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The effect of bilayer composition on calcium ion transport facilitated by fluid shear stress

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Abstract

Passive calcium ion permeability across liposome bilayers is increased during exposure to fluid shear forces attainable in the mammalian vasculature. In this study, liposomes prepared from three different lipid mixtures (phosphatidylcholine alone; phosphatidylcholine and cholesterol; a mixture of anionic and cationic phospholipids plus cholesterol) are exposed to uniform shear stress in a rotational viscometer. Liposome permeability to calcium ion is estimated from continuous measurement of free intraliposome calcium ion concentration using a fluorescence technique. Calcium ion permeability in the absence of fluid force and susceptibility to shear-induced permeability modulation are positively correlated with estimated bilayer compressibility. Fluid shear forces are presumed to influence bilayer packing and modulate defect formation in proportion to bilayer compressibility. Bilayer defects produced by fluid forces may increase liposome permeability.

Keywords: Liposome; Fluid force; Calcium ion permeability; Shear stress; Fluorescence; Ion transport

1. Introduction

Much attention has been focused on the relationship between surface active agent performance and monolayer packing. The molecular configuration and physical properties of surface active agent monolayers can be modified by the application of an external force. The monolayer molecular area (A_M) is directly related to the applied lateral pressure (F_{LP}). Monolayer compressibility, a proportionality constant, relates A_M and F_{LP}. Monolayer permeability has been shown to be a function of both the compressibility and A_M [1].

Similar force-performance relationships presumably exist for lipid bilayers, but experimental difficulties make these measurements relatively uncommon. The relationship between lipid bilayer structure and function under external force has been examined in terms of the compositional effects on membrane compressibility modulus, critical deformation to failure, critical stress to failure and the integrated parameter of total energy absorbed to failure [2]. The passive transport of small solutes and ions across bilayers occurs primarily through local, transient defects in the membrane structure. Although the mechanism responsible for defect formation remains unclear [3–5], the frequency and size of these defects are influenced by the bilayer composition and the forces applied. The first direct observation of force-induced pores in lipid bilayers [6] supports the role of force in determining bilayer permeability.

Applications of lipid bilayers formed as liposomes may involve forces sufficient to modify molecular packing and bilayer properties. Liposomes travelling through the mammalian cardiovasculature, for example, experience fluid forces in addition to biological interactions. Calcium ion permeability of phosphatidylcholine liposomes increases significantly as a function of applied fluid stresses in the range developed in the cardiovasculature [7].

The mechanism of shear force-enhanced liposome permeability has not been clearly demonstrated, but is believed to involve the formation of an increased number and/or frequency of bilayer defects through which passive ion transport may occur. The effect of liposome composition on shear-induced calcium ion permeability is unknown, but may be used to help elucidate the mechanism.

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Presumably, hydrocarbon structure, headgroup size, headgroup charge and molecular packing efficiency all may play a role in the susceptibility of a bilayer to force-induced permeability alterations.

In this study, liposomes prepared from three different lipid mixtures (phosphatidylcholine alone; phosphatidylcholine and cholesterol; a mixture of anionic and cationic phospholipids plus cholesterol) are exposed to uniform shear stress in a rotational viscometer. Liposome permeability to calcium ion is estimated from continuous measurement of free intraliposome calcium ion concentration ($[Ca^{2+}]_i$) using a fluorescence technique.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine (PC), cholesterol (CHOL), L-α-phosphatidylethanolamine (PE), sphingomyelin (SM), L-α-phosphatidyl-L-serine (PS), L-α-phosphatidylinositol (PI), ethylenediaminetetraacetic acid (EDTA) and *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (Hepes) were obtained from Sigma. All lipids were purchased in chromatographically purified form, each greater than 98% pure and maintained at −10°C where appropriate. Chloroform, $MgCl_2$, $CaCl_2$, KCl, Na_2HPO_4, NaCl, NaN_3 were purchased from Fisher. Indo-1^{5-}, a fluorescent indicator of free Ca^{2+} concentration, was obtained from Molecular Probes.

