed as described in Fig. 1, with the addition of fatty intercalants into membranes. The fluorescence measurements were similarly processed to fit a sigmoidal curve, from which the cholesterol threshold concentration was determined. (Individual plots containing error bars for each experiment are provided in the Appendix section, along with the equation from which we derived the cholesterol threshold concentrations (Fig. S3))

PE/PS vesicles containing membrane intercalators require the least amount of cholesterol for PFO binding.

A different set of PFO binding patterns were observed when the triglycerides, diglycerides and fatty alcohols were present in POPE/POPS vesicles lacking DOPC (Fig. 3). Both types of triglycerides – triolein and tripalmitin significantly reduced the cholesterol threshold, down to 1.9 and 2.8 mol% cholesterol, respectively (Fig. 3A). Upon inclusion of 5% triolein, the threshold value of ~ 4 mol% cholesterol in PE/PS membranes (control) was brought down by 50% to a value of ~ 2 mol% cholesterol. This was the lowest cholesterol concentration we have managed to achieve for PFO binding. Interestingly, the effect conferred by the degree of acyl chain unsaturation was reversed for the diglycerides. Instead of PO glycerol conferring a lower sterol requirement in PC/PE/PS membranes, we found dipalmitin to have a greater threshold-reducing effect (Fig. 3B) with ~ 1 – 2 mol% less cholesterol needed for PFO-vesicle association. This is equivalent to a threshold-lowering effect of 12% (PO glycerol) and 47% (dipalmitin) from their control values. Due to the very low molar requirement of cholesterol for PFO binding, samples incorporating 5% dipalmitin did not exhibit a sigmoidal curve. In cases where a sigmoidal curve would not fit, a 1-site ligand curve was selected to fit the sample points. Assuming that Trp emission intensity no longer increases after 10 mol% cholesterol, the midpoint between the lowest (Trp fluorescence observed at 0 mol% cholesterol) and the highest (saturated) y-value was selected, and the corresponding x-value was quantitated as the threshold concentration. Amongst the fatty alcohols, tetradecanol enhanced cholesterol exposure most profoundly. 5% tetradecanol shifted the threshold concentration by 30%, from ~ 4 to 2.9 mol% cholesterol (similar to dipalmitin, results for samples containing 5% tetradecanol also required a non-sigmoidal 1-site ligand fit). Octadecanol exhibited less of an effect, decreasing the value to 3.5 mol% (an 18% decrease from the control), while oleoyl alcohol displayed no significant effect (Fig. 3C). In summary, without PC inclusion in membranes, PE and PS phospholipids facilitated PFO binding at lower cholesterol concentrations and provided additional freedom for certain membrane intercalators to exert a threshold-reducing effect.

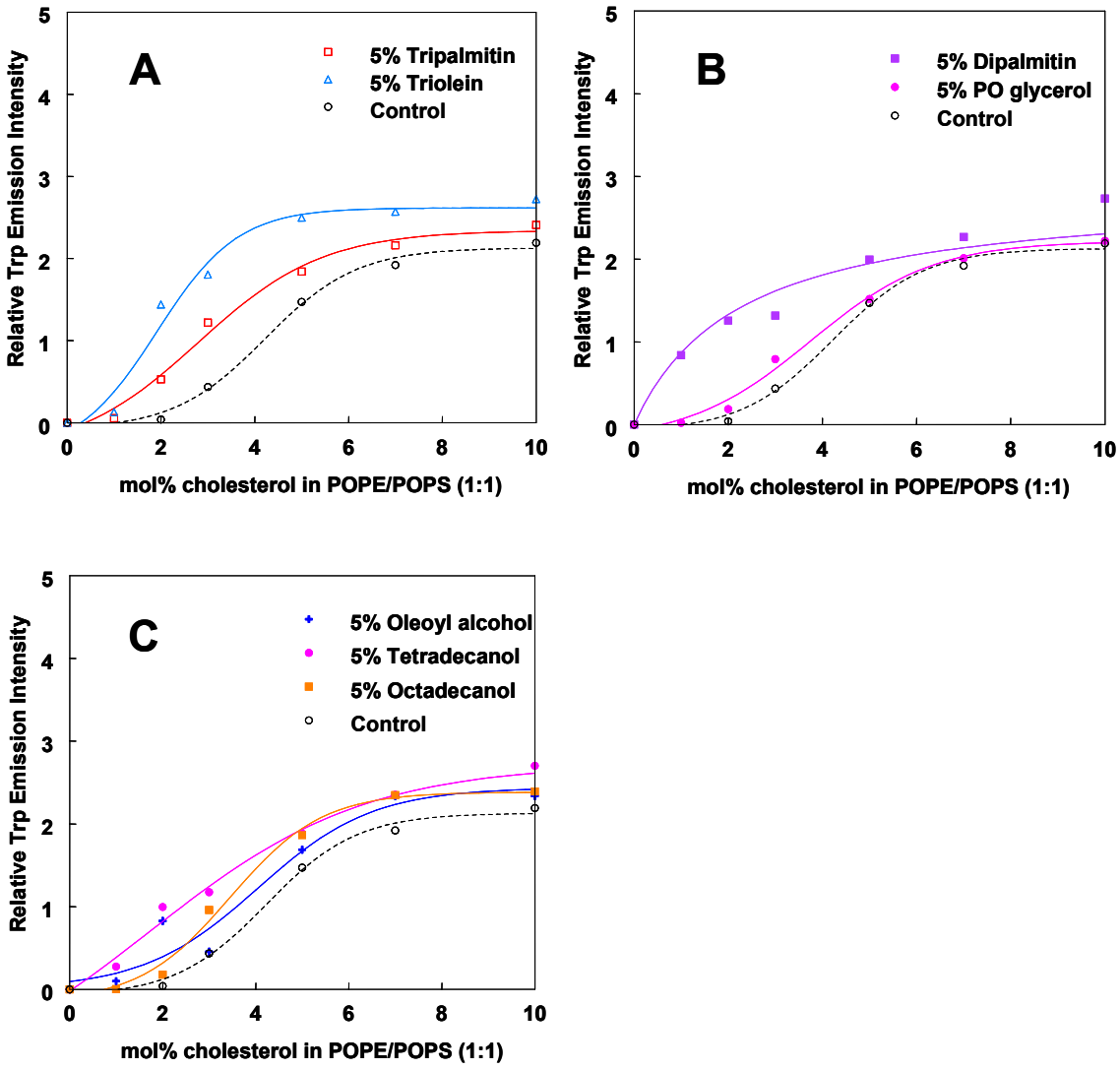

Figure 3 – PFO vesicle binding experiments with triglycerides, diglycerides, and fatty alcohols in POPE/POPS (1:1) SUVs. Threshold-reducing effects of 5 mol% (A) tripalmitin or triolein, (B) dipalmitin or PO glycerol, and (C) of oleoyl alcohol, tetradecanol or octadecanol, in comparison to their respective controls. Experiments were carried out as specified in Fig. 1, and relative Trp emission intensities were similarly derived for data sets that matched to a sigmoidal curve. In cases where the sample points did not fit a sigmoidal curve (dipalmitin and tetradecanol), a 1-site ligand curve was used to assess the threshold value. To adhere to the requirements of a 1-site ligand curve, all relative Trp emission intensities were subtracted with the value obtained from cholesterol-free samples, resulting in a value of zero in the absence of cholesterol. By finding the midpoint (on y-axis) between zero and the saturated value for Trp emission intensity, the corresponding x-value for the midpoint was determined as the cholesterol threshold concentration. (Individual plots containing error bars for each experiment are provided in the Appendix section, along with the equations from which we derived the cholesterol threshold concentrations (Fig. S5))

Triglycerides, diglycerides and fatty alcohols exhibit no threshold-lowering effects in DOPC vesicles.

The effects of including the described intercalators into vesicles composed of large-headed phospholipids (DOPC) seemed to be none or small (Fig. 4). Instead of reducing the threshold concentration, these molecules seem to have no effect or raised the concentration at which PFO binds. In the case of triolein (Fig. 4A), there was a 33% increase from the control threshold for the Trp emission intensity to increase (experiments for tripalmitin were not performed). However, this may be inaccurate given that the 15 mol% cholesterol point was not tested for the effects of triolein. For diglycerides, no significant deviations from the control threshold concentration were observed (Fig. 4B). While tetradecanol raised the onset value by 21%, oleoyl alcohol and octadecanol did not demonstrate any substantial change in the cholesterol threshold concentration (Fig. 4C). Whether the presence of 5% triolein or tetradecanol have a significant adverse effect on the threshold or not, it is evident from these results that the tested intercalators have no threshold-lowering effects in DOPC membranes.

