Viscoelasticity of BLM from Choline Plasmalogen, Alkylacyl- and Diacyl-Glycerophosphocholines

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Abstract. Mechanical characteristics of bilayer lipid membranes (BLM) composed of alkenylacyl-, alkylacyl-, and diacyl-glycerophosphocholines were studied by measuring modulus of elasticity in the direction normal to BLM plane, $E_\perp$, and coefficient of dynamic viscosity η. Alkenylacyl-glycerophosphocholine (choline plasmalogen) BLM typically show larger values of $E_\perp$ and η as compared to their analogs, suggesting a tighter packing of their hydrophobic regions. Increasing cholesterol concentrations are associated with monotonically increasing values of parameters $E_\perp$ and η of plasmalogen BLM, whereas a nonmonotonic dependence of these parameters with a maximum around $c = 66$ mol% cholesterol is typical for the diacyl analog POPC. This may be due to the formation of cholesterol clusters at $c > 66$ mol%.

Key words: Ether lipids — Bilayer lipid membranes — Phospholipid-cholesterol interaction — Viscoelasticity

Introduction

Plasmalogens (1-O-alkenyl-2-acyl-glycerophospholipids) are important constituents of most human and animal cell membranes (Paltauf 1983). The role they play in these membranes remains unknown. The study of physical characteristics of membranes which contain plasmalogens and related lipids may contribute to our understanding of this role. Model membrane systems are advantageous tools for this purpose. Significant results could be obtained in studying properties of monolayers, multilayers and unilamellar vesicles. Plasmalogens in monolayers showed

higher ordering and lower dipole surface potentials compared with their alkylacyl and diacyl analogs (Smaby et al. 1983). Studies of fluorescence anisotropy of unilamellar vesicles with the fluorescent probe trimethylammonium-DPH suggested reduced lipid mobility in membranes composed of choline plasmalogen as compared to diacyl glycerophospholipid bilayers (Hermetter 1988). On the other hand, recent ^{2}H-NMR studies with artificial membranes containing 1-O-alkenyl or 1-acyl-2-(α, α'-^{2}H) oleoyl-sn-glycero-3 phosphocholine showed a greater flexibility of the α-methylene segments of sn-2-acyl chains as compared to the diacyl analogs (Malthaner et al. 1987). Studies of cholesterol effects on physical properties of membranes prepared from plasmalogens and/or their analogs can supply significant information as the presence of plasmalogens was detected just in the cell membranes which contain a high percentage of cholesterol. No significant differences in the cholesterol effects on monolayer characteristics (Smaby et al. 1983), membrane ion permeability (Hermetter and Paltauf 1981) or on the interaction of the respective glycerophospholipids with blood serum (Paltauf 1983) could be observed between plasmalogens and their diacyl analogs. However the presence of 30 mol% cholesterol significantly enlarged the diameter of plasmalogen vesicles, whereas no changes were observed with vesicles from alkyl and diacyl analogs (Hermetter et al. 1985).

Planar bilayer lipid membranes (BLM) are another model suitable to be used to study structural and functional role of plasmalogens. BLM have widely been used as an adequate model of biomembranes (Tien 1974). Contrary to monolayers, BLM account for the interactions of hydrocarbon chains in the inner membrane region. Thanks to their flatness, they represent a more suitable model of biomembranes than do small, strongly curved unilamellar vesicles, with different lipid distributions in the inner and outer monolayer. BLM allow the use of highly informative experimental methodology which cannot be employed with other model systems. The methods include the study of mechanical characteristics in the direction normal to the membrane plane. Mechanical properties of membranes in this direction are characterized by the modulus of elasticity $E_\perp$ and the coefficient of dynamic viscosity η. The above parameters provide information on the degree of membrane deformability and on the dynamical properties which are closely associated with the structural state and the lipid composition of the membrane.

The present work was focused on the measurement of mechanical characteristics of BLM of varying lipid composition including: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC); 1-oleyol-2-palmitoyl-sn-glycero-3-phosphocholine (OPPC); 1-O-hexadecyl-2-O-octadecenyl-glycerophosphocholine (diether-PC); 1-O-hexadec-1′-enyl-2-oleoyl-sn-glycero-3-phosphocholine (choline plasmalogen), cholesterol-containing BLM, and those composed of a mixture of POPC and choline plasmalogen at various molar ratios.

Materials and Methods

Choline glycerophospholipids

POPC, OPPC (Hermetter et al. 1989), diether-PC (Hermetter and Paltauf 1983) and choline plasmalogen (Hermetter and Paltauf 1982) were prepared according to the methods described elsewhere. Cholesterol (Merck AG, FRG) was repeatedly recrystallized from absolute ethanol prior to use.