Unilamellar vesicles were prepared with a liposome extruder purchased from Lipex Biomembranes. Polycarbonate membrane filters were obtained from Poretics. Centrifugation of the liposome suspension was carried out with a Beckman model GPC tabletop centrifuge fitted with a swinging bucket rotor. An electronic particle counter (Particle Data; model Elzone 280 PC) was used for liposome counting and sizing.

2.2. Liposome sample preparation

Liposomes were prepared by microporous membrane filtration as described in detail previously [7]. A thin lipid film produced by rotary vacuum evaporation of the solvent/lipid solution was hydrated in Ca^{2+}-free Hepes-buffered saline (1 mM $MgCl_2$, 5 mM KCl, 0.85 mM Na_2HPO_4, 154 mM NaCl, 10 mM Hepes) at a pH of 7.4, containing indo-1^{5-} (1 mM). The multilamellar vesicle (MLV) suspension produced was equilibrated at room temperature for 3 to 4 h.

The MLV suspension was extruded through two stacked microporous membrane filters (1.0 μm and 0.8 μm diameter filter pores) with nitrogen gas. The extrusion process was repeated 10 times to obtain well characterized, uniform unilamellar vesicles (ULV) [8,9]. Liposome size distribution has been shown to be unaffected by the shear forces applied in this study [7].

Unencapsulated indo-1^{5-} was removed from the ULV suspension by centrifugal separation of the liposomes and supernate. Final resuspension volume was adjusted to provide a suspension containing 0.2% liposomes by volume. The controlled liposome diameter and concentration provide uniform collision rates among all liposome compositions at the same fluid shear stress.

2.3. Liposome compositions

Experiments were carried out with three different liposome compositions. PC liposomes were prepared from phosphatidylcholine only. PC-CHOL liposomes contain PC (76%) and cholesterol (24%). PC-MIX liposomes were composed from PC (29%), cholesterol (24%), PE (21%), SM (14%), PS (8%) and PI (4%). The PC-MIX formulation mimics the protein-free composition of many natural mammalian cell membranes, including blood platelets [10].

2.4. Calcium flux measurement

Studies of Ca^{2+} flux across liposomes during shear stress exposure were done in an optically modified cone and plate viscometer described previously [7]. Briefly, an aliquot of the essentially Ca^{2+}-free ULV suspension was contained between the cone and plate of the viscometer. Sample fluorescence is monitored continuously through an optical cable mounted flush with the flat, stationary viscometer platen. Cone rotation was preset to the desired speed (4, 225 or 450 revolutions per min, resulting in shear rates of 27, 1350, 2700 s^{-1}, respectively). A fluorescence measurement was performed before shear exposure for subsequent subtraction of background fluorescence. A concentrated $CaCl_2$ solution was added to the exterior aqueous space to obtain a final concentration of 1 mM Ca^{2+} as cone rotation was initiated. The sample was recovered from the viscometer at the conclusion of the experiment for calibration of the indo-1^{5-} fluorescence. The recovered ULV sample was sonicated for 30 seconds in an ultrasonic homogenizer and returned to the optically modified viscometer for measurement of the maximum fluorescence ratio (R_{max}). The minimum fluorescence ratio (R_{min}) was obtained by adding a final concentration of 2 mM EDTA to the sonicated sample. All additions were carried out in Hepes-buffered saline at pH 7.4. The minimum and the maximum fluorescence ratios are required in the calculation of free calcium ion concentration by the method of Grynkiewicz [11]:

$$[Ca^{2+}]_i = K_d\left(\frac{(R - R_{min})}{(R_{max} - R)}\right)$$

where K_d is the dissociation constant (250 nM) and R is

the fluorescence ratio ($I_{410\ nm}/I_{490\ nm}$). $[Ca^{2+}]_i$ was estimated as a function of time. The $[Ca^{2+}]_i$ flux across the liposomes was then obtained from the unsteady Ca^{2+} balance:

$$\text{transmembrane } Ca^{2+} \text{ flux} = \left(\frac{V}{SA}\right)\frac{d[Ca^{2+}]_i}{dt}$$

where V is the total liposome encapsulated volume (cm^3), SA is the total liposome surface area (cm^2) and $d[Ca^{2+}]_i/dt$ is the time rate of change of $[Ca^{2+}]_i$ (determined as the slope of $[Ca^{2+}]_i$ vs. time). The volume and surface area of the ULV sample were obtained directly from the particle counter. The liposome size distribution was not significantly altered as a result of shear exposure, suggesting negligible shear-induced liposome damage or fusion [7]. Control experiments reveal total indo-1^{5-} leakage (presumably due to liposome-liposome collisions, liposome-wall collisions and/or liposome fusion) to average 1.4% per h during the first five hours of continuous fluid shear exposure [7]. The fluorescence intensity correction applied to compensate for indo-1^{5-} leakage is small compared to the measured sample fluorescence. Indo-1^{5-} photobleaching has also been shown to be negligible in this system [7].

2.5. Bilayer permeability estimation

The total resistance to passive solute transport across the liposome membrane includes the inner and outer convective resistances ($1/k_i$ and $1/k_o$, respectively, (s/cm)) and the diffusive resistance of the bilayer (D_m, (cm^2/s)). The driving force for passive transport is provided by the solute concentration difference between the inner ($C_{a,i}$, (nmol/cm^3)) and outer ($C_{a,o}$, (nmol/cm^3)) aqueous phases. The trans-bilayer Ca^{2+} flux (J_a/A, (nmol/cm^2 per s)) is described by the ratio of driving force to total resistance:

$$\frac{J_a}{A} = \frac{(C_{a,o} - C_{a,i})}{\frac{\delta}{D_m} + \frac{1}{k_i} + \frac{1}{k_o}}$$

where δ is the bilayer thickness (cm). The bilayer permeability, P_m (s/cm^2), is defined as $1/D_m$. Because the inner convective resistance is negligible compared to the other resistances [7], the total resistance to solute transport ($1/k_t$, (s/cm)) reduces to

$$\frac{1}{k_t} = \frac{\delta}{D_m} + \frac{1}{k_o}$$

The outer convective resistance can be estimated from mass transfer correlations at the surface of a sphere [12,13]. The trans-bilayer Ca^{2+} flux can be calculated from measurements recorded during liposome shearing experiments. With a suitable value of the bilayer thickness [14], the remaining parameter, D_m, is estimated. Ca^{2+} coordination with lipid headgroups would increase the effective bilayer surface Ca^{2+} concentration ($C_{a,o}$) and proportionally decrease both k_t and D_m.

3. Results

3.1. Calcium transport across liposomes exposed to uniform shear stress

Liposome sample fluorescence was measured continuously during application of uniform, controlled fluid shear at rates attained in the normal mammalian cardiovasculature (2700, 1350, and 27 s^{-1}). Duplicate experiments were performed with each independently prepared liposome sample.

Intraliposomal free calcium ion concentration ($[Ca^{2+}]_i$) is shown as a function of time after 1 mM Ca^{2+} addition to the exterior aqueous phase in Fig. 1. Initial $[Ca^{2+}]_i$ ranged between 75 nM and 90 nM and is believed to result from non-specific environmental Ca^{2+} contamination. The duration of each experiment was controlled to limit fluorescence measurement to the $[Ca^{2+}]_i$ range in which indo-1^{5-} is most sensitive. $[Ca^{2+}]_i$ increased continuously from the initial, background value during fluid shear exposure.