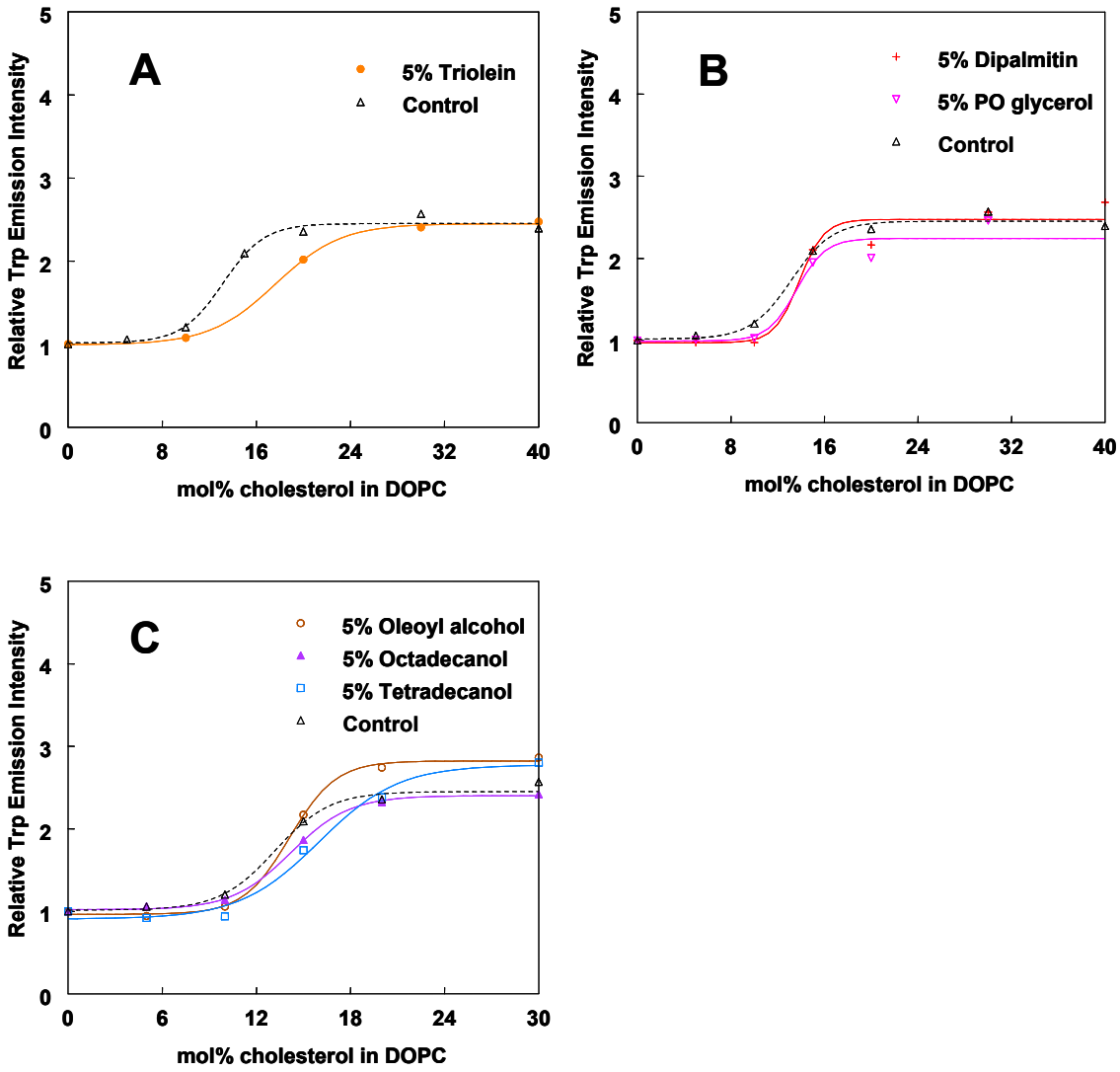

Figure 4 – PFO vesicle binding experiments with triglycerides, diglycerides, and fatty alcohols in DOPC SUVs. Effects of incorporating 5 mol% of (A) triolein, (B) dipalmitin and PO glycerol, and (C) oleoyl alcohol, octadecanol and tetradecanol into vesicles containing DOPC only. Experiments were performed as described in Fig.1, and cholesterol threshold values were similarly obtained. (Individual plots containing error bars for each experiment are provided in the Appendix section, along with the equations from which we derived the cholesterol threshold concentrations (Fig. S2))

In summary, it has been demonstrated that there is a proportional relationship between POPE/POPS levels and the extent of cholesterol exposure or accessibility. As the presence of POPE and POPS increases within membranes, there is a corresponding reduction in the cholesterol threshold concentration. The threshold value was highest in SUVs containing DOPC only, and addition of membrane-intercalating compounds was unable to further expose cholesterol to PFO monomers. When equal molar amounts of PE and PS were integrated with PC, the chemical environment of the vesicles has shown to improve PFO binding at lower cholesterol concentrations. To different degrees, cholesterol was rendered more susceptible to PFO binding in the presence of certain triglycerides, diglycerides or fatty alcohols. With the exception of tripalmitin and oleoyl alcohol, a substantial drop in the threshold concentration was observed in PC/PE/PS SUVs. Lastly, vesicles lacking PC head groups displayed the most dramatic change in cholesterol exposure. The lowest cholesterol concentration at which PFO would bind was ~ 2 mol%, and this was attained via the incorporation of 5% triolein or dipalmitin. While other compounds also exerted some influence, oleoyl alcohol once again appears to have the least effect in threshold-reduction.

	DOPC	DOPC/POPE/POPS (1:1:1)		POPE/POPS (1:1)	
	Threshold (mol%)[2]	**Threshold (mol%)[2]**	**% decrease[3]**	**Threshold (mol%)[2]**	**% decrease[3]**
Control	13.2	7.9	-	4.2	-
Triolein	17.6	5.9	25.2	1.9	54.7
Tripalmitin	nd[4]	7.8	0.9	2.8	33.8
PO glycerol	13.6	5.1	35.4	3.8	11.3
Dipalmitin	13.9	6.3	19.9	2.2	46.9
Oleoyl Alcohol	14.1	7	10.5	4	4.3
Tetradecanol	16	5.9	24.7	2.9	30.4
Octadecanol	14.2	6	23.7	3.5	18.2

Table 1 - Summary of effects upon cholesterol threshold values of 5 mol% triglycerides, diglycerides and fatty alcohols in DOPC, DOPC/POPE/POPS and POPE/POPS membrane mixtures, as well as the percentage decrease in cholesterol threshold concentration relative to the control value induced by the incorporation of 5 mol% fatty intercalants in DOPC/POPE/POPS, or POPE/POPS SUVs.

[2] Threshold (mol%): Cholesterol threshold concentration in mol%.

[3] % decrease: Percentage decrease in cholesterol threshold concentration from the control threshold value (%).

[4] Nd = not determined

Discussion

Various biosynthetic processes for complex lipids, including PC, PE, PS, cholesterol, cholesteryl esters and ceramide, all intersect at the ER membrane (*41*). Our studies have clearly demonstrated the influence of PE and PS incorporation into membranes. Phosphatidylethanolamine (PE) and phosphatidylserine (PS) are two aminophospholipids that not only play a structural role in membranes, but they are also extensively involved in important biological processes (*56*). Along with phosphatidylcholine (PC), PE is synthesized from diacylglycerols, and PS is subsequently derived from PE. Within cells, PC is the most abundant phospholipid, representing 40 – 50% of total phospholipids. PE is second, constituting 20 – 50% of total phospholipids in mammalian cell membranes, and PS is least abundant at 2 – 10% of the total. While PC predominantly occupies the outer leaflet of the plasma membrane with sphingomyelin, more than 80% of total PE and PS are observed to localize to the inner leaflet (*56*). Due to the lack of precise knowledge for the ER membrane composition, our studies assume that the cytoplasmic leaflet of the ER membrane is similar to that of the plasma membrane, by means of endocytotic processes.

To reflect this asymmetry, we constructed model membranes containing mixtures of PC, PE and PS to represent the cytoplasmic leaflet of the ER membrane (and/or plasma membrane). Vesicles of assorted lipid compositions were combined with the cholesterol binding activity of PFO to probe the chemical activity of cholesterol. Previous work by Nelson et al. established a threshold concentration of 15 – 20 mol% cholesterol, for PFO binding in DOPC membranes at pH 5.1 (*30*). A slightly lower but similar threshold value of 13 mol% was obtained with the same conditions in our experiments. This value was then used as a comparative tool to discern the threshold-reducing effects of PE and PS. A 68% decrease from the DOPC threshold was attained in vesicles made solely out of PE and PS. Inclusion of PC with the aminophospholipids gave an intermediate threshold value of ~ 7.8 mol%, which is a 40% decrease from the control (DOPC).

