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# Cholesterol Exposure at the Membrane Surface Is Necessary and Sufficient to Trigger Perfringolysin O Binding<sup>†</sup>

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Running title: Recognition and Binding of Cholesterol by PFO

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1 **Abbreviations:** PFO, perfringolysin O; CDCs, cholesterol-dependent cytolysins;  $l_o$ ; liquid-ordered; SM, sphingomyelin; PFO<sup>C459A</sup> is an example of the notation for the PFO derivative where the Cys459 was mutated to Ala; IANBD, N, N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylene diamine; DPH, 1,6-diphenyl-1,3,5-hexatriene; PC, phosphatidylcholine; SDS, sodium dodecyl sulfate; AGE, agarose gel electrophoresis; TX-100, Triton X-100; LUVs, large unilamellar vesicles; TMH, transmembrane beta-hairpin;  $l_d$ , liquid-disordered.

**ABSTRACT**

Perfringolysin O (PFO) is the prototype for the cholesterol-dependent cytolysins, a family of bacterial pore-forming toxins that act on eukaryotic membranes. The pore-forming mechanism of PFO exhibits an absolute requirement for membrane cholesterol, but the complex interplay between the structural arrangement of the PFO C-terminal domain and the distribution of cholesterol in the target membrane is poorly understood. Herein we show that PFO binding to the bilayer and the initiation of the sequence of events that culminate in the formation of a transmembrane pore depend on the availability of free cholesterol at the membrane surface, while changes in the acyl chain packing of the phospholipids and cholesterol in the membrane core, or the presence or absence of detergent-resistant domains do not correlate with PFO binding. Moreover, PFO association with the membrane was inhibited by the addition of sphingomyelin a typical component of membrane rafts in cell membranes. Finally, addition of molecules that do not interact with PFO, but intercalate into the membrane and displace cholesterol from its association with phospholipids (*e.g.*, epicholesterol), reduced the amount of cholesterol required to trigger PFO binding. Taken together, our studies reveal that PFO binding to membranes is triggered when the concentration of cholesterol exceeds the association capacity of the phospholipids and this cholesterol excess is then free to associate with the toxin.

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3 The cholesterol-dependent cytolysins (CDCs)<sup>1</sup> are a family of bacterial pore-forming  
4 toxins that act on cholesterol-containing eukaryotic membranes. There are more than 20 known  
5 proteins of this toxin family that have been identified from different genera of Gram-positive  
6 bacteria such as *Streptococcus*, *Listeria*, *Clostridium*, *Bacillus*, and *Arcanobacterium* (1). This is  
7 a growing family of toxins as new members are being discovered (2-4). Since the CDCs share a  
8 high degree of primary sequence homology, it is believed that the toxins adopt similar three-  
9 dimensional structures and have similar cytolytic modes of action (5, 6). Moreover, the recently  
10 reported crystal structures for the membrane attack complex/perforin showed that the structural  
11 and functional features of the CDCs are related to pore-forming proteins employed by eukaryotic  
12 immune systems (7, 8).  
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27 CDCs are secreted as water-soluble monomers of 50-70 kDa that form large ring- and  
28 arc-shaped homo-oligomeric pores (~35-50 monomers per oligomer; approximately 250 Å in  
29 diameter) in cholesterol-containing membranes (9-13). Disruption of the host cell membrane by  
30 these pore-forming toxins is an important step in bacterial pathogenesis (1).  
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37 As do many other bacterial and viral proteins, the CDCs take advantage of a  
38 distinguishing feature of mammalian membranes, in this case the presence of cholesterol (14,  
39 15). Since cholesterol is essential for CDC cytolytic activity, and pre-incubation of the CDCs  
40 with cholesterol inhibited cytolysis, it was long thought that cholesterol functioned as a receptor  
41 for this family of toxins. However, recent findings have challenged this long standing paradigm  
42 because not all CDCs require cholesterol to bind to the membrane. For example, intermedilysin  
43 recognizes and binds the human CD59 protein (16). However, cholesterol is still required for the  
44 cytolytic activity of intermedilysin (17).  
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3 Perfringolysin O (PFO) is a prototype of the CDC family (6, 18). Cytolysis starts when  
4 the C-terminal domain 4 of PFO (residues 391-500) first contacts the target membrane (19).  
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6 Domain 4 has a beta-sandwich structure that interacts with the membrane surface only at one end  
7  
8 via the loops that connect the two beta sheets in the domain (20-22). One of these loops, an  
9  
10 eleven amino acid-residue sequence known as the Trp-rich loop, is highly conserved among most  
11  
12 of the CDCs (1). Modifications in this conserved undecapeptide typically inhibit the hemolytic  
13  
14 activity of the toxin by blocking the conformational changes that are required to allosterically  
15  
16 trigger the insertion of the transmembrane beta-barrel (5, 23, 24). The correlation between this  
17  
18 highly conserved amino acid sequence and the absolute cholesterol dependence led researchers  
19  
20 to hypothesize that this undecapeptide represented the cholesterol binding site for the CDCs (25).  
21  
22 However, it has recently been shown that cholesterol recognition and toxin binding are mediated  
23  
24 by the other three loops, while the insertion of the undecapeptide may be coupled to the insertion  
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26 of the transmembrane beta-barrel (24). Despite these important observations, the exact  
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28 mechanism by which PFO interacts with cholesterol remains unclear.  
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36 We have recently shown that the binding of PFO solely to cholesterol is sufficient to  
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38 trigger the conformational changes that effect oligomerization and initiate pore formation (26).  
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40 However, substantial cholesterol (> 30 mole%) is required to trigger the PFO membrane-  
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42 interaction in bilayers comprised of cholesterol and phospholipids (19, 27). It was therefore  
43  
44 suggested that PFO binds to cholesterol rich-domains or membrane “rafts” (28). Yet, recent  
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46 studies showed no correlation between the sterol structures that promote formation of liquid-  
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48 ordered ( $l_o$ ) phases in liposomal membranes (or membrane “rafts” in cell membranes) and their  
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50 ability to promote PFO-membrane interactions (29). In contrast, PFO binding to membranes was  
51  
52 enhanced when the interaction between the sterol and the phospholipid acyl chains was weak  
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3 (29, 30). Taken together, these studies support the concept that there is a complex interplay  
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5 between the structural arrangement of the domain 4 loops and the distribution of cholesterol in  
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7 the target membrane (27).  
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10 To better understand the nature of this complex PFO-cholesterol interaction in the context  
11  
12 of membrane phospholipids, we have systematically examined the phospholipid dependence of  
13  
14 PFO binding. Our studies reveal that: (i) the interactions between phospholipids and cholesterol  
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16 in the bilayer dictate the cholesterol threshold for PFO binding; (ii) PFO binding to the target  
17  
18 membrane is not detectably dependent on the packing of the lipids in the membrane core; (iii)  
19  
20 PFO binding does not correlate with the presence of a detergent resistant fraction in the  
21  
22 membrane; (iv) PFO association with the membrane was inhibited by the increase in the  
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24 sphingomyelin (SM) content, that is important for the establishment of membrane “rafts” in cell  
25  
26 membranes; and (v) the cholesterol threshold required for PFO binding was reduced by the  
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28 addition of sterols that do not interact with PFO, but do intercalate between the cholesterol  
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30 molecules and interact with phospholipids. Taken together, our studies reveal that PFO  
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32 membrane-binding is triggered when the concentration of cholesterol exceeds the association  
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34 capacity of the phospholipids. Packing of lipids molecules in the bilayer will dictate whether or  
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36 not cholesterol is accessible to PFO. The excess of cholesterol is then free to associate with the  
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38 toxin and trigger the conformational changes required for toxin oligomerization and pore  
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40 formation.  
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## EXPERIMENTAL PROCEDURES

*Cloning and Mutagenesis of the PFO Gene.* The gene encoding PFO<sup>C459A</sup>, derived from PCR amplification of PFO from pRT20 (31), was cloned into the *Bgl*III and *Eco*RI sites of the expression vector pRSETB (Invitrogen) in order to remove an extra Trp residue that was introduced into the protein by the original expression vector (pAH21, see Supporting Information). The expressed PFO derivative contained a polyhistidine tag and enterokinase cleavage site at its N-terminus. When the polyhistidine tag is removed, the expressed protein consists of the mature PFO (beginning with amino acid K29) fused to six amino acids (DPSSRS) carried over from the expression vector at its N-terminus. Since no significant functional or structural differences were found between PFO derivatives containing or lacking the polyhistidine tag, the pAH21 derivative of PFO was used (data not shown).

*Expression, Purification, and Labeling of PFO Derivatives.* The expression, purification, and labeling with N, N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylene diamine (IANBD, Invitrogen) of recombinant PFO derivatives were done with some modifications of previous procedures (31), as detailed in the Supporting Information.