BLM preparation

The standard method according to Mueller et al. (1962) was used. The membranes were raised over a circular aperture (0.5 or 1.3 mm in diameter) in the wall of a teflon cup or at the tip of a teflon tube. As the electrolyte, 0.1 mol/l NaCl + 5 mmol/l Tris-HCl (pH 7.2) in redistilled water was used. BLM were formed from a stock solution of the lipids in *n*-heptane (Kodak, USA) (20 mg/ml).

Determination of modulus of elasticity $E_\perp$, coefficient of dynamic viscosity η, and of specific capacity C_s

BLM elasticity in the direction normal to the membrane plane is represented by the modulus of elasticity $E_\perp = -p/(\Delta h/h)$; it characterizes the capacity of a membrane to change its thickness h upon the application of pressure p. The other parameter is the coefficient of dynamic viscosity η, which is connected with energy dissipation processes resulting from internal friction of the hydrocarbon chains. The values of the parameters $E_\perp$ and η were determined using the electrostriction method developed by Passechnik and Hianik (1977). According to this method, alternating voltage $V = V_0 \sin 2\pi ft$ with amplitude V_0 and frequency f, applied to a membrane, will compress the membrane with pressure $p = C_s V^2/2h$, where C_s is the specific membrane capacity. Due to nonlinear relationship of capacity and voltage ($C = C_0(1 + \alpha V^2)$, where α is the coefficient of electrostriction), the membrane current $i = \mathrm{d}(CV)/\mathrm{d}t$ will contain components with frequencies f and $3f$ and amplitudes A_1 and A_3. Parameter $E_\perp$ can be obtained from

$$E_\perp = 3C_s V_0^2 A_1/4hA_3 \tag{1}$$

This means that to determine parameter $E_\perp$ it is sufficient to measure amplitudes A_1 and A_3 of the membrane current harmonics, e.g. by using resonance amplifiers (see Passechnik and Hianik 1977, 1991). As shown previously (Passechnik and Hianik 1991), for many simple systems, parameter η can be determined from

$$\eta = E_\perp \sin\varphi/2\pi f \tag{2}$$

where φ is the phase shift. Phase shift expresses the delay in membrane deformation following pressure application, due to the existence of internal friction; the parameter φ can be determined using a phasometer. Amplitude A_1 can also be used to determine the specific electric capacity of a membrane:

$$C_s = A_1/2\pi f V_0 S, \tag{3}$$

where S is the surface area of the lipid bilayer. Specific capacity was measured using BLM formed at the tip of a teflon tube with a large inner diameter (1.3 mm) in order to

increase the ratio of the BLM area to the area of the Gibbs-Plateau border region (torus). This provides a possibility to minimize the effects of torus on the capacity measurements (Benz and Janko 1976). The membrane thickness was determined according to

$$h = \epsilon\epsilon_0/C_s, \tag{4}$$

where $\epsilon_0 = 8.85 \times 10^{-12}$ F/m is the dielectric permittivity of vacuum, and $\epsilon \cong 2.1$ is the relative dielectric permittivity of the hydrophobic part of the membrane (Kruglyakov and Rovin 1978). Experiments were performed as follows. The lipid solution was applied to the hole in a teflon cup, and approx. 10 min were allowed for the membrane to equilibrate. This interval was sufficient for the formation of a lipid bilayer (the membrane turned black). Then, calomel electrodes were used to apply alternating voltage $V_0 = 100 - 130$ mV with frequency $f = 1$ kHz, and the kinetics of changes of parameters A_1, A_3 and φ were recorded and on-line computer processed over 10 min, to yield the kinetics of $E_\perp$, η and C.

All experiments were done at $T = 20$–22 °C, i.e. temperatures exceeding those of the phase transition of the lipids employed.