To explain this behavior, two models for lipid packing – the umbrella model and the condensed complexes model can be employed. The small polar head group of cholesterol structurally requires neighboring lipids to help prevent unfavorable contacts between the large hydrophobic ring system and the surrounding polar environment (*34*). In this context,

phospholipids equipped with a large head group may be the preferred candidate for cholesterol interactions. Thus, acting as an umbrella to shield the fused hydrophobic rings, the large-headed PC lipid can provide protection for cholesterol. Because cholesterol molecules remain unexposed by hiding under PC head groups, PFO binding would not occur until the cholesterol concentration surpasses the umbrella capacity of PC. In the case of phospholipids containing small head groups, PE and PS lack the ample shielding capacity of PC. They may shield one cholesterol molecule, but this protection is minimal (*9*). Thus, reduced protection translates into more cholesterol exposure, and this quality is exploited by PFO monomers to bind at lower cholesterol concentrations.

According to the condensed complex model, cholesterol preferentially forms reversible condensed complexes with PC than with PE or PS (*31*, *35*). There is a threshold complexing capacity that sets the limit for cholesterol-phospholipid interactions. When sterol concentrations are below the threshold value, cholesterol is part of a condensed complex and its chemical activity is low. Once cholesterol levels exceed the threshold, active cholesterol arises. Additionally, cholesterol displacement by other lipid membrane components can also free cholesterol from their complexes (*36*, *39*, *40*). In our studies, this model would state that cholesterol has shown a low chemical activity when tightly complexed with PC molecules. The lack of free cholesterol in this case reduces substrate availability for PFO monomers and thus raises the threshold concentration for PFO association.

On the contrary, when PC components are reduced or removed, the remaining uncomplexed cholesterol molecules bind to PE or PS with lower affinities. Reduced cholesterol miscibility in PE- or PS- (to a lesser extent) containing membranes was previously observed and reasoned with the tendency of aminophospholipids to associate with each other through inter-head group electrostatic and hydrogen bonding interactions (*31*, *57*). Electrostatic attraction between the opposite charges of PE and PS phospholipid head groups may account for this preference. However, favored PE-PE or PS-PS interactions (over PE/PS-cholesterol associations) may only be explained by hydrogen bonding. To test for this idea, an additional experiment may involve the assessment of cholesterol accessibility to PFO binding in vesicles containing PE and PC lipids in a 2:1 ratio, and compare the results to that obtained from PS/PC (2:1) membranes. This may clarify the strength of PE-PE and PS-PS interactions and may provide new insights to

the individual importance of PE and PS in lowering the cholesterol threshold concentration. As a result, when PE and PS dominate the membrane composition, cholesterol becomes empowered with a higher chemical activity, which facilitates PFO binding at lower cholesterol concentrations. Our studies do not distinguish between these different models for cholesterol-lipid interaction.

With regard to relevance to cholesterol regulation via the SREBP pathway, we suggest that previous in vivo observations of a 5 mol% ER cholesterol threshold (*23*, *24*) are mainly shaped by the high occurrence of PE and PS phospholipids in the ER membrane. We have attained a threshold as low as ~ 4 mol% in PE/PS vesicles, which is fairly close to the reported 5 mol% value. Our experimental conditions used pH 5.1, the difference induced by lowering the pH from 7.4 to 5.1 was previously reported to be a 50 – 67% decrease in the threshold value (*30*). Derived from this ratio, a 50% increase from ~ 4 mol% cholesterol is expected to be ~ 6 mol% if our experiments were conducted in pH 7.4 conditions. Given the complex and diverse composition of lipids in membranes, it is likely that this behavior is not completely due to the presence of PE and PS. Rather, there are likely to be other ER membrane lipids working in conjunction with PE and PS to molecularly organize cholesterol exposure and activity. Whether these include some of the intercalator lipids we studied or other lipids is not settled by our studies. The precise ratio of PE and PS (along with other membrane components) that generates this response has yet to be determined. Another unknown variable that can be further investigated is whether PE or PS has more of a threshold-reducing effect than the other.

In any case, we believe cholesterol exposure and binding to SCAP in the SREBP pathway is indirectly simulated with PFO as a mimic of SCAP. At or above the 5 mol% cholesterol set point, the unique membrane lipid composition of the ER offers more cholesterol exposure and activity for SCAP associations. Upon direct binding of cholesterol to SCAP, conformational changes are induced to inhibit subsequent steps involved in activating the SREBP-responsive genes. Consequently, cholesterol biosynthesis is downregulated to reset the homeostatic sterol environment.

Proceeding to the second set of related experiments, cholesterol-displacement effects were observed for biologically relevant molecules – triglycerides, diglycerides, and alkanols. Some membrane intercalators gradually exerted more or less profound effects as elevating

concentrations of PE and PS in artificial membranes enhanced cholesterol activity. The intercalator concentration of amphipath molecules required for cholesterol activation was previously estimated to be ~ 1 mole of amphipaths for 1 mole of cholesterol (*50*). In our results, the amount of cholesterol displaced was on average ~ 1 – 2 mol% when 5 mol% of intercalator exerted an influence. Thus, our results indicate that a higher concentration of intercalator is required for the activation of cholesterol. The difference may be attributed to our use of symmetric vesicles for experiments, whereas asymmetric red blood cell membranes are applied for producing a 1:1 ratio.

Not all intercalators activated cholesterol when PC was excluded from membranes. One intercalator may display no (or minimal) effect in PC/PE/PS membranes, yet it may drastically lower the threshold in PE/PS mixtures. Similarly, the opposite pattern may be detected. For experiments intercalating triolein, tripalmitin, dipalmitin, and tetradecanol, a greater degree of threshold-reduction was observed as membrane mixtures transitioned from PC/PE/PS to PE/PS. In contrast, less cholesterol displacement effect was generated in vesicles containing PO glycerol and octadecanol when PC was no longer incorporated. The only membrane intercalator that did not exhibit a significant effect in either case was oleoyl alcohol. Differential threshold-lowering effects of certain intercalators across different membrane compositions may be explained by intercalators first disrupting the weakest cholesterol complexes (*50*). As described above, PE and PS form the weakest associations with cholesterol in membranes, and are thus the easiest targets for cholesterol displacement. This explains for our findings that intercalators exert no influence in PC-only membranes, but once PE and PS are incorporated, we observe the reduced cholesterol requirement for PFO binding. In other words, uptake of intercalators we used into membranes can only displace cholesterol from their weakly complexed partners. This gives rise to exposed and active cholesterol, facilitating PFO binding at low cholesterol levels.

A problem that has not yet been addressed is the proportion of intercalators that are incorporated. With alkanols as a representative, red blood cell plasma membranes no longer have the capacity to assimilate intercalators once their concentration exceeds 8 – 9 mol% of lipids (*50*, *58*, *59*). We have maintained an intercalator concentration of 5 mol%. Nevertheless, this issue was of particular concern for the incorporation of triglycerides. With the highest degree of hydrophobicity, it is possible that the triglycerides were too hydrophobic to be incorporated

efficiently. Instead, many of the triglyceride molecules might cluster into droplets outside of the membrane region. This would decrease the effect observed on the threshold value. (Another factor decreasing the effect on threshold values may involve the incorporation of triglycerides into the interleaflet region of the bilayer membranes, and this may limit their effect on the threshold concentration.) Previous studies have shown triglycerides to incorporate into membranes. Medium chain triacylglycerols (TAG) were shown to most optimally incorporate into phospholipid bilayers in contrast to long chain TAGs (*60*). Nevertheless, very minimal amounts of long chain TAGs were still incorporated and saturating concentrations of triolein were described to compete with medium chain TAGs for incorporation (*60*). This suggests the possibility of triglyceride uptake into artificial membranes, yet the exact percentage of incorporation in our samples requires further assessment and quantitation. The effect of 2 mol% intercalators (data shown in Appendix, Fig. S4 and Fig. S6) was also tested and found to show smaller effects on shifting the threshold than 5 mol%. Thus, it is likely that at 5 mol%, a large fraction of the intercalators were membrane associated.

Our results revealed no significant patterns of correlation between the structural characteristics of intercalation and the extent of cholesterol threshold reduction. This is consistent with the statement from Lange et al. that the effectiveness per molal concentration of amphipaths is similar once assimilated into membranes (*50*). Nevertheless, within the same phospholipid compositions, we do observe different threshold-lowering effects caused by the various intercalators. Intercalators such as hexadecanol and ceramide were demonstrated to competitively displace cholesterol from ordered domains and act as cholesterol mimics to promote raft formation in membranes containing co-existing ordered and disordered domains (*47*, *48*). Furthermore, intercalators containing a small polar head group and two saturated acyl chains were reported to be effective at cholesterol substitution in sterol/sphingomyelin domains. However, only half of the tested single-chain amphipaths displaced cholesterol from these ordered domains (*51*). Thus, a small head group and saturated acyl chains were assumed to be features that facilitated cholesterol displacement from ordered domains. Our membranes have no ordered domains and this may explain why we see no clear relationship between intercalator saturation and cholesterol activation.