*Liposome Preparation and Incorporation of DPH. Liposome Preparation.* 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), bovine brain sphingomyelin (SM), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl (dansyl-PE) were obtained from Avanti Polar Lipids. Cholesterol (5-cholesten-3 $\beta$ -ol) and 3-epicholesterol (5-cholesten-3 $\alpha$ -ol) were obtained from Steraloids. To quantify the percent



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3 recovery of both phospholipid and cholesterol during liposome preparations, trace amounts of  
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5  $^{14}\text{C}$ - phospholipid and  $^3\text{H}$ -cholesterol were added to each batch of liposomes. For POPC-,  
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7 DOPC-, and SOPC- cholesterol liposomes, L-3-phosphatidylcholine-1,2-di[ $^{14}\text{C}$ ]oleoyl (114  
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9 mCi/mmol; GE Healthcare) and [1,2- $^3\text{H}$ (N)]cholesterol (48.3 Ci/mmol; NEN, Boston, MA) were  
10  
11 added. For DMPC-cholesterol, DPPC-cholesterol, and DSPC-cholesterol liposomes, L-3-  
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13 phosphatidylcholine-1-palmitoyl-2[1- $^{14}\text{C}$ ]palmitoyl (110 mCi/mmol; PerkinElmer) and [1,2-  
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15  $^3\text{H}$ (N)]cholesterol were added. Mixtures of phospholipid and cholesterol were dried under a  
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17 stream of nitrogen at a temperature approximately  $10^\circ\text{C}$  above the  $T_m$  of the phospholipid. The  
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19 dried lipid mixture was then dried further under vacuum for 3 h. The dried  
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21 phospholipid/cholesterol mixture was resuspended in 0.55 ml of buffer A and vortexed until the  
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23 dried lipid was fully hydrated. LUVs comprised of POPC-sterol or DOPC-sterol were generated  
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25 using an Avestin Liposofast extruder as described previously (31, 32). For preparation of  
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27 DMPC-sterol, DPPC-sterol, DSPC-sterol, and DOPC-SM-sterol liposomes, an Avanti mini  
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29 extruder and heat block were used to ensure the liposomes were maintained at least  $10^\circ\text{C}$  above  
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31 the  $T_m$  of the major phospholipid during generation of the vesicles. The percentage recovery of  
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33 phospholipid and cholesterol ranged from 88-100% for all liposomes prepared. For 1,6-diphenyl-  
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35 1,3,5-hexatriene (DPH) incorporation into liposomes, 1  $\mu\text{l}$  of a 280  $\mu\text{M}$  DPH (dissolved in  
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37 dimethylformamide) solution was added to 550  $\mu\text{l}$  of liposomes in buffer A [50 mM HEPES (N-  
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39 2-hydroxyethyl piperazine-N-2-ethanesulfonic acid) (pH 7.5), 100 mM NaCl] (100  $\mu\text{M}$  total  
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41 lipid). These conditions created a molar ratio of DPH:lipid of 1:200 while also keeping the  
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43 amount of organic solvent added to the liposomes at a minimum ( $\ll 1\%$  of final volume). The  
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45 liposome-DPH mixture was incubated for 1 h. at 20-23  $^\circ\text{C}$  in the dark to prevent any  
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47 photodegradation of the fluorescent probe.  
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3 *Steady-state Fluorescence Spectroscopy.* Fluorescence equipment and measurements  
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5 procedures were done as detailed previously with some modifications described in the  
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7 Supplemental Section. For Trp intensity measurements, the excitation and emission wavelengths  
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9 were 295 nm and 348 nm, respectively, with a 4 nm band-pass. For DPH anisotropy  
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11 measurements, the excitation and emission wavelengths were 350 nm and 452 nm, respectively,  
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13 with a 4 nm band-pass.  
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17 *Trp Fluorescence.* For the spectroscopic analysis of PFO-membrane interactions, 250  $\mu$ l  
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19 aliquots of 50 nM purified PFO<sup>C459A</sup> in buffer A were distributed into phosphatidylcholine (PC)-  
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21 coated quartz microcuvettes. After measuring the initial net (after blank subtraction) fluorescence  
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23 intensity ( $F_0$ ) of each sample, each cuvette received 13.5  $\mu$ l of 1 mM liposomes (total lipid). The  
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25 PFO-liposome samples were mixed and incubated at 37 °C for 30 min. After re-equilibration  
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27 back to 25 °C, the net (after blank subtraction and dilution correction) emission intensity ( $F$ ) of  
28  
29 each sample was measured. The change in the Trp emission intensity was expressed as  $F/F_0$ .  
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35 *Anisotropy.* The steady-state anisotropy of DPH in liposomes was measured in the L-  
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37 format using Glan-Thompson prism polarizers in both the excitation and emission beams. The  
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39 emission intensity measured when a 250- $\mu$ l sample was excited by vertically polarized light and  
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41 the signal was detected through a horizontal polarizer was designated as  $I_{VH}$ .  $I_{HH}$ ,  $I_{HV}$ , and  $I_{VV}$   
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43 were defined analogously. The component intensities of a DPH-free liposome sample were  
44  
45 subtracted from the corresponding component intensities of a DPH-containing liposome sample  
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47 to obtain the net DPH emission intensities of a given sample. The fluorescence anisotropy was  
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49 then calculated from  
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$$r = [\text{net}I_{VV} - G(\text{net}I_{HV})] / [\text{net}I_{VV} + 2G(\text{net}I_{VH})]$$

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54 where the grating factor  $G = \text{net}I_{VH} / \text{net}I_{HH}$ .  
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3 *SDS-AGE Analysis of PFO Oligomers.* Denaturing sodium dodecyl sulfate agarose gel  
4 electrophoresis (SDS-AGE) was carried out as previously described (33). Briefly, a 20- $\mu$ l sample  
5 containing 0.5  $\mu$ M NBD-labeled PFO and 0.5 mM liposomes (total lipid) was incubated for 30  
6 minutes at 37 °C. After the incubation, one-third volume of sample buffer [0.1% (w/v)  
7 bromophenol blue, 45% (v/v) glycerol, 6% (w/v) SDS, 150 mM Tris-HCl (pH 6.8), 300 mM  $\beta$ -  
8 mercaptoethanol] was added. The reaction mixture was then loaded onto a 1.5% (w/v in buffer  
9 A) agarose gel (10 cm x 6 cm x 0.5 cm) and run at 100V for 40 min. After electrophoresis, the  
10 gel was scanned using a BioRad Molecular Imager FX with the external laser set to 488 nm to  
11 excite the NBD-labeled PFO.  
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25 *Triton X-100 solubility.* For detergent-insolubility assays, 30  $\mu$ l of 5 mM liposomes (total  
26 lipid) were added to 170  $\mu$ l of chilled 0.6% (v/v) TX-100 in buffer A. The detergent-liposome  
27 reactions were incubated on ice for 30 min, and then the entire reaction (200  $\mu$ l) was centrifuged  
28 in a TLA 100.2 rotor (Beckman) at 217,000g for 1 h at 4 °C. The supernatant was carefully  
29 removed and the amount of [ $^{14}$ C]phosphatidylcholine and/or [ $^3$ H]cholesterol in both the  
30 supernatant and the pellet (if any) was determined after detergent extraction and  
31 ultracentrifugation.  
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## RESULTS

*Phospholipid Dependence of Cholesterol Requirement for PFO binding to Liposomes.* It was shown previously that the interaction of PFO with cholesterol-containing liposomes requires a considerable amount of cholesterol to be present in target bilayers (19, 34). We found this observation striking since PFO can form holes in mammalian endoplasmic reticulum membranes where the cholesterol content is known to be quite low (35-39). These apparently conflicting observations led us to examine more closely the relationship between PFO's cytolytic mechanism and the cholesterol content in model membrane systems.

Fluorescence spectroscopy was used to quantify the cholesterol dependence of PFO binding to large unilamellar vesicles (LUVs) composed of a simple binary mixture of cholesterol and PC. These LUVs consisted of increasing amounts of cholesterol with different PC species that varied both in the length and degree of saturation of their acyl chains (Table 1). The extent of PFO binding to different liposome preparations was monitored by the increase in PFO Trp emission intensity when Trp residues in domain 4 were exposed to the hydrophobic core of the membrane bilayer (19, 21).

PFO showed a remarkable range of ability to bind membranes composed of different PC species. PFO bound to liposomes containing di-unsaturated DOPC at ~5 mole% lower cholesterol concentrations than to liposomes containing mono-unsaturated SOPC (Fig. 1A) or saturated DSPC (Fig. 1B). Thus, the extent of PC acyl chain unsaturation is an important variable in determining the cholesterol content required for PFO binding.