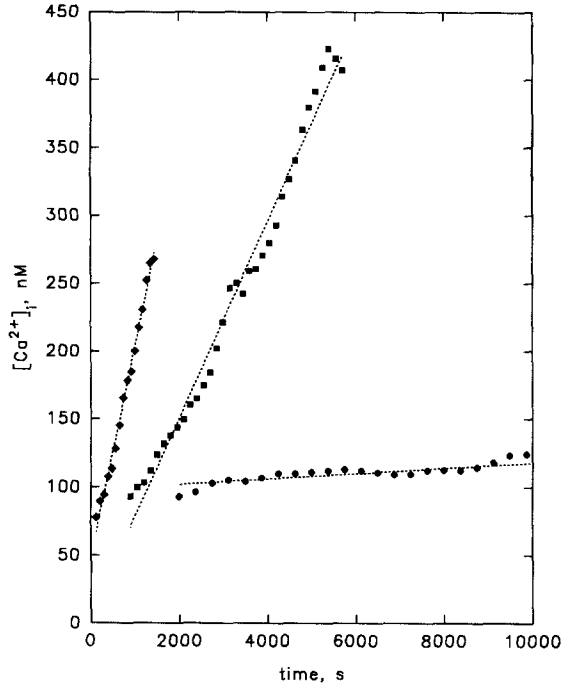

Fig. 1. Intraliposome free Ca^{2+} concentration as a function of fluid shear exposure time. Shear rate (27, ●; 1350, ■; 2700, ◆; s^{-1}) is shown as a parameter. Points represent results calculated from fluorescence data; dashed lines are linear approximations based on minimization of the least squares summation. Results shown here are for PC liposomes; results for PC-CHOL and PC-MIX liposomes are similar.

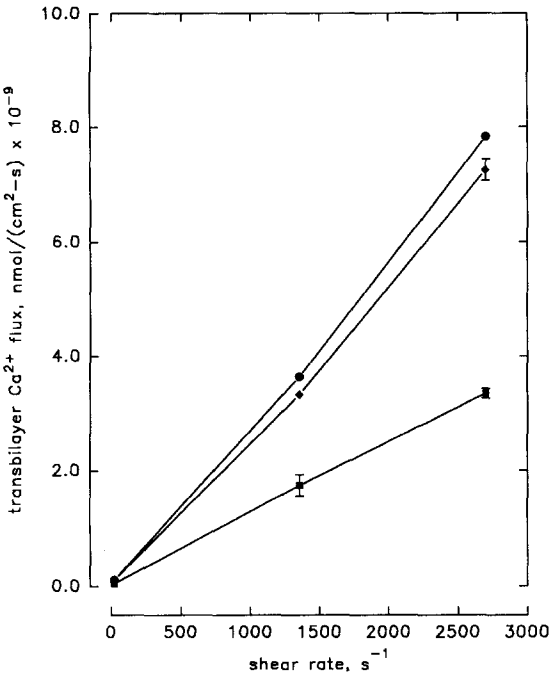

Fig. 2. Specific transbilayer Ca^{2+} flux as a function of applied fluid shear intensity. Bilayer composition is shown as a parameter (PC, ●; PC-CHOL, ■; PC-MIX, ♦). Points are averaged from four independent trials and error bars indicate standard deviations of the means. Specific transbilayer Ca^{2+} flux (means ± S.D.) under nearly static conditions (27 s^{-1}) was $1.08 \cdot 10^{-10} \pm 2.05 \cdot 10^{-12}$ (PC); $4.84 \cdot 10^{-11} \pm 1.27 \cdot 10^{-12}$ (PC-CHOL); $1.05 \cdot 10^{-10} \pm 5.94 \cdot 10^{-12}$ (PC-MIX).

The rate of Ca^{2+} transport, as indicated by the slope of the linear approximations to the data in Fig. 1, increased as a function of fluid shear intensity. Estimated $[Ca^{2+}]_i$ plotted as a function of time for PC-CHOL and PC-MIX liposomes is qualitatively similar to that for PC liposomes shown in Fig. 1.