As a side note, these results may have practical applications for studies of PFO. A mixture of lipids with PE and PS may enhance the binding affinity of PFO to artificial membranes, and may increase the binding efficiency of poorly bound mutant PFO constructs, with which our lab has been working.

Our conclusions suggest that PE and PS are two of the major phospholipids responsible for setting the 5 mol% cholesterol threshold value in the ER membrane. In accordance with our cellular demands, ER cholesterol concentrations are carefully balanced and regulated around this homeostatic set point to either activate or turn off the SREBP pathway. Though the exact proportion of PE and PS content in relation to the wide diversity of lipids in membranes is still unknown, our results indicate that these small-headed aminophospholipids have a significant effect in producing the 5 mol% threshold value. A number of membrane-intercalating intercalators were tested for their ability to further lower the sterol concentration required for PFO-cholesterol association (i.e. SCAP binding). The lowest threshold concentration (~ 2 mol%) was attained in membranes composed of PE/PS (1:1) intercalated with 5 mol% of triolein or dipalmitin. These results may imply that significant amounts of fats (triglycerides and diglycerides) in the ER membrane may have an influence cellular cholesterol levels. Hence, we have demonstrated a means to hypothetically manipulate the threshold for activation/deactivation of the SREBP pathway, and by simply varying the lipid membrane composition, we have achieved a threshold value for activation of cholesterol lower than the physiological set point of 5 mol%.

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Appendix

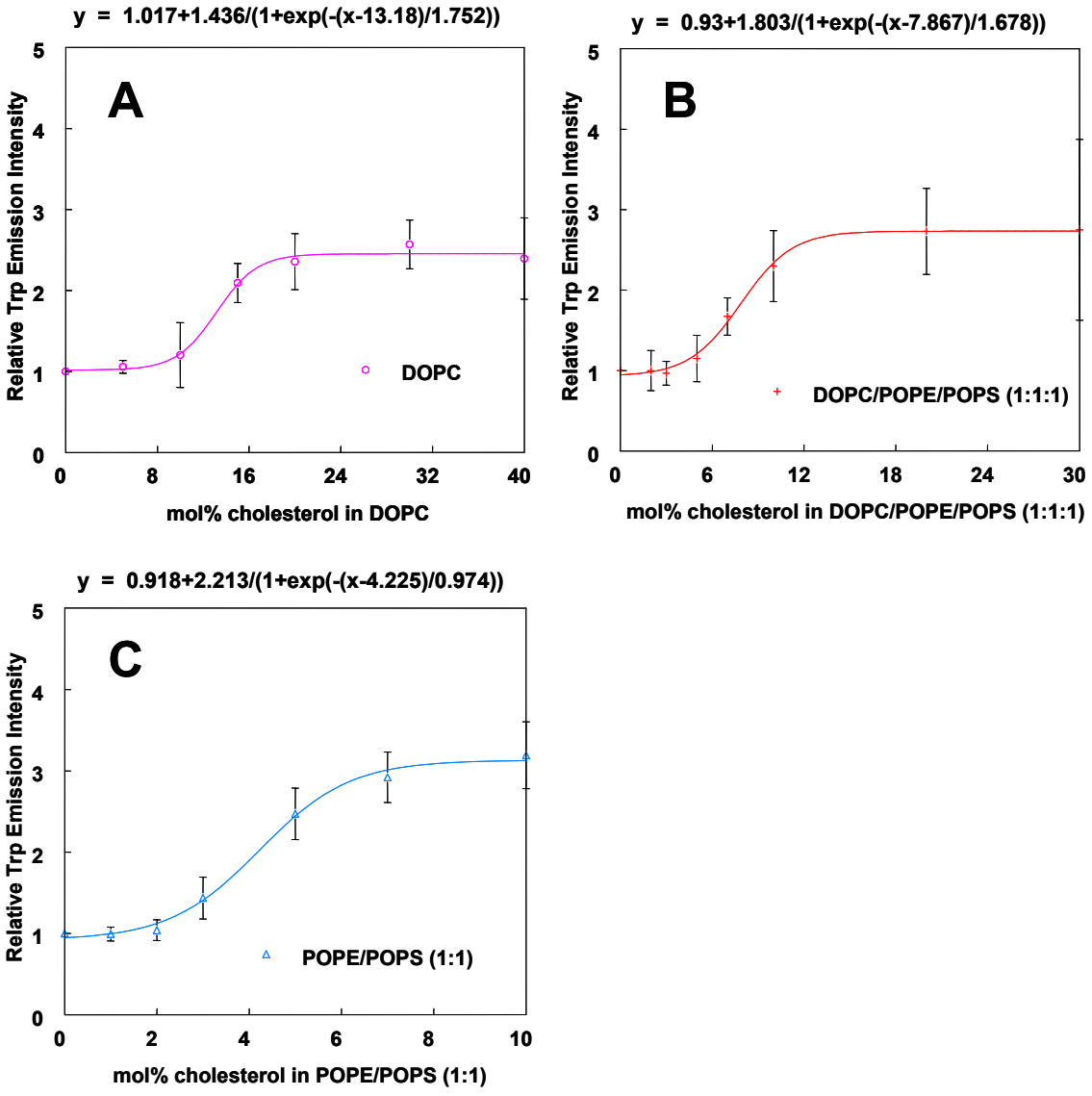
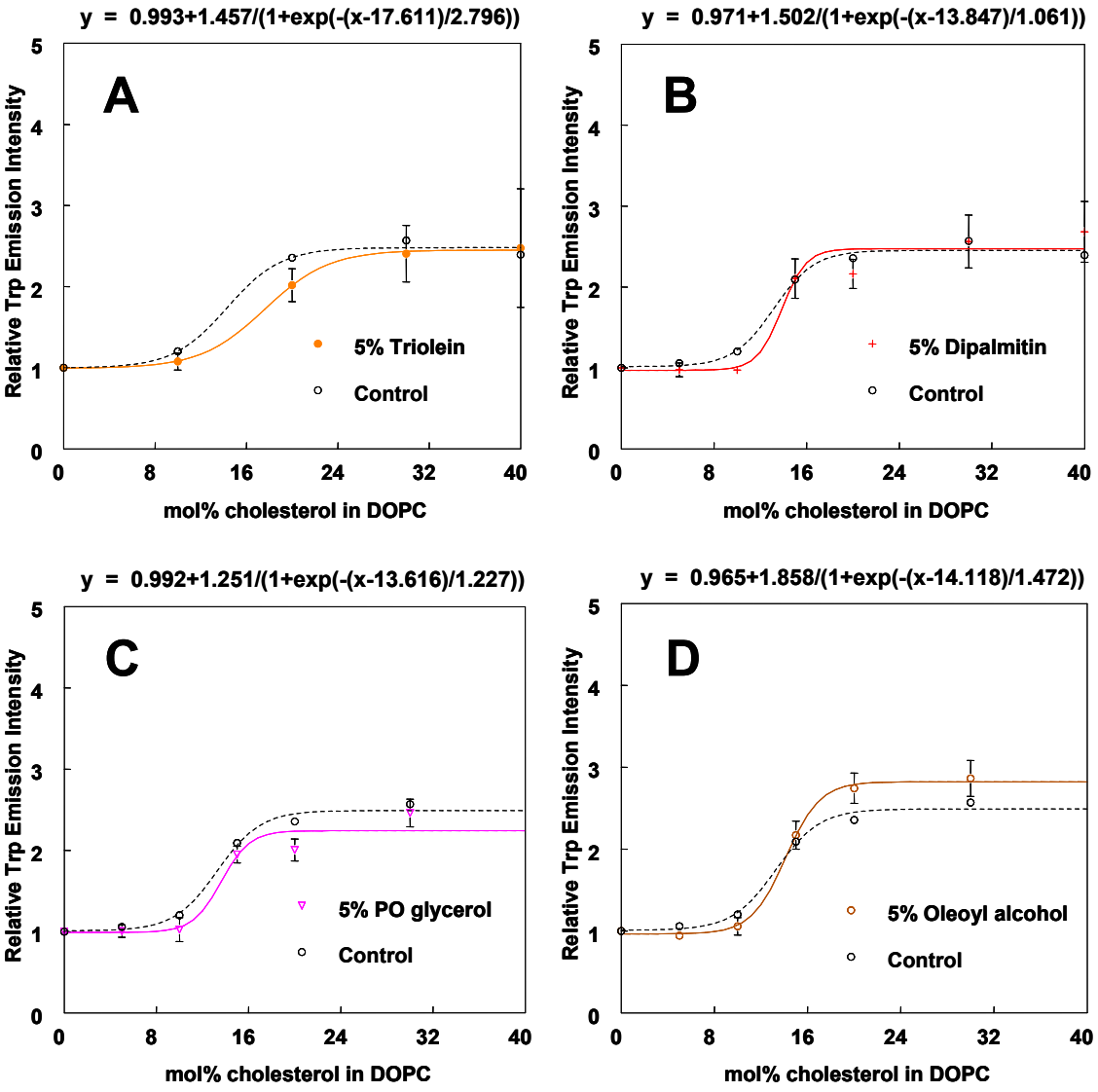