The phospholipid acyl chain length also affects PFO binding, though less dramatically than acyl chain unsaturation. Increasing the length of the saturated acyl chain at the sn-1 position by 2 carbons (SOPC, 18 carbons vs POPC, 16 carbons; Table 1) lowered the cholesterol content

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3 required for PFO binding for mono-unsaturated PCs (Fig. 1A), albeit only modestly. To further  
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5 examine how the phospholipid acyl chain length influences PFO's ability to recognize  
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7 cholesterol in membranes, binding was measured using cholesterol-containing liposomes and  
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9 PCs with completely saturated acyl chains that were 14, 16, or 18 carbons in length (Table 1).  
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11 As in the above experiments, the change in Trp emission intensity was used to monitor PFO  
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13 binding to the LUVs containing saturated PCs and cholesterol. In contrast to PFO binding to  
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15 liposomes having at least one unsaturated acyl chain, a greater concentration of cholesterol was  
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17 required for PFO to interact with saturated PC/cholesterol liposomes (Fig. 1B). Only minimal  
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19 PFO binding to DMPC/cholesterol liposomes is observed, unless the cholesterol concentration is  
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21 higher than 50 mole%. But as the acyl chains were lengthened by 2 or 4 carbons (DPPC or  
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23 DSPC, respectively), the amount of cholesterol needed for PFO binding was lowered, and the  
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25 cholesterol dependence profiles were shifted toward the one for POPC/cholesterol.  
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32 *Cholesterol Requirement for PFO Oligomerization on PC-liposomes.* The above  
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34 fluorescence experiments examined the binding of PFO to cholesterol-containing membranes.  
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36 However, the experiments did not address whether the subsequent steps of the PFO cytolytic  
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38 mechanism, oligomerization and transmembrane beta-hairpin (TMH) insertion (19, 32, 33, 40),  
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40 had the same cholesterol dependence. Since oligomerization and TMH insertion occur after PFO  
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42 binds to the membrane, it is conceivable that the cholesterol requirement would not be the same  
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44 for these steps.  
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49 To test whether or not oligomerization occurred on the different PC-liposomes, we  
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51 employed a technique known as SDS-AGE to monitor PFO oligomerization because PFO  
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53 oligomers exhibit significant or complete resistance to dissociation by SDS. This technique has  
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55 already been used to show that PFO forms large, oligomeric prepore complexes prior to insertion  
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3 of its membrane-spanning TMHs (33). Therefore, the extent (if any) of PFO oligomerization on  
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5 the various cholesterol-containing membranes can be determined by the presence of an SDS-  
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7 resistant oligomeric band.  
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10 The degree of PFO oligomerization was determined by incubating cholesterol-containing  
11 liposomes with NBD-labeled PFO<sup>D397C, C459A</sup> (a PFO derivative labeled with a fluorescent NBD  
12 dye at position 397, hereafter termed NBD-PFO) and resolving the PFO samples on a 1.5%  
13 agarose gel. The Asp397 residue is water-exposed in the PFO monomer, and after  
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15 oligomerization remains exposed to the aqueous solvent. Substitution of Asp397 for Cys and  
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17 labeling with NBD did not modify the pore-forming properties of the toxin (21). For these  
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19 experiments, we focused on POPC- and DOPC-cholesterol liposomes since these liposome  
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21 compositions showed a large disparity in their cholesterol dependence curves of PFO binding  
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23 (Fig. 1A). NBD-PFO oligomerization on POPC- and DOPC- cholesterol liposomes (Fig. 2, lanes  
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25 A-F and G-L, respectively) showed the same dependence on cholesterol concentration as Trp-  
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27 detected PFO binding to liposomes (Fig 1A).  
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36 In addition to examining the cholesterol concentration dependence of PFO binding to and  
37 oligomerization on various PC liposomes, we observed that the same cholesterol dependence  
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39 held true for both TMH insertion (19) and pore formation (data not shown). Therefore, the above  
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41 results, taken in total, indicate that the length and degree of saturation of the phospholipid acyl  
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43 chains comprising the bilayer, along with the amount of cholesterol, dictate when cytolysis  
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45 occurs. If PFO cannot bind to the membrane, then the subsequent steps (oligomerization, TMH  
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47 insertion and pore formation) in the cytolytic mechanism will not occur. Since the primary effect  
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49 of changing phospholipids is to alter the amount of cholesterol required, it suggests that the  
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51 ability of PFO to recognize and/or interact with cholesterol at the membrane surface is  
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3 influenced by changing the physicochemical and/or steric properties of the phospholipid acyl  
4 chains and membranes.  
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8 *The Relative Ordering of the Phospholipid Acyl Chains does not Influence PFO Binding*  
9 *to Membranes.* One of the major concerns when changing the phospholipid and sterol  
10 compositions of a membrane is the effect the change may have on the dynamics of the lipid  
11 bilayer. Generally, membrane fluidity reflects the motion and order (*i.e.*, packing) of the various  
12 lipids within a membrane bilayer. To assess membrane fluidity, small fluorescent probes are  
13 often incorporated into bilayers to measure the acyl chain packing of the phospholipids. A large  
14 body of work on model membrane systems demonstrates that the molecular order and dynamics  
15 of the lipid chains are drastically affected by the introduction of double bonds into the fatty acid  
16 chains of the lipid. In addition, the presence of cholesterol in the membrane has an impact on the  
17 physical state and order of the surrounding lipids (*41, 42 and references cited therein*).  
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32 To measure the relative acyl chain packing of the various PC/cholesterol liposomes used  
33 in this study, we incorporated the fluorescent probe DPH into our cholesterol-containing  
34 liposomes and measured the steady-state anisotropy of the probe. Figure 3 summarizes these  
35 results for DPH incorporated at 0.5 mol% into the liposomes. For DPPC- and DSPC-cholesterol  
36 liposomes at 25 °C, the steady-state anisotropy is high (0.33 – 0.34) and nearly constant from 30-  
37 52 mol% cholesterol. These results are consistent with a high degree of acyl chain order that is  
38 independent of the high amount of cholesterol present in the membrane.  
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48 We also compared the steady-state anisotropy of DPH in monounsaturated POPC and  
49 diunsaturated DOPC as a function of cholesterol concentration. For these cholesterol-containing  
50 membranes at 25 °C, DPH steady-state anisotropies were lower throughout the entire cholesterol  
51 range when compared to the disaturated PCs, consistent with more freedom of motion of the  
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3 DPH probe in the unsaturated acyl chain environment of POPC or DOPC. Increasing the  
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5 cholesterol concentration results in an approximately linear increase in DPH anisotropy,  
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7 indicating that cholesterol restricts the molecular motion of the acyl chains, though to a lesser  
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9 extent than in the presence of disaturated phospholipids.  
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13 When comparing anisotropy values obtained at the same cholesterol concentration for the  
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15 various phospholipids:cholesterol mixtures, the difference in DPH anisotropy values can be  
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17 attributed to the presence of the double bonds in the unsaturated acyl chains that tend not to pack  
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19 together as tightly as the saturated acyl chains. These results clearly show that the relative acyl  
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21 chain packing in the hydrophobic core of the membrane is sensitive both to the  
22  
23 phospholipid:cholesterol ratio and to the degree of unsaturation of the phospholipid acyl chains.  
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25 However, it is also clear that when comparing the liposome compositions that support PFO  
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27 binding (Figs. 1A, 1B) to the DPH anisotropy values of those liposomes (Fig. 3), there is no  
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29 correlation between the DPH-detected lipid packing and the ability of PFO to recognize the  
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31 cholesterol molecules in the liposomes. .  
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37 The above DPH anisotropies were measured at 25 °C, yet PFO was incubated with  
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39 liposomes at 37 °C in the above Trp binding experiments. Since changes in temperature may  
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41 affect the properties of a membrane, we also measured the DPH anisotropy of the liposomes at  
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43 37 °C and found no significant change in the anisotropy profiles obtained at these temperatures  
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45 (data not shown) (43). Therefore, we conclude that the packing of the phospholipids acyl chains  
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47 and cholesterol into the membrane core does not dictate whether or not PFO binds to the bilayer  
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49 surface.  
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53 *PFO Binds to Membranes Containing High Cholesterol Content Independently of Their*  
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55 *Detergent Solubilization Properties.* The above DPH anisotropy experiments provided some  
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3 information regarding the overall behavior of the lipids in the liposomes (*i.e.*, degree of lipid  
4 order). To determine if PFO binding to membranes correlated with the presence of detergent-  
5 resistant membranes in PC/cholesterol liposomes, we determined the extent of detergent-  
6 insoluble lipid material in various liposomes and compared the results to the PFO binding data.  
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8 The PC/cholesterol liposomes were made with trace amounts of [<sup>3</sup>H] cholesterol and [<sup>14</sup>C]  
9 phosphatidylcholine so that we could determine the amount of insoluble membrane material by  
10 performing solubilization experiments with cold TX-100. The PC/cholesterol liposomes were  
11 then subjected to extraction with 0.6% (v/v) TX-100 at 0 °C, followed by ultracentrifugation to  
12 pellet any insoluble lipid material (44). The data revealed that the percentage of insoluble PC and  
13 cholesterol (amount of pelleted material) varied depending on the degree of saturation of the PC.  
14  
15 At concentrations of ~30 mole% and above the saturated PC lipids, DPPC (Fig. 4) or DMPC  
16 (data not shown), had the highest percentage of cholesterol and PC (92 – 100 %) in the pellet  
17 regardless of the amount of cholesterol in the liposomes. The large amount of insoluble lipid in  
18 DPPC- and DMPC-cholesterol liposomes correlates with the lipids being in the *l<sub>o</sub>* phase, as  
19 suggested by their respective lipid/cholesterol phase diagrams (45, 46). These *l<sub>o</sub>* phases have  
20 been shown to be more resistant to solubilization by detergents than *l<sub>d</sub>* ones (47).  
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42 In contrast to the saturated PC/cholesterol liposomes, DOPC/cholesterol liposomes were  
43 completely solubilized in cold TX-100, as shown by the lack of insoluble PC or cholesterol (Fig.  
44 4). The poor interaction between DOPC and cholesterol prevented the packing of the acyl chains,  
45 making the lipids to be more susceptible to detergent solubilization. Interestingly, the detergent  
46 insolubility data with POPC/cholesterol liposomes revealed insolubility properties that were  
47 intermediate between those of the saturated- and diunsaturated-cholesterol liposomes. In POPC  
48 liposomes, the percentage of insoluble cholesterol and PC in the pellet increased with increasing  
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3 cholesterol concentrations (Fig. 4). Since POPC contains both a saturated acyl chain and an  
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5 unsaturated acyl chain, membranes containing POPC can form the  $l_o$  phase when mixed with  
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7 sufficient amounts of cholesterol (44, 48). Therefore, increasing the cholesterol concentration in  
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9 POPC liposomes appears to contribute to the packing of the acyl chains making the lipids more  
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11 resistant to detergent solubilization. Since the ratio of  $^{14}\text{C}/^3\text{H}$  in the pellet was constant, cold TX-  
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13 100 did not preferentially solubilize either cholesterol or phospholipid from the liposomes.  
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17 If PFO binding to membranes was based solely on whether the membranes are either  
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19 detergent-resistant, detergent-soluble, or a combination of both properties, then there should be a  
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21 correlation between the presence of these solubilization properties and PFO binding. However,  
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23 PFO was able to bind to membranes being detergent-resistant (DMPC, DPPC, POPC) or  
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25 detergent-soluble (DOPC), albeit at different cholesterol concentrations. For example,  
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27 DOPC/cholesterol liposomes are detergent-soluble at either 30- or 50-mole% cholesterol (Fig.  
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29 4), yet PFO binds only to the liposomes containing 50 mole% cholesterol (Fig 1A). Similarly,  
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31 DPPC/cholesterol liposomes are mostly detergent-insoluble at all cholesterol concentrations (Fig.  
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33 4), yet PFO binds only to the liposomes containing at least 50 mole% cholesterol (Fig 1B). Thus,  
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35 the detergent solubility properties of membranes do not appear to be associated with PFO  
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37 recognition of and binding to cholesterol-containing membranes.  
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#### 43 *Sphingolipid-cholesterol Interaction Reduces the Ability of PFO to Recognize*