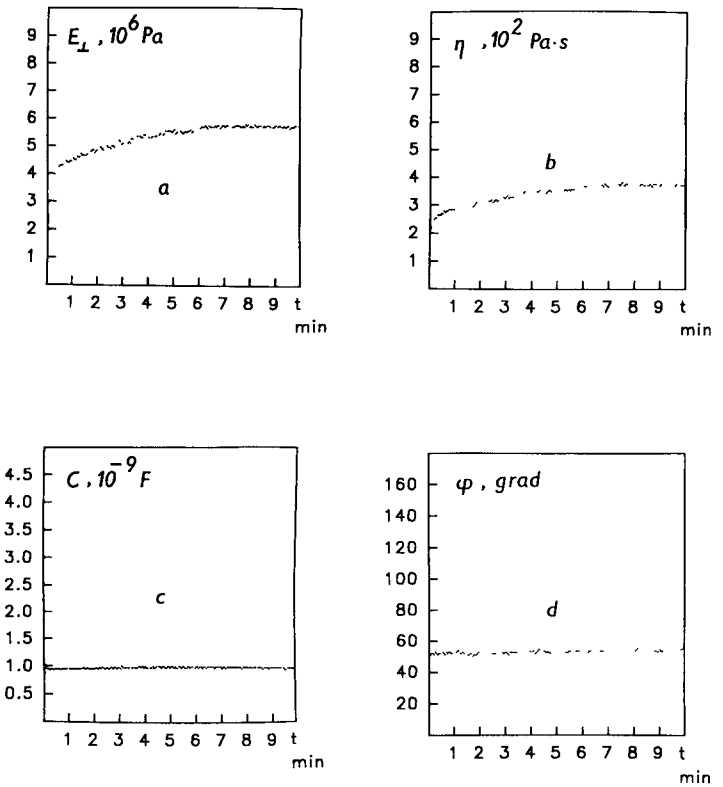

Figure 1. An illustration of the kinetics of changes in modulus of elasticity $E_\perp$ (a), coefficient of dynamic viscosity η (b), electrical capacitance C (c) and phase shift φ (d) for BLM prepared from OPPC in n-heptane. $V_0 = 110$ mV, $f = 1$ kHz.

Results

Fig. 1 illustrates typical kinetics of changes in modulus of elasticity $E_\perp(a)$, coefficient of dynamic viscosity $\eta(b)$, electrical capacitance $C(c)$ and phase shift $\varphi(d)$ for BLM from OPPC. The values of $E_\perp$ and η showed relatively rapid increases during the first 1–4 min with almost steady state levels reached gradually thereafter. The kinetics of changes in $E_\perp$ and η were very similar for BLM from POPC, diether-PC and choline plasmalogen. Capacitance showed only very small changes in all cases (see Fig. 1*c*). It could be shown previously (Passechnik et al. 1984) that the initial increases in the values of mechanical parameters were due to structural transitions occurring in the lipid bilayer, and reflected the process of formation of ordered bilayer structures. The kinetics of changes in $E_\perp$, η and C showed very similar time courses for BLM of different compositions, although absolute values of $E_\perp$ and η occasionally showed considerable differences. Table 1 summarizes the absolute values of $E_\perp$, η and C_s for BLM of various compositions, as calculated from kinetic curves at steady state after approx. 10 min following voltage application. With the exception of POPC and OPPC BLM, membranes from all other lipids tested showed significant differences in $E_\perp$ and η (by the Student's t-test at the $p < 0.01$ level). C_s values showed only weak, insignificant differences in dependence on the lipid composition. This is in agreement with a previous report (Hermetter et al. 1985), demonstrating weak dependence of the lipid bilayer thickness on the lipid type employed.

Next, the effects of cholesterol on parameters $E_\perp$, η and C_s were studied in BLM prepared from POPC and/or choline plasmalogen. Fig. 2*a* shows the dependence of modulus of elasticity $E_\perp$ on cholesterol concentration in the membrane for BLM from POPC (curve 1) and those from choline plasmalogen (curve 2). Fig. 2*b* shows the corresponding dependences of coefficient of dynamic viscosity η. The cholesterol concentration dependences of $E_\perp$ and η are nonmonotonic for POPC BLM. At cholesterol concentrations exceeding 66 mol%, the values of parameters $E_\perp$ and η start decreasing. Choline plasmalogen BLM show an entirely different pattern of these dependences: the values of both parameters monotonically grow with the increasing cholesterol concentration. This means that cholesterol markedly affects the parameters $E_\perp$ and η, while interacting differently with POPC and choline plasmalogen. In contrast to considerable changes of parameters $E_\perp$ and η, specific membrane capacity increases weakly with the increasing cholesterol concentration.

Choline plasmalogens make up a significant portion of the lipids in many animal cell membranes (Paltauf 1983). It is therefore interesting to know how various choline plasmalogen concentrations affect mechanical properties of BLM composed of a mixture of lipids, such as POPC and choline plasmalogen. Fig. 3 shows the re-

lationship of parameters $E_\perp$ (curve 1), η (curve 2) and C_s (curve 3), and molar concentrations of choline plasmalogen in the membrane. A nonmonotonic dependence is obvious for $E_\perp$, with a maximum at around 10–15 mol% choline plasmalogen, i.e. at a ratio of 10 POPC molecules per 1–2 molecules of choline plasmalogen.