Figure S1: Increase in Trp Emission intensity versus molar percentage of cholesterol in (A) DOPC, (B) DOPC/POPE/POPS (1:1:1), and (C) POPE/POPS (1:1)


y = 0.993+1.457/(1+exp(-(x-17.611)/2.796))
A
Relative Trp Emission Intensity
mol% cholesterol in DOPC
5% Triolein
Control
y = 0.971+1.502/(1+exp(-(x-13.847)/1.061))
B
Relative Trp Emission Intensity
mol% cholesterol in DOPC
5% Dipalmitin
Control
y = 0.992+1.251/(1+exp(-(x-13.616)/1.227))
C
Relative Trp Emission Intensity
mol% cholesterol in DOPC
5% PO glycerol
Control
y = 0.965+1.858/(1+exp(-(x-14.118)/1.472))
D
Relative Trp Emission Intensity
mol% cholesterol in DOPC
5% Oleoyl alcohol
Control

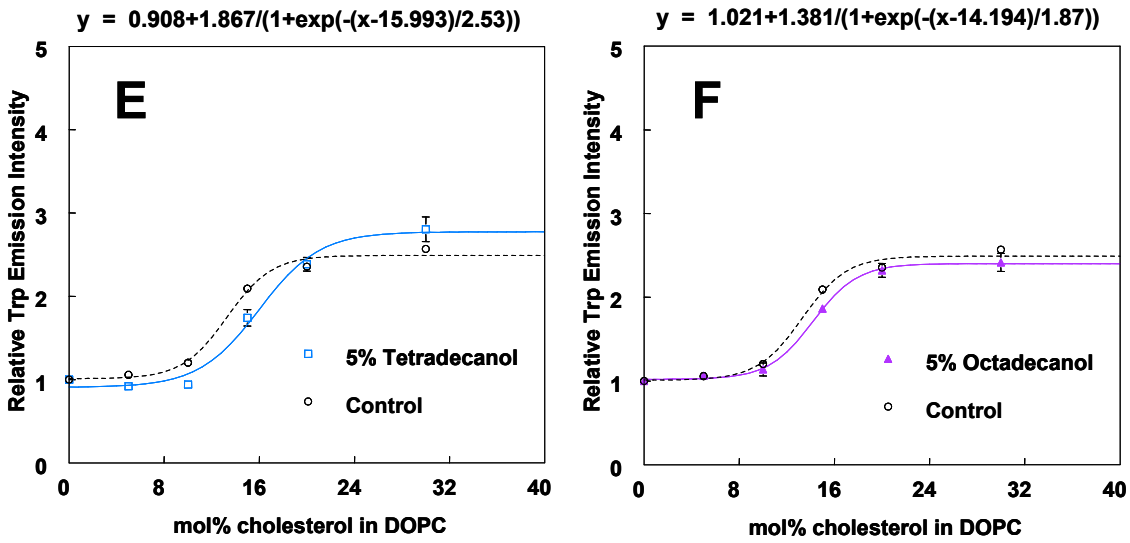
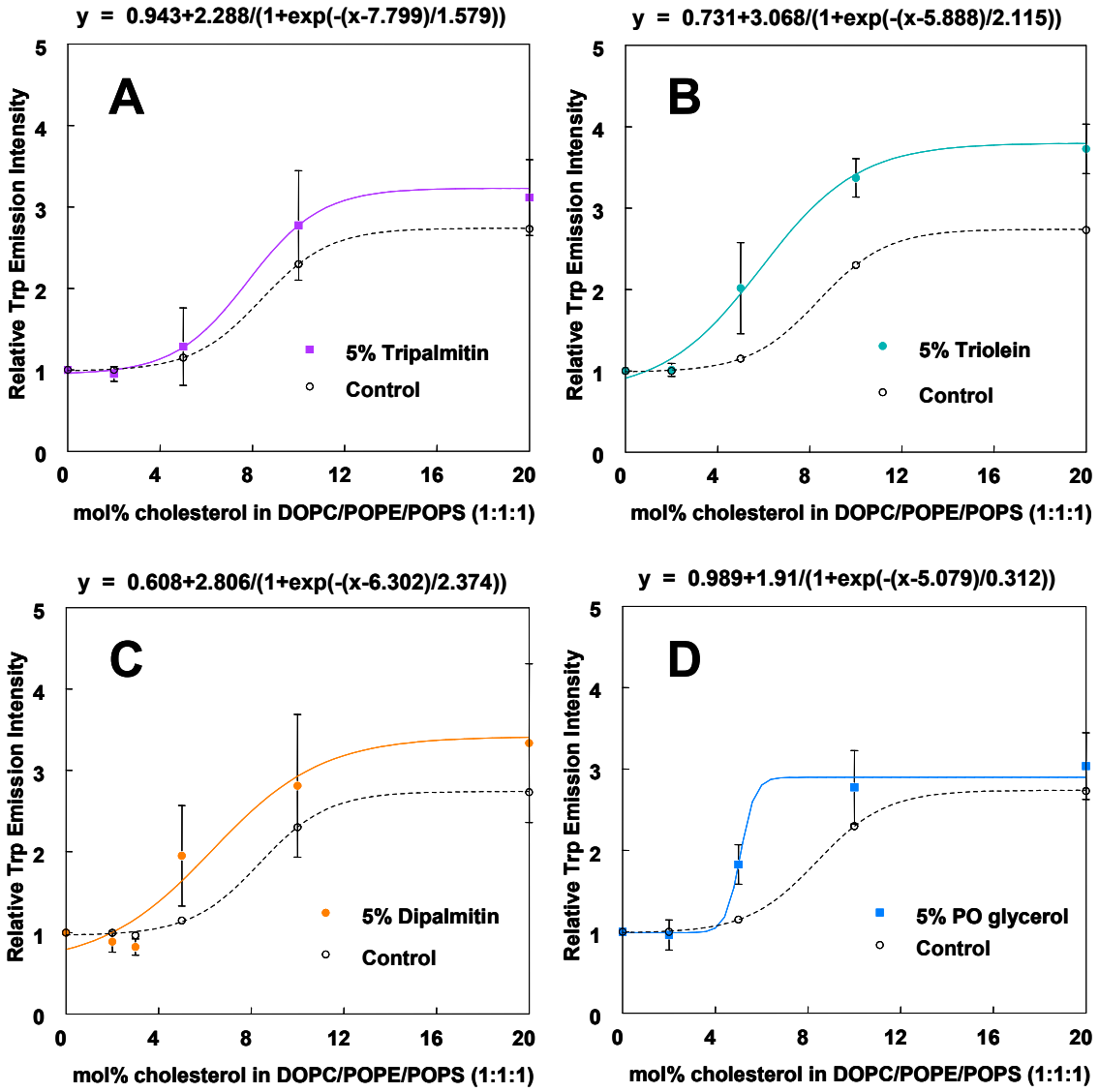


Figure S2: Cholesterol threshold-lowering effects of 5 mol% (A) triolein, (B) dipalmitin, (C) PO glycerol, (D) oleoyl alcohol, (E) tetradecanol, and (F) octadecanol, in DOPC membranes. The control curve is generated in the same conditions with the absence of fatty intercalants.


y = 0.943+2.288/(1+exp(-(x-7.799)/1.579))
A
Relative Trp Emission Intensity
5% Tripalmitin
Control
mol% cholesterol in DOPC/POPE/POPS (1:1:1)
y = 0.731+3.068/(1+exp(-(x-5.888)/2.115))
B
Relative Trp Emission Intensity
5% Triolein
Control
mol% cholesterol in DOPC/POPE/POPS (1:1:1)
y = 0.608+2.806/(1+exp(-(x-6.302)/2.374))
C
Relative Trp Emission Intensity
5% Dipalmitin
Control
mol% cholesterol in DOPC/POPE/POPS (1:1:1)
y = 0.989+1.91/(1+exp(-(x-5.079)/0.312))
D
Relative Trp Emission Intensity
5% PO glycerol
Control
mol% cholesterol in DOPC/POPE/POPS (1:1:1)