3.2. Specific calcium ion flux across liposomes exposed to uniform shear stress

Specific trans-bilayer Ca^{2+} flux is shown as a function of applied shear rate in Fig. 2 with liposome composition as a parameter. As suggested in Fig. 1 for PC liposomes, calcium ion flux increases with applied fluid shear rate. Transbilayer Ca^{2+} flux is a strong function of both bilayer composition and applied shear rate (Fig. 2). The slopes of the specific transbilayer Ca^{2+} flux versus shear rate data are significantly different from zero (ANCOVA, $P < 0.01$) for all bilayer compositions.

Intrinsic calcium ion permeability of the bilayer is measured under essentially stagnant fluid conditions ($\dot{\gamma} = 27\ s^{-1}$, Fig. 2). PC liposomes provided the least resistance to calcium ion transport. Addition of 24% cholesterol to PC produced a bilayer with the greatest resistance to calcium ion transport. Calcium ion permeability of the PC-MIX liposomes was intermediate, but closer to that of the pure PC liposomes.

3.3. Bilayer permeability to calcium ion as a function of applied shear stress

Effective bilayer permeability to Ca^{2+} increases with applied fluid stress intensity for all liposome compositions (Table 1). Intrinsic calcium ion permeability increases with composition as PC-CHOL < PC-MIX < PC.

Permeability of PC liposomes to passive Ca^{2+} transport increases from $4.88 \cdot 10^{-20}$ cm^2/s at 27 s^{-1} to $8.52 \cdot 10^{-18}$ cm^2/s at 2700 s^{-1}. Bilayer permeabilities estimated for other liposome compositions and applied shear rates appear in Table 1. Addition of 24% cholesterol reduces the PC bilayer permeability by more than 50%. Permeability of the PC-MIX liposomes at all shear rates was between that of PC and PC-CHOL preparations.

3.4. Convective and diffusional resistances

Outer convective mass transfer coefficients shown in Fig. 3 were estimated from correlations under conditions which provide a minimum limiting value. The total mass transfer coefficients, k_t, are estimated from experimental data using Eq. (4) and are presented with liposome composition as a parameter. Both k_o and k_t increase with applied shear rate, in agreement with observed shear-induced increases in trans-bilayer Ca^{2+}. The total mass transfer coefficient is more than ten orders of magnitude smaller than the outside mass transfer coefficient, suggesting that bilayer permeability is the limiting step in passive trans-bilayer Ca^{2+} transport. The outside convective mass transfer coefficient increases by a factor of three over the range of shear rates used in this study; the total mass transfer coefficient increases by approximately two orders of mag-

Table 1
Bilayer permeability ($1/D_m$, cm^2/s) to Ca^{2+} as a function of applied fluid shear rate for each of three liposome compositions

Shear rate (s^{-1})	PC	PC-CHOL	PC-MIX
27	$4.86 \cdot 10^{-20} \pm 9.35 \cdot 10^{-22}$	$2.18 \cdot 10^{-20} \pm 5.72 \cdot 10^{-22}$	$4.72 \cdot 10^{-20} \pm 2.67 \cdot 10^{-21}$
1350	$1.64 \cdot 10^{-18} \pm 5.46 \cdot 10^{-21}$	$7.84 \cdot 10^{-19} \pm 8.33 \cdot 10^{-20}$	$1.41 \cdot 10^{-18} \pm 1.38 \cdot 10^{-19}$
2700	$3.52 \cdot 10^{-18} \pm 3.03 \cdot 10^{-21}$	$1.51 \cdot 10^{-18} \pm 3.79 \cdot 10^{-20}$	$2.76 \cdot 10^{-18} \pm 2.83 \cdot 10^{-19}$

Each value shown is the mean of four measurements ± S.D.