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45 *Cholesterol on the Membrane Surface*—All of the above experiments were performed on  
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47 liposomes composed of a binary mixture of lipids (*i.e.*, cholesterol and PC), yet biological  
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49 membranes contain a variety of lipid species possessing different lengths of acyl chains, head  
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51 groups, and number of double bonds. Since one of the objectives of this work is to elucidate the  
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53 role of phospholipids in PFO binding to cholesterol-containing membranes, we decided to  
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3 examine PFO binding to liposomes composed of a more complex lipid mixture. In doing so, we  
4 prepared a series of liposomes containing a mixture of DOPC, cholesterol, and SM to see if the  
5 presence of sphingolipids influenced PFO binding and oligomerization. Since liposomes  
6 containing 60 mole% DOPC and 40 mole% cholesterol supported PFO binding (Fig. 1A),  
7 liposomes were prepared with 40 mole% cholesterol and varying proportions of DOPC and SM  
8 in the 60 mole% of nonsterol lipid.  
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18 As the amount of SM was increased in the nonsterol fraction from 17 mole% to 50 mole%  
19 total lipids, the change in PFO Trp emission intensity decreased, thereby suggesting that PFO  
20 does not recognize or bind cholesterol in membranes that contain a high percentage of  
21 sphingolipids (Fig. 5A). However, since the presence of SM in the membrane modifies the  
22 spectral properties of the membrane-bound Trp residues (data not shown), a direct correlation  
23 between PFO binding and the total SM content cannot be ascertained from this data. The extent  
24 of PFO binding as the concentration of SM increase in the membrane, was also determined by  
25 measuring formation of oligomers by NBD-PFO on DOPC/SM/cholesterol. Oligomerization is a  
26 good reporter for the cholesterol-dependent PFO-membrane interaction because PFO does not  
27 oligomerize in solution (49) or in the absence of cholesterol (26). The amount of PFO  
28 oligomerization decreased as the percentage of SM in the nonsterol fraction increased (Fig. 5B).  
29 Thus, replacing DOPC molecules in the bilayer with SM caused a reduction in PFO binding to  
30 and oligomerization on the membrane.  
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49 Although the mole% of membrane cholesterol remained constant, replacing the  
50 unsaturated DOPC with the saturated sphingolipid species clearly affected PFO's ability to  
51 recognize and bind to cholesterol in the membranes. Since it has been shown previously that  
52 sphingolipids preferentially interact with sterols in both biological and model membranes and  
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3 form detergent-resistant membranes (50), we measured the amount of these insoluble material  
4 present in the DOPC/SM/cholesterol membranes by performing TX-100 insolubility assays. The  
5 DOPC/SM/cholesterol membranes contained a trace amount of [<sup>3</sup>H]cholesterol in order to  
6 determine the amount of cholesterol present in the detergent-resistant fraction. After incubation  
7 with cold non-ionic TX-100, the samples were pelleted, and the amount of radioactive  
8 cholesterol recovered in the pellet was plotted as a function of the initial percentage of SM in the  
9 nonsterol fraction. When the membranes contained no or very little SM, most of the  
10 [<sup>3</sup>H]cholesterol was found in the supernatant and no lipid pellet was observed after incubation  
11 with TX-100 (Fig. 5C). However, as the amount of SM increased in the cholesterol-containing  
12 membranes, a detergent-resistant pellet started to appear, and the percentage of [<sup>3</sup>H]cholesterol  
13 found in the pellet increased (Fig. 5C). When the nonsterol fraction of the membranes contained  
14 an equimolar amount of DOPC and SM (30 mole% apiece), approximately 40% of the  
15 [<sup>3</sup>H]cholesterol could be found in the detergent-resistant pellet. The amount of [<sup>3</sup>H]cholesterol  
16 found in the detergent-resistant pellet increased to roughly 80% when the nonsterol fraction was  
17 entirely SM. Since the formation of a detergent-resistant sphingolipid-cholesterol phase  
18 coincided with a decrease in PFO binding to the DOPC/SM/cholesterol membranes, it appears  
19 that PFO does not recognize or bind to cholesterol that is sequestered into sphingolipid-  
20 cholesterol rich domains.  
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46 *Only a Limited Number of the Cholesterol Molecules in the Membrane are Accessible to*  
47 *Interact with PFO*— Early experiments demonstrated that some sterols that are structurally related  
48 to cholesterol had an inhibitory effect on the hemolytic properties of the CDC bacterial toxins  
49 (51, 52). From those inhibition studies, the structural and stereospecific requirements for the  
50 sterol molecule were identified. One such requirement was the presence of a hydroxyl group in  
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3 the  $\beta$ -configuration on carbon-3 of ring A of the cyclopentanoperhydrophenanthrene ring of  
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5 cholesterol. However, if the hydroxyl group was in the  $\alpha$ -configuration, as in epicholesterol, then  
6  
7 the sterol did not inhibit toxin activity because the toxin presumably could not recognize the  
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9 sterol derivative (26, 53).  
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13 To examine how cholesterol packing in the bilayer affects PFO interactions with the  
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15 membrane, we took advantage of the inability of PFO to recognize epicholesterol and performed  
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17 competition experiments with these two sterols. Since the stereochemistry of the polar OH group  
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19 is the only difference between cholesterol and epicholesterol, it has been suggested that the flat  
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21 four-ring portions of the two sterols pack similarly with phospholipid acyl chains (54). Since  
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23 PFO bound to and oligomerized on POPC membranes containing 48 mole% or more cholesterol  
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25 (Fig. 1A, 3), the total sterol concentration was fixed at 48 mole% of total lipids and POPC was  
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27 used as the phospholipid in the following experiments. NBD- PFO was incubated with the  
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29 various liposome preparations, and the extent of PFO binding and oligomerization was detected  
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31 by visualization of SDS-resistant oligomeric bands. When the membrane was composed of only  
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33 POPC and epicholesterol, no PFO oligomerization was detected (Fig. 6, lane A). This result  
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35 confirms previous data regarding PFO's inability to interact with a sterol having the OH group in  
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37 the  $\alpha$ -configuration (1, 26). As the ratio of cholesterol:epicholesterol increased (Fig. 6, lanes B-  
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39 F), PFO oligomers began to form until the majority of PFO was present as an oligomeric species.  
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41 Interestingly, when only 19 mole% cholesterol was present in the membrane, SDS-resistant PFO  
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43 oligomers appeared (Fig. 6, lane C). This result appears to conflict with our earlier findings that  
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45 PFO required a minimum of 45 mole% cholesterol in POPC membranes to bind and form  
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47 detectable oligomers. However, the membranes used in this sterol supplementation experiment  
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49 contained a substantial amount of epicholesterol to maintain the total sterol concentration at 48  
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3 mole% in the membranes. Thus, while at least 45 mole% cholesterol was required for PFO  
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5 binding when POPC/cholesterol liposomes were examined (Fig. 1A), more than half of those  
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7 sterol molecules were not directly involved in PFO-membrane interactions. The simplest  
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9 explanation for this observation is that most of the sterol molecules in the bilayer are tightly  
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11 packed with the phospholipids and hence not available to interact with PFO.  
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## DISCUSSION