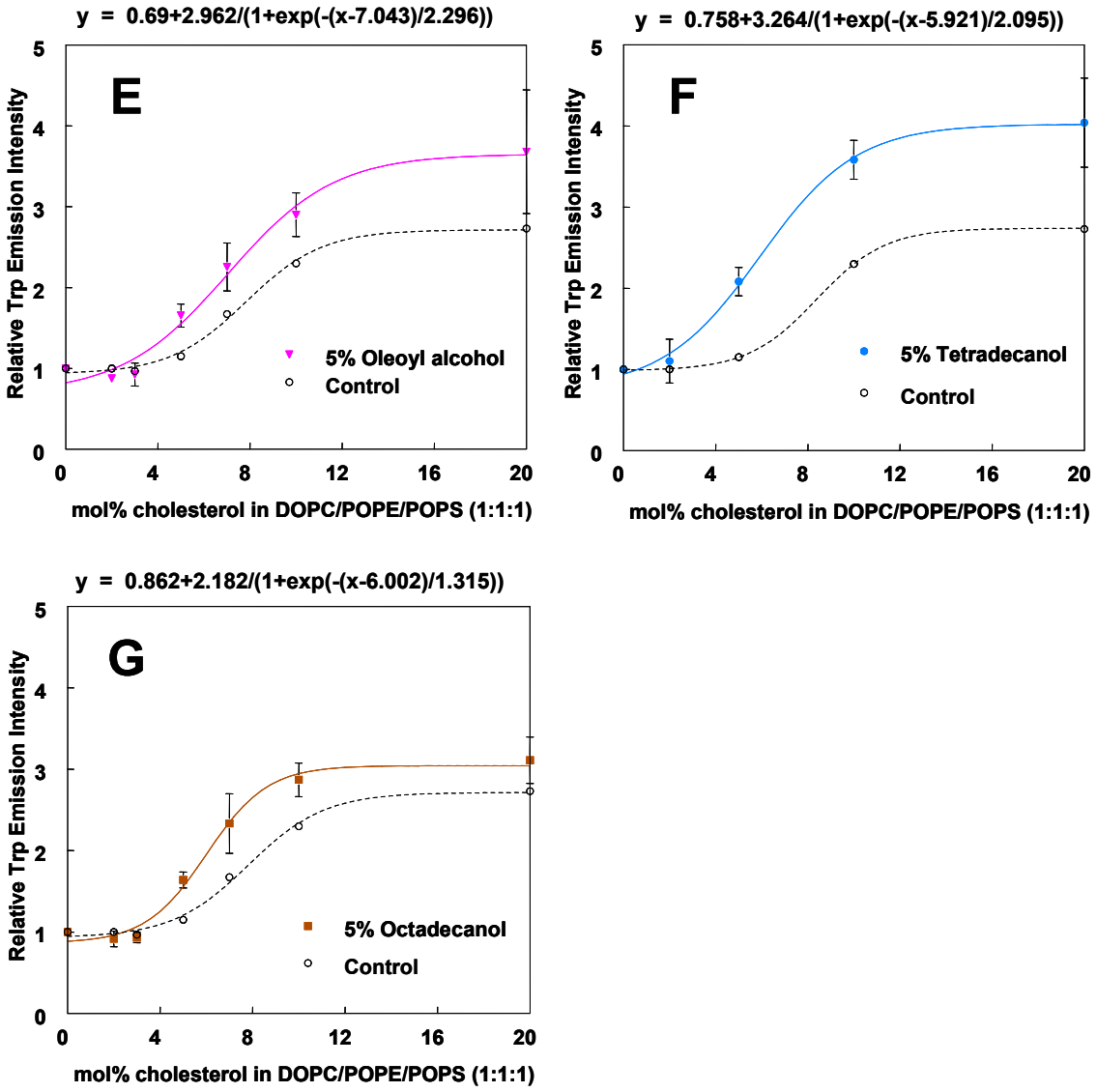
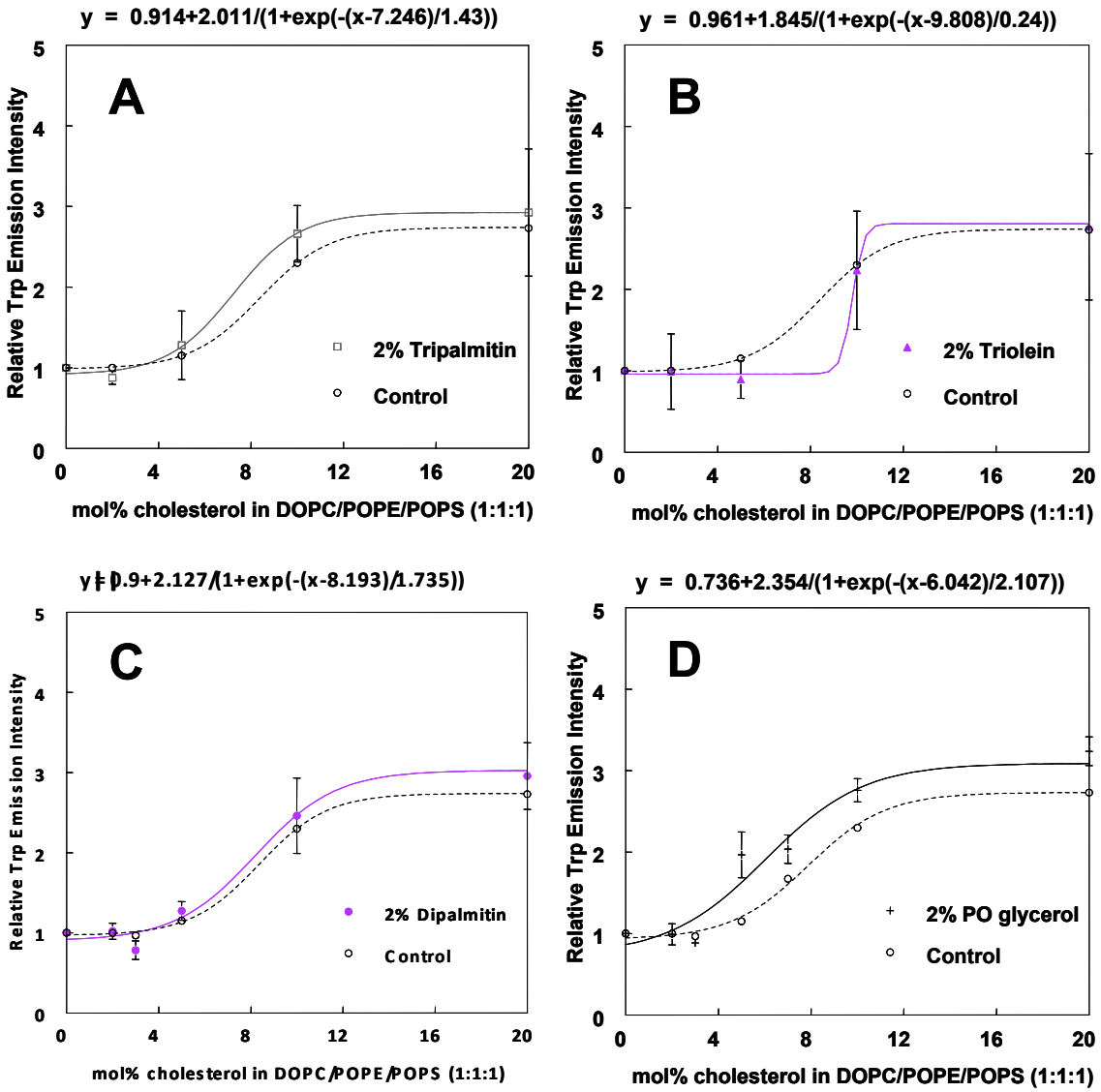


Figure S3: Cholesterol threshold-lowering effects of 5 mol% (A) tripalmitin, (B) triolein, (C) dipalmitin, (D) PO glycerol, (E) oleoyl alcohol, (F) tetradecanol, and (G) octadecanol in DOPC/POPE/POPS (1:1:1) vesicles, compared to the situration in which fatty intercalants are not incorporated (control).


y = 0.914+2.011/(1+exp(-(x-7.246)/1.43))
A
Relative Trp Emission Intensity
2% Tripalmitin
Control
mol% cholesterol in DOPC/POPE/POPS (1:1:1)
y = 0.961+1.845/(1+exp(-(x-9.808)/0.24))
B
Relative Trp Emission Intensity
2% Triolein
Control
mol% cholesterol in DOPC/POPE/POPS (1:1:1)
y = 0.9+2.127/(1+exp(-(x-8.193)/1.735))
C
Relative Trp Emission Intensity
2% Dipalmitin
Control
mol% cholesterol in DOPC/POPE/POPS (1:1:1)
y = 0.736+2.354/(1+exp(-(x-6.042)/2.107))
D
Relative Trp Emission Intensity
2% PO glycerol
Control
mol% cholesterol in DOPC/POPE/POPS (1:1:1)