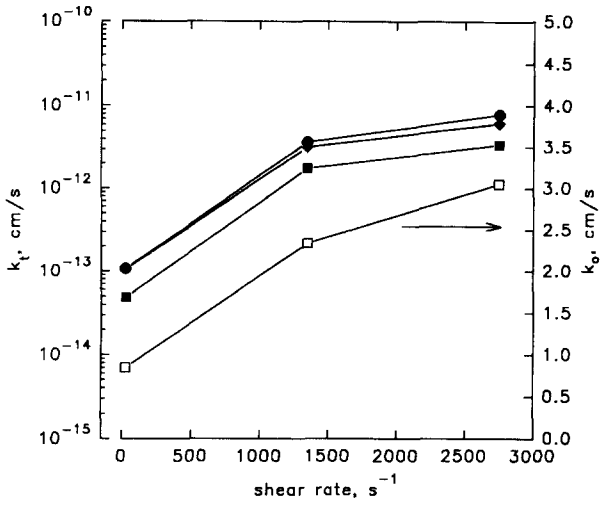

Fig. 3. Outer convective mass transfer coefficient (k_o, □) and total mass transfer coefficients (k_t: PC, ●; PC-CHOL, ■; PC-MIX, ♦) for passive Ca^{2+} trans-bilayer transport as a function of applied shear rate. k_o is estimated from numerical correlations for mass transfer from a submerged sphere; k_t is calculated from the experimentally obtained trans-bilayer Ca^{2+} flux.

nitude over the same shear rate range. Fluid shear modulates liposome permeability primarily through alteration of the bilayer with little convective contribution.

4. Discussion

The influence of fluid shear on bilayer permeability to Ca^{2+} for liposomes prepared from three different compositions follows the same pattern as reported previously for phosphatidylcholine liposomes [7]. The rate of passive transbilayer Ca^{2+} transport is significantly increased during exposure to physiologically obtainable fluid forces. Fluid shear enhances Ca^{2+} flux by reducing the effective bilayer permeability, not through decreasing the convective mass transfer coefficient.

The intrinsic bilayer permeability, measured at nearly static fluid conditions ($\dot{\gamma} = 27\ s^{-1}$), was a significant function of liposome composition. Addition of cholesterol to the phosphatidylcholine bilayer decreases the intrinsic Ca^{2+} permeability by 55%. The experiments were conducted at 22°C, a temperature well above the gel-to-fluid transition of phosphatidylcholine (T_g(PC) = −10°C [15]). Cholesterol addition under these conditions increases membrane mechanical stiffness and toughness [2]. Acyl chain region stabilization induced by cholesterol addition is generally believed to reduce overall bilayer permeability. Phosphatidylcholine bilayer permeability reduced by the addition of cholesterol has been previously reported under static conditions [3,16,17]. The current work confirms the fundamental relationship between bilayer stability and permeability and extends the observation to fluid shear conditions similar to those in the mammalian cardiovasculature.

Calcium ion permeability has been measured in red blood cells (RBCs) exposed to fluid shear stress [18]. The reported Ca^{2+} flux in sheared RBC is $(1.1–1.9) \cdot 10^{-7}$ nmol Ca^{2+}/s per cm^2; the Ca^{2+} flux in sheared liposomes reported here is $(1.7–7.8) \cdot 10^{-9}$ nmol Ca^{2+}/s per cm^2. Shear stress induced a dose-dependent increase in the Ca^{2+} permeability of RBCs in qualitative agreement with the results of this study. The difference between RBC and liposome permeability to Ca^{2+} might result from a variety of factors including residual active Ca^{2+} transport in the RBC, compositional and/or structural effects and membrane defects at protein/lipid interfaces in the RBC.

The mechanism of shear-induced bilayer permeability increase is unknown, but is presumed to be related to lipid bilayer structure modifications induced by fluid force application [7]. The fluid force intensities used in this study have been shown to produce fractional change in membrane area much less than the critical fractional membrane increase before failure [2,7]. The role of liposome collisions in the formation of transient lipid bilayer structure modifications remains uncertain. Possible artifacts which might confound analysis of the data presented here, including indo-1^5 photobleaching and leakage, have been shown to be negligible in this system.