Our examination of the cholesterol dependence of PFO binding to and interaction with membranes has provided insights into the mechanisms by which PFO accomplishes the initial cholesterol recognition step during pore formation. By applying a number of different experimental approaches, we were able to assess several different possibilities for the nature of the PFO-cholesterol interaction and ultimately identify the most important factor in determining whether PFO binds to the membrane surface. This factor, the presence of free cholesterol in the membrane, is critical for triggering the PFO-membrane interaction, while the packing of the acyl chains of the phospholipids, and the nature of the phospholipids in the bilayer are only important to the extent they influence cholesterol chemical activity or the exposure of cholesterol to the surface (55, 56).

This conclusion is based primarily on the epicholesterol data that revealed that the efficacy of PFO binding to the membrane surface was dictated by both the presence of cholesterol and the total sterol content in the bilayer (Fig. 6). PFO binds directly to pure cholesterol (26), but PFO-membrane recognition and binding requires PFO to access cholesterol that is membrane-embedded, yet surface-exposed. Cholesterol interacts more favorably with saturated acyl chain lipids than with unsaturated acyl chains lipids (41 and references cited therein), and recent studies with monolayers and bilayers have demonstrated the existence of cholesterol-enriched regions in membranes whose formation is heavily dependent on the degree of phospholipid acyl chain unsaturation, head group structure, and acyl chain length (56-59). Since epicholesterol has similar membrane packing characteristics as cholesterol (60), but is not functional in PFO pore formation [Fig. 6, lane A; (26)], the ability to partially replace cholesterol with epicholesterol without blocking PFO binding and oligomerization shows that some sterols

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3 in the membrane associate with phospholipids while others are free to interact directly with PFO.  
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5 Hence, PFO binding was observed to membranes containing as little as 19 mole% cholesterol  
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7 because the added epicholesterol (Fig. 6, lane C) associates and interacts with phospholipids in  
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9 the membrane allowing some of the cholesterol molecules to be free to interact with PFO.  
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11 Epicholesterol apparently intercalates in the bilayer and competes with cholesterol for  
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13 association with phospholipids, as reported for other membrane intercalating agents (61). These  
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15 data therefore reveal that there are at least two distinctive states of cholesterol in a typical  
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17 membrane bilayer: one in which cholesterol is readily accessible for binding to proteins such as  
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19 PFO (free cholesterol), and one in which the sterol is associated with surrounding membrane  
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21 components that reduce its exposure to the surface (*e.g.*, phospholipid headgroups may obscure  
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23 access to sterols associated with phospholipid acyl chains).  
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29 Consistent with our view, recent work done on two other cytolysins that bind cholesterol  
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31 (Streptolysin O and *Vibrio cholerae* toxin), indicated that reducing the size of the phospholipid  
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33 head group caused an increase in cholesterol exposure and consequently an increase in the  
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35 binding of the cytolysins (62). We have confirmed these observations by replacing some of the  
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37 choline-containing POPC with ethanolamine-bearing POPE, a phospholipid with the same acyl  
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39 chains but a smaller head group. The addition of POPE reduced the amount of cholesterol  
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41 required for PFO binding (data not shown). In contrast, the presence of 10 mole% POPS in a  
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43 POPC:POPS:cholesterol mixture did not affect the binding profile of PFO when cholesterol is  
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45 varied from 34 mole% to 55 mole%, either in the presence or absence of 2 mM Ca<sup>2+</sup> (data not  
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47 shown).  
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53 Similarly, PFO binding requires less cholesterol when the liposomal phospholipids  
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55 contain unsaturated rather than saturated acyl chains [Fig. 1; (29, 30)], presumably because acyl  
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3 chain kinks introduced by the double bonds occupied more space within the bilayer and therefore  
4 pushed adjacent sterols out from under the head group where they were less accessible to PFO.  
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8 We also observed that less cholesterol was required for PFO binding when the phospholipid acyl  
9 chain length increased up to 18 carbons (Fig.1). It have been shown that sterol/phospholipid  
10 interactions are affected by the hydrophobic mismatch between these two lipids because the  
11 hydrophobic “length” of cholesterol is equivalent to the length of a PC with 17 carbon saturated  
12 acyl chains (41). Phospholipid acyl chains with more than 17 carbons must bend to  
13 accommodate the shorter cholesterol molecule, and as a consequence the cholesterol molecules  
14 are pushed out from under the head group and become more exposed to PFO. Thus, the steric  
15 demands and interactions of lipid molecules in the bilayer will influence whether or not  
16 cholesterol is accessible to PFO. These findings agreed with those reported previously (34),  
17 though no clear conclusions evolved from their more limited investigation.  
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32 We also examined other membrane properties that did not correlate with the cholesterol  
33 dependence of PFO binding to the membrane. The relative acyl chain packing was one such  
34 property, as PFO was just as capable of binding to membranes displaying a low amount of lipid  
35 order (*i.e.*, DOPC/cholesterol) as to membranes having a more restricted lipid motion (*i.e.*,  
36 DPPC/cholesterol) (Fig. 3). In addition, we also investigated whether PFO had a preference for  
37 binding to detergent-resistant or detergent-soluble membranes. Using cold TX-100 as an assay  
38 for membrane resistant to solubilization, we found the PFO bound both to DOPC/cholesterol  
39 liposomes that were completely soluble in TX-100 and to DPPC/cholesterol liposomes that were  
40 insoluble in TX-100 (Fig. 4). These data indicate that the detergent solubility properties of a  
41 liposome comprised of cholesterol and a single phospholipid does not correlate with the binding  
42 of PFO to the membrane.  
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However, when we examined more complex lipid mixtures containing DOPC, SM, and a constant amount of cholesterol, we discovered that the ratio of DOPC:SM in the membranes affected PFO binding and oligomerization (Fig. 5). Increasing the SM content of the membranes enhanced their resistant to cold TX-100 solubilization and reduced the ability of PFO to recognize and/or bind to cholesterol. PFO binding to membranes therefore appeared to be negatively influenced by the presence of SM. More importantly, this result suggests that PFO does not recognize or bind to cholesterol that is sequestered in SM rich microdomains, structures that are commonly associated with the presence of “rafts” in cell membranes (63).