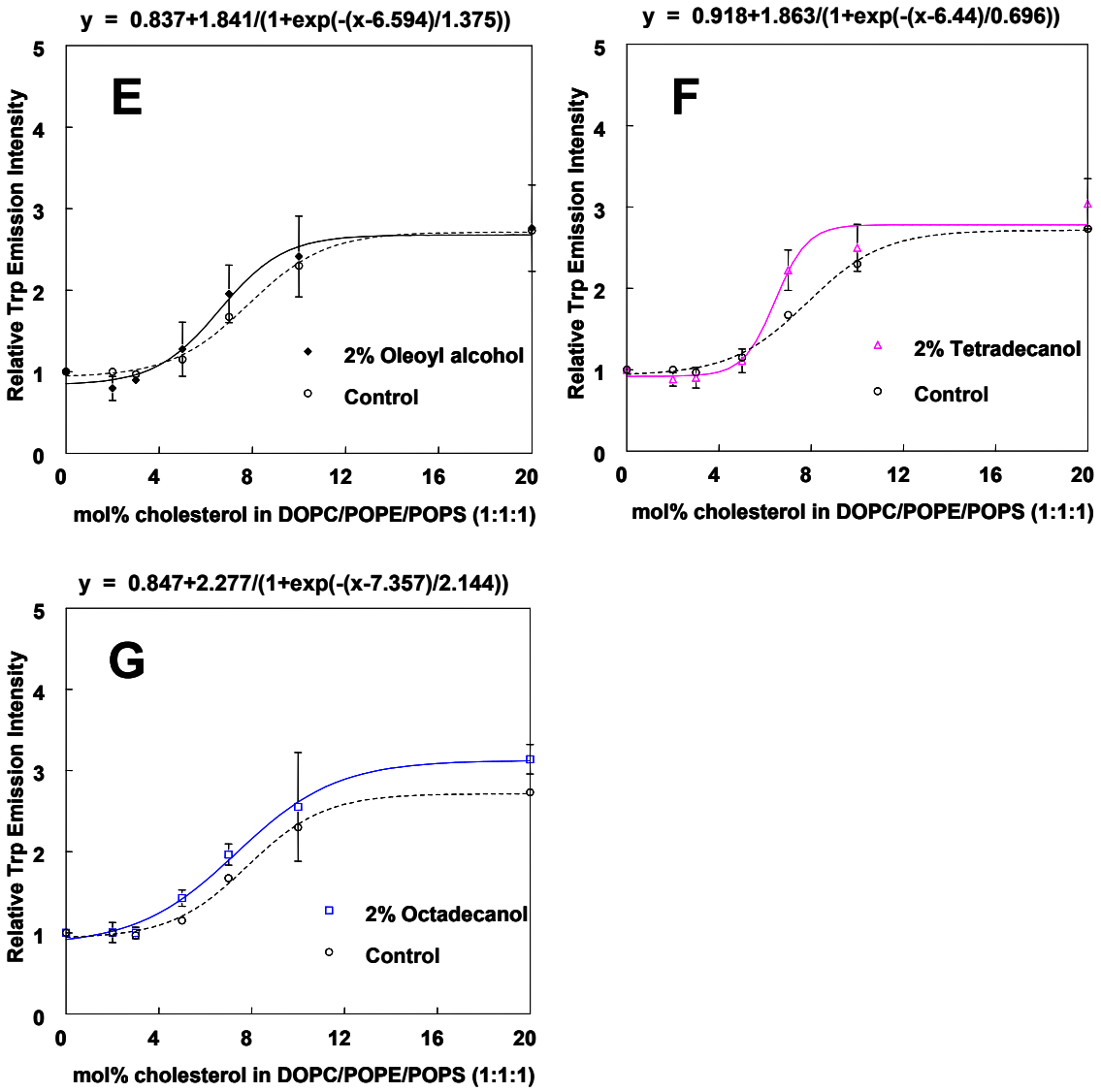
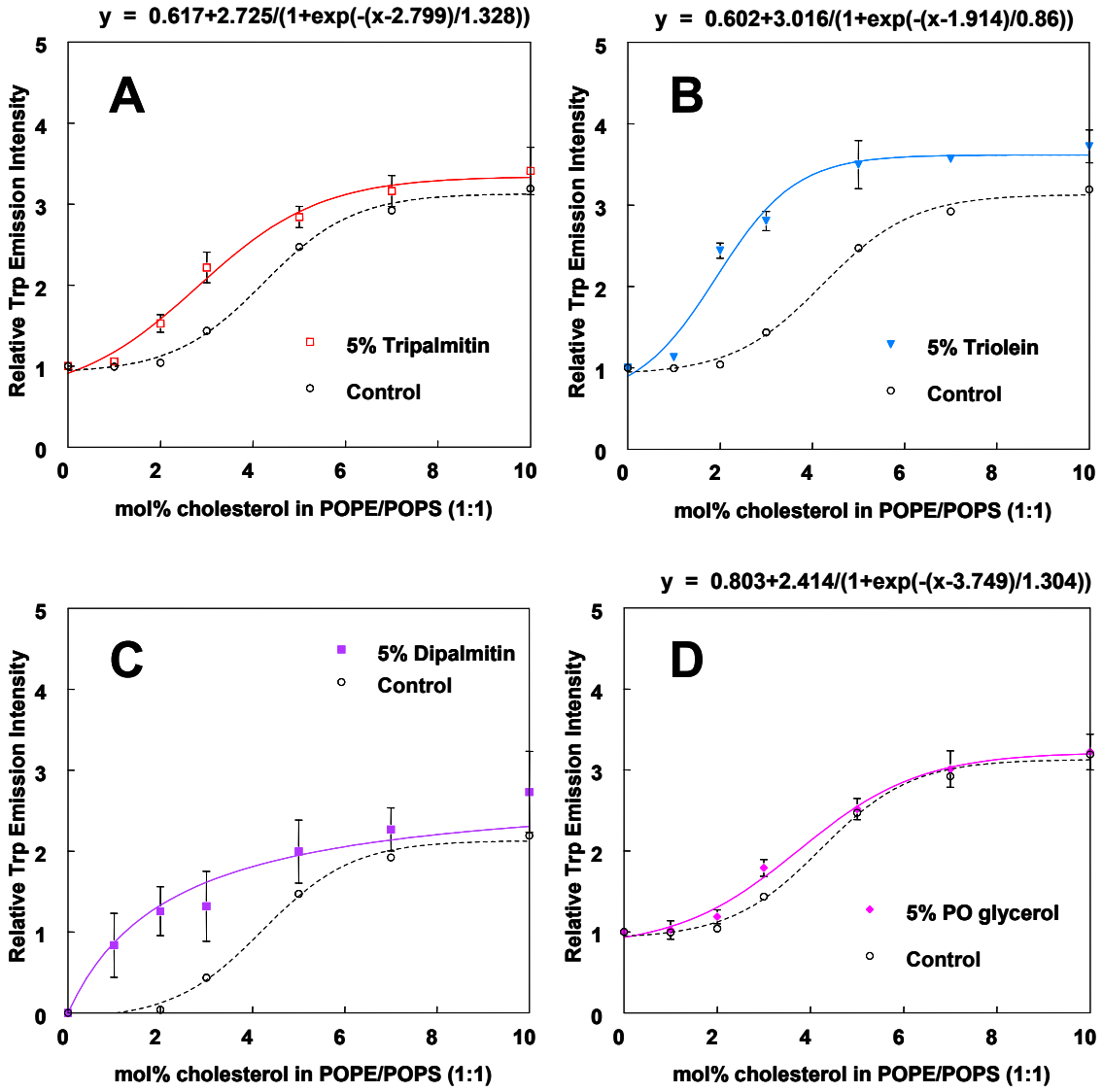


Figure S4: Comparison in threshold-reducing effects of 2 mol% (A) tripalmitin, (B) triolein, (C) dipalmitin, (D) PO glycerol, (E) oleoyl alcohol, (F) tetradecanol, (G) octadecanol in DOPC/POPE/POPS (1:1:1), with the control curve (PC/PE/PS membranes without fatty intercalants).


y = 0.617+2.725/(1+exp(-(x-2.799)/1.328))
A
5% Tripalmitin
Control
Relative Trp Emission Intensity
mol% cholesterol in POPE/POPS (1:1)
y = 0.602+3.016/(1+exp(-(x-1.914)/0.86))
B
5% Triolein
Control
Relative Trp Emission Intensity
mol% cholesterol in POPE/POPS (1:1)
C
5% Dipalmitin
Control
Relative Trp Emission Intensity
mol% cholesterol in POPE/POPS (1:1)
y = 0.803+2.414/(1+exp(-(x-3.749)/1.304))
D
5% PO glycerol
Control
Relative Trp Emission Intensity
mol% cholesterol in POPE/POPS (1:1)