The rate of bilayer permeability increase with applied shear rate describes the susceptibility of a liposome preparation to fluid force-induced structure modifications. A linear relationship between bilayer permeability and shear rate is well-correlated ($r^2 = 0.999$ for all preparations, data from Table 1). PC bilayer permeability increases at a rate of $1.30 \cdot 10^{-21}$ cm^2/s per s^{-1} of applied shear rate. PC-CHOL bilayer permeability increases at a rate of $5.56 \cdot 10^{-22}$ cm^2/s per s^{-1} of applied shear rate. Addition of cholesterol reduces the susceptibility of PC bilayers to fluid shear-induced permeability increase. Since cholesterol is believed to stabilize the PC bilayer through hydrocarbon region interactions, this work suggests that hydrocarbon region properties play an important role in modulating bilayer permeability changes induced by fluid shear stress.

The addition of PE, SM, PS and PI to the PC-CHOL liposomes resulted in an increase in both the intrinsic bilayer permeability to passive Ca^{2+} transport ($\dot{\gamma} = 27\ s^{-1}$) and the susceptibility to increased permeability induced by fluid force. Lipids added to the PC-CHOL preparation are expected to increase bilayer packing disorder by headgroup steric and charge effects [17]. Hydrocarbon phase packing modifications are also expected. Sphingomyelin, for example, is reported to partition preferentially in the acyl chain region and disrupt hydrocarbon phase packing. Cholesterol and sphingomyelin also strongly associate, creating additional hydrocarbon phase disorder [2]. The overall effect of non-PC lipid addition to the PC-CHOL preparation is presumed to be an increase in

both polar and hydrocarbon region disorder. Lipids used in this study were prepared from natural sources and, as such, possess a range of acyl chain compositions and configurations. The respective roles of polar region and acyl chain effects can not be decoupled in the PC-MIX studies described here. PC-MIX bilayer permeability increases at an intermediate rate of $1.01 \cdot 10^{-21}$ cm^2/s per s^{-1} of applied shear rate.

Bilayer compressibility has been studied in cell [19] and liposome [1] systems. These studies provide theoretical and experimental evidence for a positive correlation between bilayer compressibility and passive solute permeability under static conditions. Anthroyloxy fatty acid probes have been used to determine bilayer compressibility as a function of membrane depth and direction of force [20]. A 2-fold increase in bilayer compressibility was obtained by substituting an unsaturated lipid for a fully saturated lipid. The implication is that hydrocarbon region packing defects increase bilayer compressibility. This finding is consistent with the measured increase in bilayer permeability on addition of PE, SM, PS and PI to the PC-CHOL preparation. Bilayer compressibility was also determined to be much greater in the two directions in-plane with the bilayer as compared to the direction orthogonal to the bilayer [20]. The shearing system used in this study produces fluid forces which act primarily tangential to the bilayer plane.

A relationship between bilayer compressibility and shear-induced permeability is an appealing postulate supported by evidence in this study. Probable bilayer compressibility of the PC-CHOL is less than the other bilayer compositions used here. Estimation of the relative compressibilities of PC and PC-MIX is complicated by the unknown, and possibly competing, effects of cholesterol and non-PC interactions in both the acyl chain and polar regions of the bilayer. Passive solute permeability under nearly static conditions, however, suggests that the PC-MIX bilayer is less compressible than PC alone. Shear-induced bilayer permeability also increases as PC-CHOL < PC-MIX < PC, providing evidence for a correlation between bilayer compressibility and susceptibility to shear induced permeability increase.

Potential new applications of injectable liposome preparations promise to play important roles in the future of human therapy and disease diagnosis. Fluid shear force modulation of liposome properties may be a significant factor in controlling the performance of such formulations.

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