Our data therefore conflict with an earlier report that PFO interacts preferentially with cholesterol located in lipid “rafts” in natural membranes (64, 65). Their conclusions were based on the co-localization of a protease-treated PFO fragment with lipid raft-associated proteins during TX-100 detergent-insolubility assays. In our experience, such an experimental approach is susceptible to multiple interpretations and this may explain the difference between their conclusions and ours. Using the detergent and pelleting conditions reported in their work to examine PFO localization in “rafts”, we found that the majority of the PFO was located in the pellet after treatment with TX-100 when either DOPC- or DPPC-liposomes containing 50 mole% cholesterol were used (data not shown). Since DOPC/cholesterol membranes were soluble in TX-100 (Fig. 4), the PFO in the pellet was not bound to any detergent-resistant membranes. This result demonstrated that the membrane-inserted PFO oligomer was resistant to dissociation by either SDS or TX-100 since oligomeric PFO was found in the pellet. The resistance of the PFO oligomer to TX-100 solubilization may therefore complicate the interpretation of the sedimentation data because PFO oligomers may sediment in the presence of TX-100 even if they were formed on non-raft membrane surfaces. Since our data were obtained with full-length PFO

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3 and uniform chemically-defined liposomes, we believe that PFO does not necessarily bind to  
4 lipid “rafts” in membranes. But we also recognize that the conflicting conclusions may reflect  
5 limitations in the TX-100 assay as applied to natural and/or synthetic membranes (47, 50).  
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10 It is clear from the data presented in this work that the total amount of cholesterol  
11 required to trigger PFO binding to a particular membrane will be influenced by the lipid  
12 composition of the bilayer. Moreover, the total amount of cholesterol required to initiate PFO  
13 binding will also be affected by the presence of membrane proteins due to specific lipid binding  
14 to, association with, or intercalation into the transmembrane segments of these proteins. We  
15 speculate that the latter effect may be the reason that PFO binds to and forms pores in ER  
16 membranes (37-39) even though their total cholesterol content is very low, because the non-  
17 sterol lipid components are more likely to pack within the membrane proteins than are the  
18 relatively rigid cholesterol molecules. One effect of this selective partitioning is that the mole  
19 fraction of cholesterol in the bulk lipid in the exposed membrane surface area will increase (35).  
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21 Whatever the explanation for the sensitivity of ER membranes to PFO, one should be cautious  
22 when using PFO or any PFO derivatives to track the total cholesterol content in cellular  
23 membranes. PFO binding may depends on the “free cholesterol” or cholesterol chemical activity,  
24 rather than on the total amount of cholesterol present in the membrane, and the chemical activity  
25 will be influenced by the lipid composition and the presence of other membrane components.  
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30 In summary, exposure of free cholesterol at the membrane surface is essential for PFO  
31 binding to the bilayer and the initiation of the sequence of events that culminate with the  
32 spontaneous formation of a transmembrane pore. Bending of the phospholipids acyl chains by  
33 introducing double bonds or reducing the size of the phospholipid head groups decreased the  
34 threshold of cholesterol required to trigger binding. In contrast, changes in the relative packing of  
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3 lipids in the membrane core or the presence or absence of detergent-resistant membranes did not  
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5 correlate with PFO binding. Finally, addition of molecules that do not interact with PFO, but  
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7 intercalate into the membrane and displace cholesterol from its association with phospholipids  
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9 (*e.g.*, epicholesterol), reduced the amount of cholesterol required to trigger PFO binding. Since  
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11 exposure of free cholesterol at the surface of membranes is necessary for the binding of CDCs  
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13 and other bacterial and viral proteins (15, 66-70), the results reported here may be generally  
14  
15 applicable to other protein-membrane interactions involved in human pathogenesis.  
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## 22 **ACKNOWLEDGMENTS**

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24 We thank Dr. Paul Moe for critically reading the manuscript.  
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## 29 **SUPPORTING INFORMATION AVAILABLE**

30  
31 Detailed protocols for the expression, labeling, and purification of PFO derivatives, Förster  
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33 resonance energy transfer (FRET) measurements, and steady-state fluorescence spectroscopy  
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35 procedures. FRET analysis of the PFO binding to LUVs containing different amount of  
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37 cholesterol. A figure describing the primary sequence for commonly used PFO derivatives. This  
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39 material is available free of charge via the Internet at <http://pubs.acs.org>.  
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## TABLES

Table 1. Names and acyl-chain properties of PCs used to prepare liposomes

Abbreviation	1-Acyl	Carbons : Double bonds	2-Acyl	Carbons : Double bonds
POPC	Palmitoyl	16 : 0	Oleoyl	18 : 1 ( $\Delta 9$ )
DOPC	Oleoyl	18 : 1 ( $\Delta 9$ )	Oleoyl	18 : 1 ( $\Delta 9$ )
SOPC	Stearoyl	18 : 0	Oleoyl	18 : 1 ( $\Delta 9$ )
DMPC	Myristoyl	14 : 0	Myristoyl	14 : 0
DPPC	Palmitoyl	16 : 0	Palmitoyl	16 : 0
DSPC	Stearoyl	18 : 0	Stearoyl	18 : 0

**FIGURE LEGENDS**

Fig. 1. **Cholesterol dependence of PFO binding to liposomes comprised of cholesterol and various phospholipids.** Changes in the PFO Trp emission intensity are shown as a function of the cholesterol content of the different liposomes. The net  $F / F_0$  was calculated as described in Experimental Procedures. (A) DOPC,  $\blacklozenge$ ; SOPC,  $\blacksquare$ ; POPC,  $\bullet$ . (B) DSPC,  $\blacktriangle$ ; DPPC,  $\blacksquare$ ; DMPC,  $\circ$ . The cholesterol dependence curve for POPC ( $\bullet$ ) is shown for reference. Error bars indicate the standard deviation observed for 3 independent measurements per data point.

Fig. 2. **Cholesterol dependence of PFO oligomerization on POPC- or DOPC-cholesterol liposomes.** The amount of cholesterol present in the PC-liposomes is shown below each lane. Lanes A-F show PFO oligomerization with POPC/cholesterol liposomes; while lanes G-L contain DOPC/cholesterol liposomes. The NBD-PFO monomer and oligomer bands were visualized with a BioRad Molecular Imager FX as described in the Experimental Procedures.

Fig 3. **Effect of cholesterol concentration and phospholipid unsaturation on lipid order in liposomes as measured by DPH anisotropy.** DPH was incorporated into the PC/cholesterol membranes as described in Experimental Procedures. DPPC,  $\blacktriangle$ ; DSPC,  $\circ$ ; POPC,  $\bullet$ ; DOPC,  $\blacksquare$ .

Fig. 4. **Detergent insolubility of various PC/cholesterol liposomes.** Liposomes with trace amounts of  $[^3\text{H}]$ cholesterol and  $[^{14}\text{C}]$ PC were extracted with TX-100 at 0 °C. Radioactivity in the pellet fractions was measured after ultracentrifugation, and the percent of radioactivity in the pellet was plotted as a function of cholesterol concentration. (A) Fraction of  $[^3\text{H}]$ cholesterol

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3 found in pellet. (B) Fraction of [ $^{14}\text{C}$ ]PC found in pellet. In both (A) and (B), the PC species are  
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5 the following : DOPC, ○; POPC, ●; and DPPC, ■.  
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10 **Fig. 5. PFO binding to DOPC/SM/cholesterol membranes.** (A) Changes in the Trp emission  
11 intensity of PFO are shown as a function of the percentage of SM in the nonsterol fraction of the  
12 membranes. The net  $F / F_0$  was calculated as described in Experimental Procedures. Error bars  
13 indicate the standard deviation observed for 3 independent measurements per data point. (B)  
14 NBD-PFO oligomerization on DOPC/SM/cholesterol membranes. After incubation of PFO with  
15 the membranes, samples were solubilized with sample buffer and separated by 1.5% SDS-AGE.  
16 The NBD-PFO monomer and oligomer bands were visualized with a BioRad Molecular Imager  
17 FX as described in the Experimental Procedures. In both (A) and (B), the membranes were  
18 composed of 50 mole% cholesterol and various proportions of DOPC and SM comprising the  
19 remaining 50 mole% lipid in the nonsterol fraction. For example, when SM is at 50% in the  
20 nonsterol fraction, there is an equimolar amount of SM and DOPC (25 mole% each). (C).  
21 Formation of detergent-resistant domains in DOPC/SM/cholesterol membranes. The liposomes  
22 were composed of 40 mole% cholesterol and various proportions of DOPC and SM comprising  
23 the remaining 60 mole% lipid in the nonsterol fraction. Liposomes with trace amounts of  
24 [ $^3\text{H}$ ]cholesterol were extracted with TX-100 at 0 °C. Radioactivity in the pellet fractions was  
25 measured after ultracentrifugation, and the percent of radioactivity in the pellet was plotted as a  
26 function of the percentage of SM in the nonsterol fraction of the membrane.  
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53 **Fig. 6. NBD-PFO oligomerization on membranes containing cholesterol and epicholesterol.**