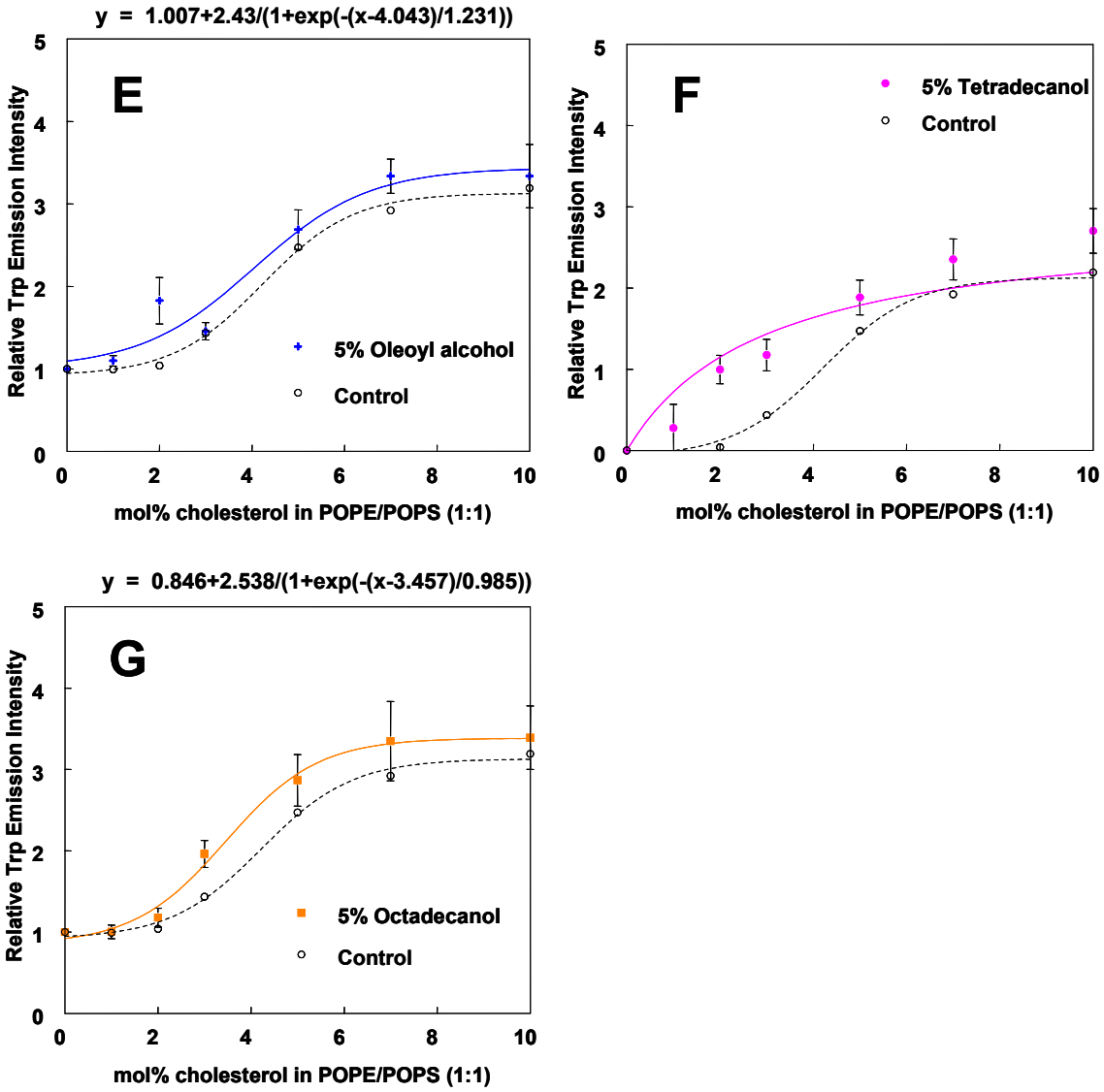


Figure S5: In POPE/POPS (1:1) vesicles, threshold-reducing effects were compared between the control (no fatty intercalants in PE/PS) and 5 mol% of (A) tripalmitin, (B) triolein, (C) dipalmitin, (D) PO glycerol, (E) oleoyl alcohol, (F) tetradecanol, and (G) octadecanol. Quantitation of the threshold concentration where Trp emission intensity is half-maximal for 1-site ligand fits: (C) the curve reached a saturation value of 2.822. The corresponding x-value for half-maximal activity (y = 1.411) is 2.244 mol% cholesterol. For (F), the same method of calculation was applied to yield a threshold value of 2.942 mol% from a half-maximal Trp emission intensity value of 1.419 (saturation value was 2.839).

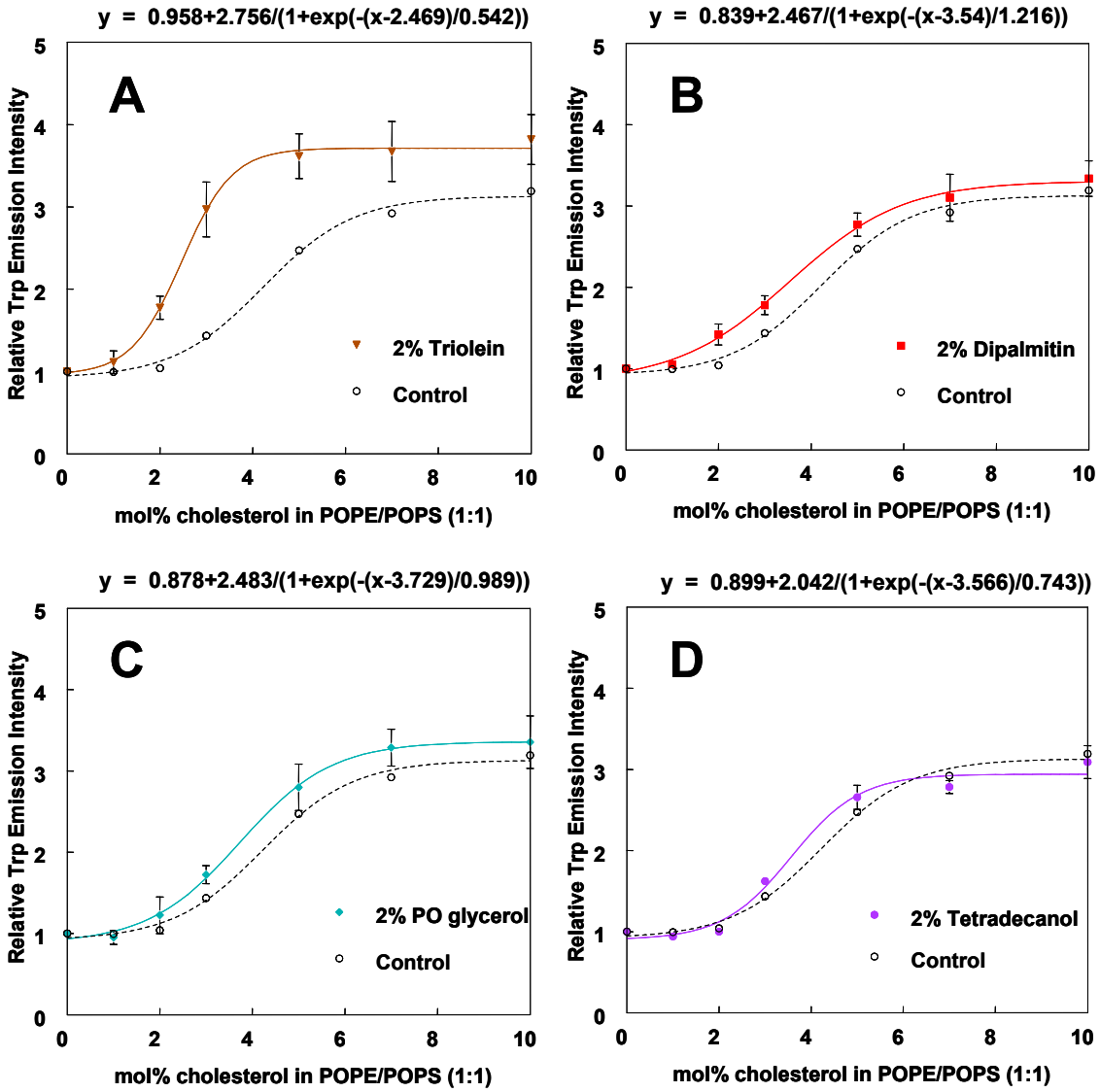


Figure S6: In POPE/POPS (1:1) membranes, the threshold-reducing effects of 2 mol% of (A) triolein, (B) dipalmitin, (C) PO glycerol, and (D) tetradecanol, were compared to the threshold values generated from PE/PS vesicles that did not incorporate fatty intercalants.