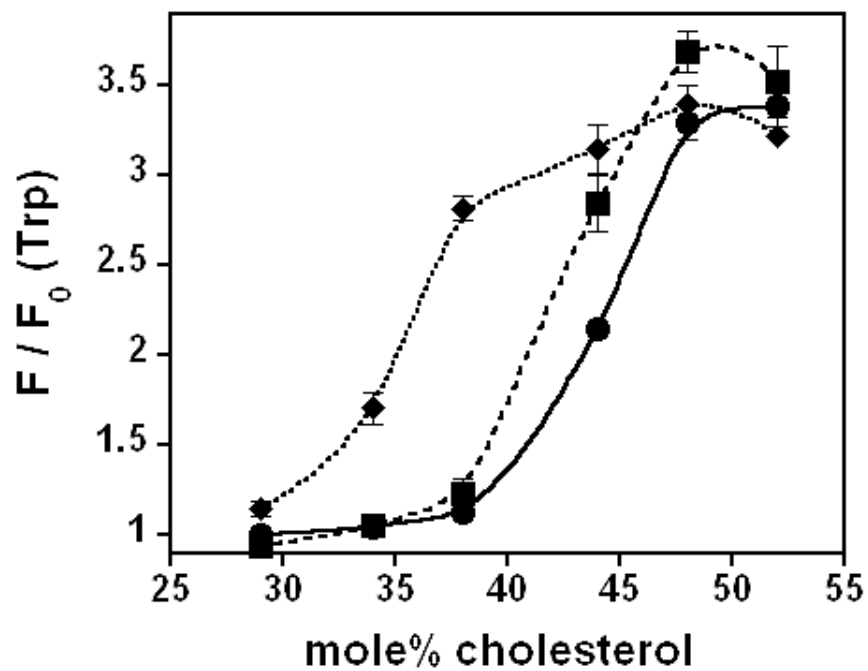
54 The liposomes contained 52 mole% POPC and 48 mole% sterol. The mole% of the individual  
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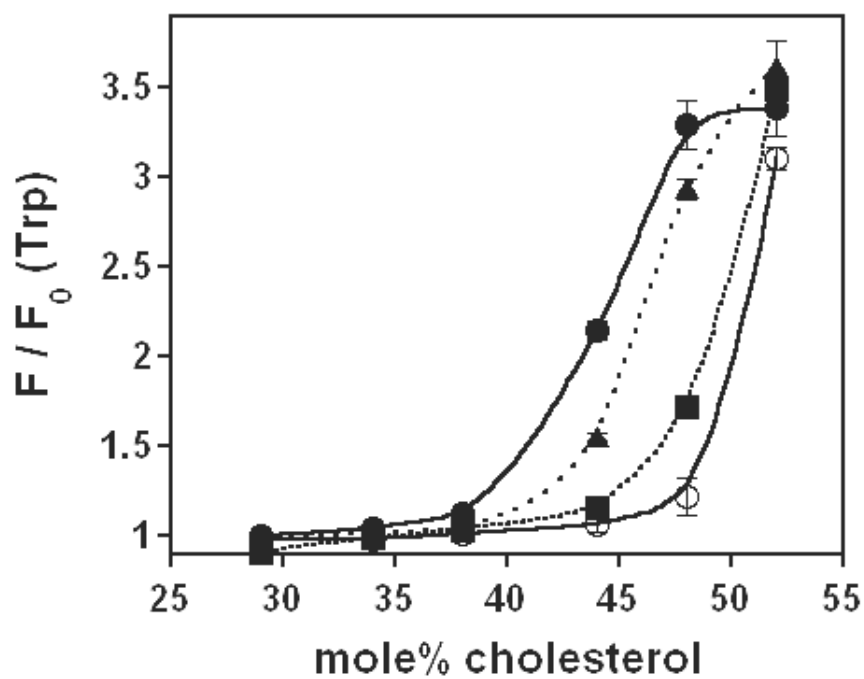
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3 sterols comprising the sterol fraction of the liposome is indicated below each lane in the above  
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5 figure. After incubation of PFO with the membranes, samples were solubilized with sample  
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7 buffer and separated by 1.5% SDS-AGE. The NBD-PFO monomer and oligomer bands were  
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9 visualized with a BioRad Molecular Imager FX as described in the Experimental Procedures.  
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Figure 1

A



B



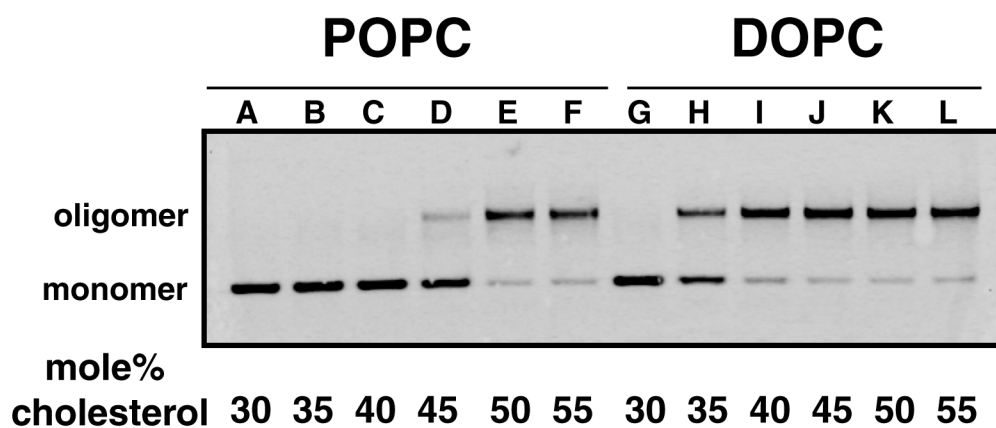
**Figure 2**

Figure 3

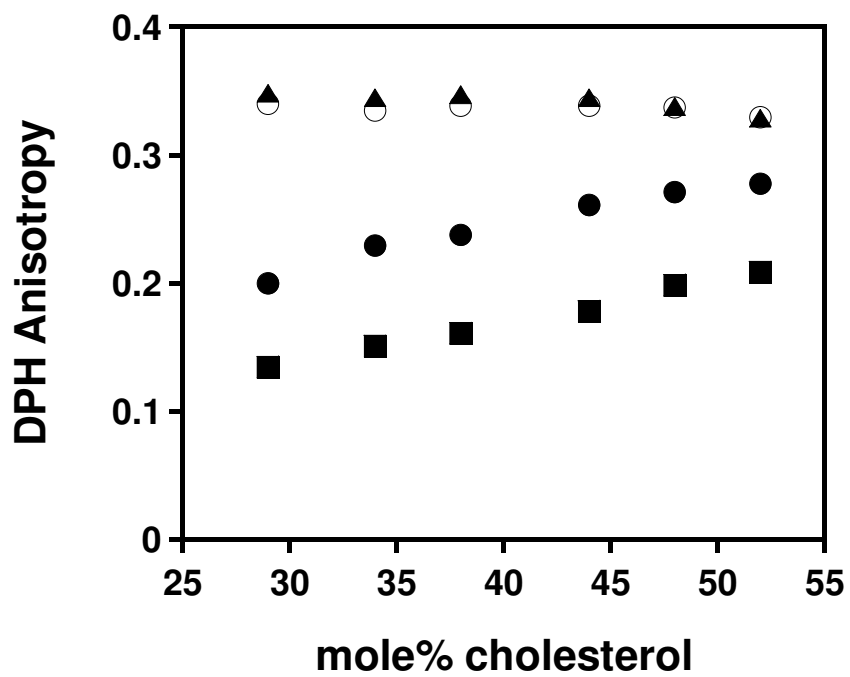


Figure 4

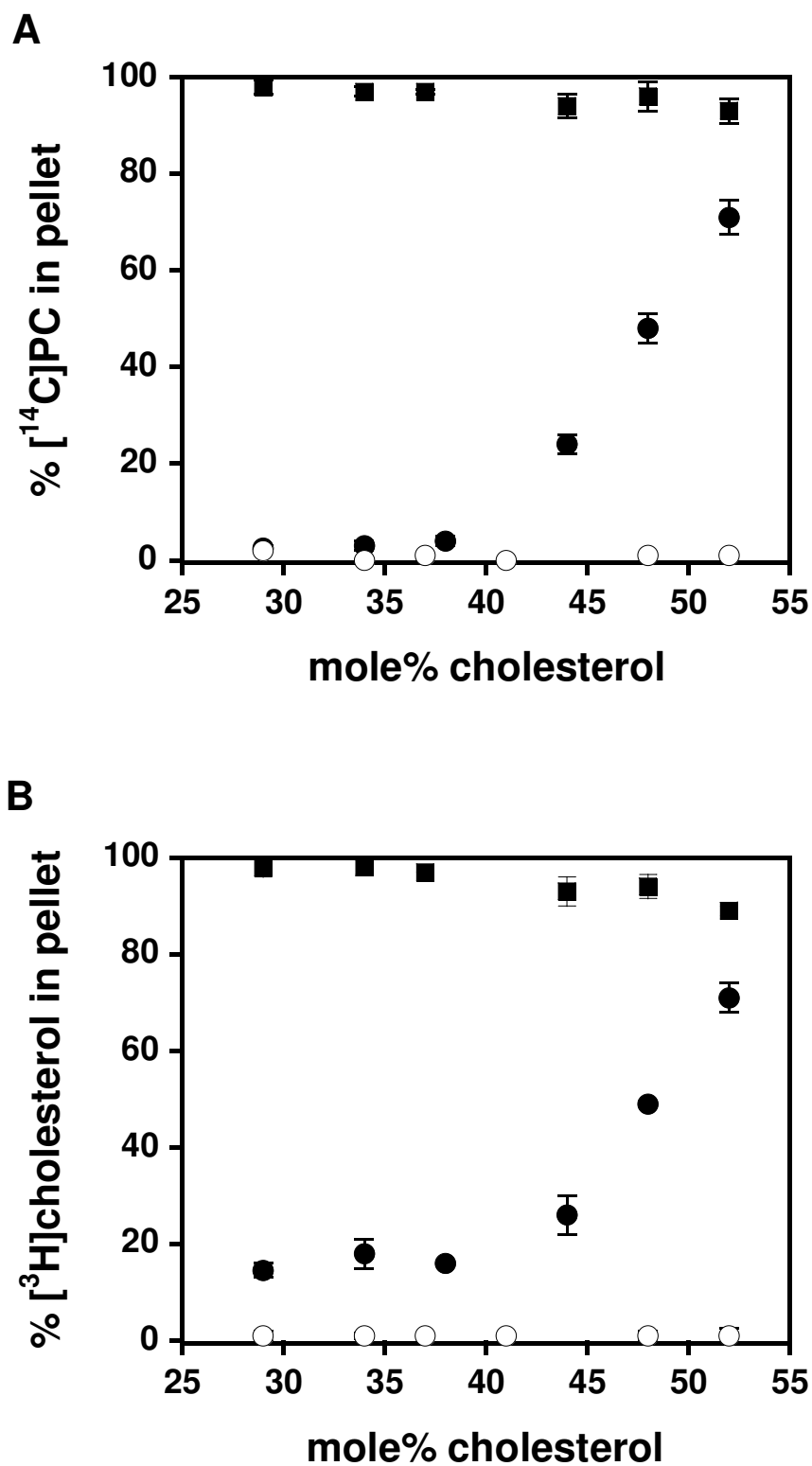
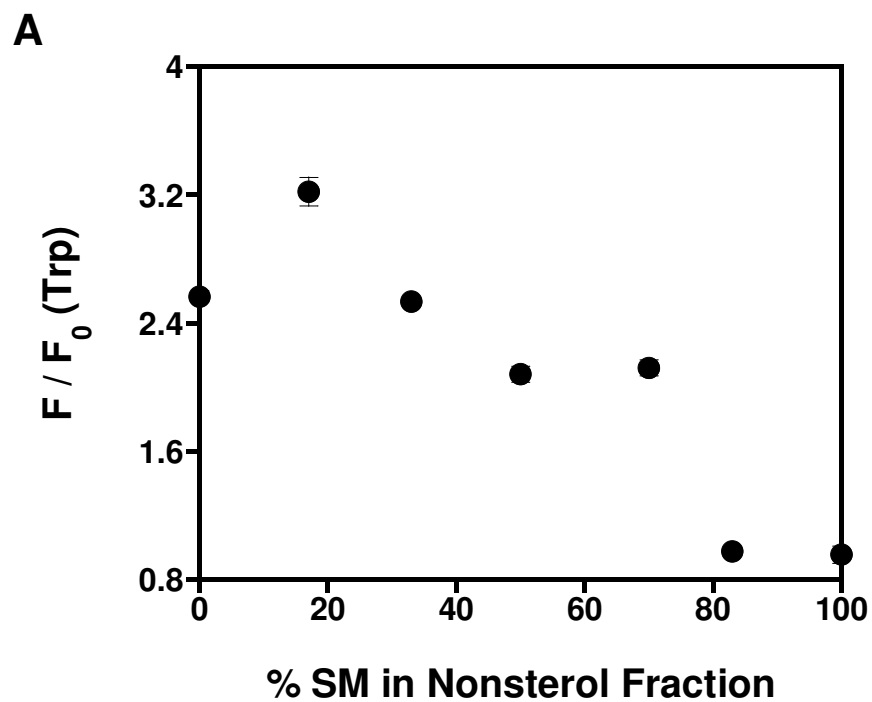
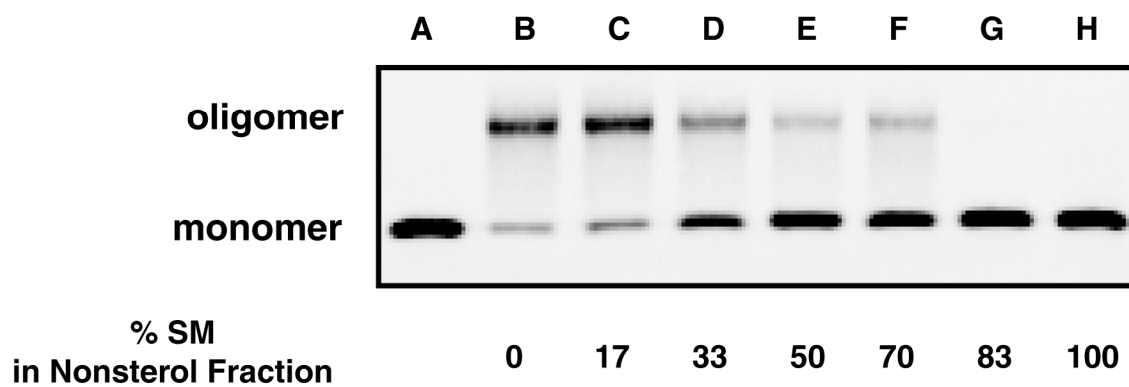
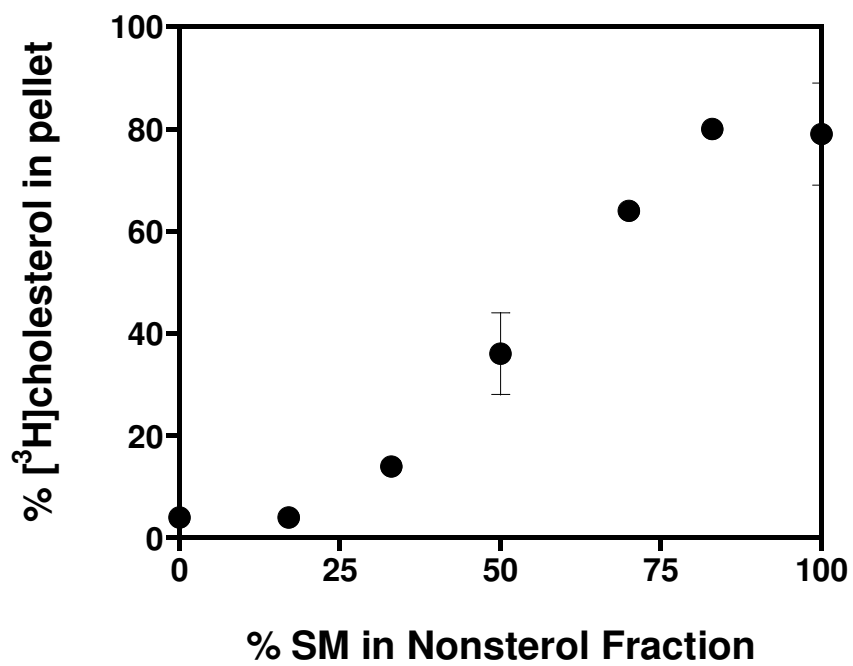
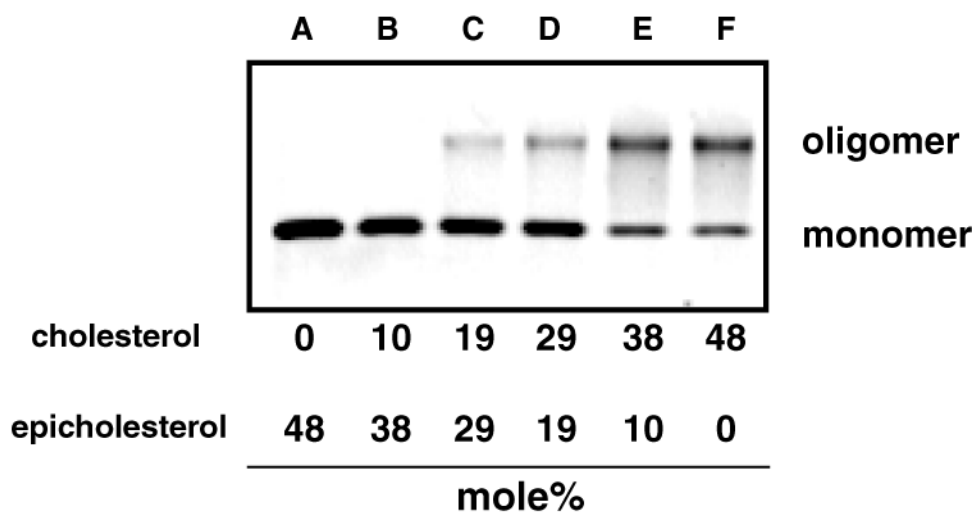


Figure 5

**B**

C



**Figure 6**



Graphic image to represent the paper within the Table of Contents

PFO