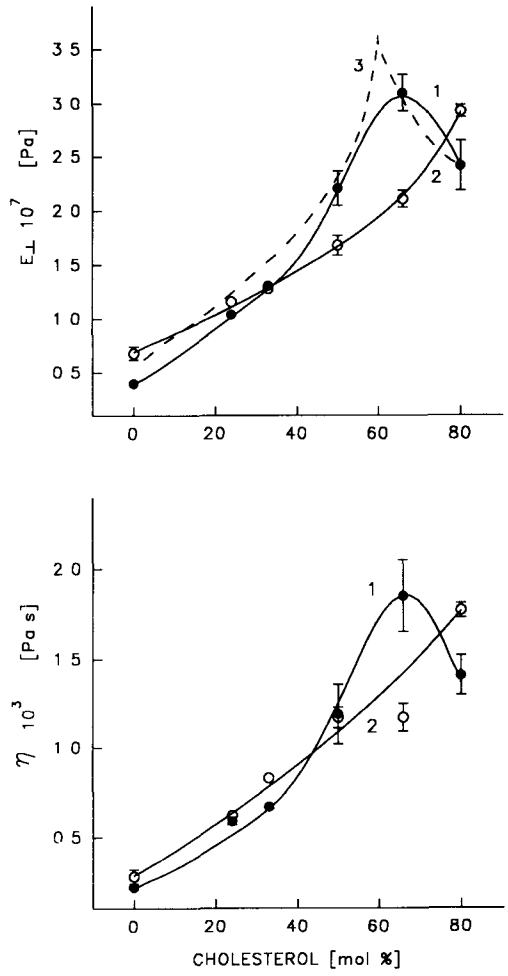

Figure 2. The dependences of $E_\perp$ (a) and η (b) on cholesterol concentration. BLM prepared from: 1 – POPC, 2 – choline plasmalogen, 3 – theoretical dependence of $E_\perp$ for POPC, as calculated by relationships (6) and (7) (see Discussion). Each point represents mean ± S.E. for 7 membranes.

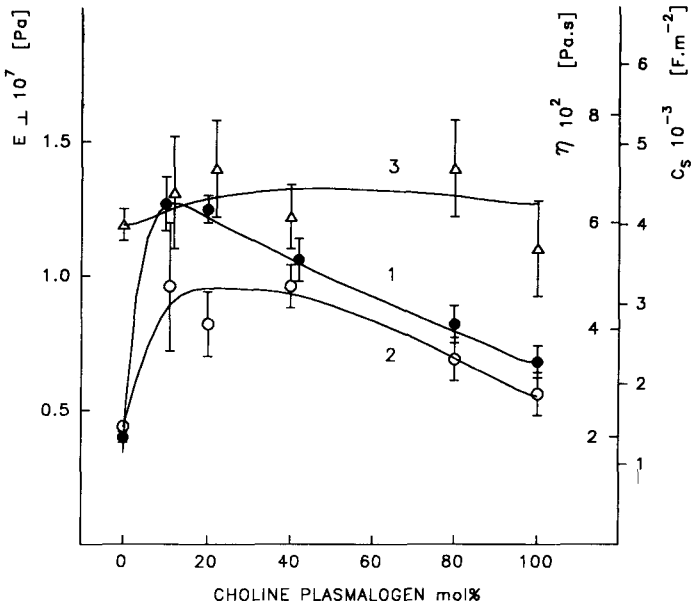

Figure 3. The dependences of $E_\perp$ (curve 1), η (curve 2) and C_s (curve 3) on choline plasmalogen concentration for BLM prepared from POPC-choline plasmalogen mixture. Each point represents mean ± S.E. for 5–7 membranes.

Table 1. Elasticity modulus $E_\perp$, coefficient of dynamic viscosity η and specific capacity C_s for BLM of different composition; *n*-heptane; $T = 20$–$22\,^\circ$C. $n = 7$ each

Lipid composition	$E_\perp \pm$ S.E. 10^6 Pa	$\eta \pm$ S.E. 10^2 Pa · s	$C_s \pm$ S.E. 10^{-3} F/m^2
Choline plasmalogen	6.8 ± 0.6	2.8 ± 0.4	3.7 ± 0.6
OPPC	4.9 ± 0.5	3.7 ± 0.4	4.0 ± 0.2
POPC	4.0 ± 0.2	2.2 ± 0.1	4.0 ± 0.2
Diether-PC	1.8 ± 0.3	1.3 ± 0.2	3.7 ± 0.3

Discussion

Table 1 shows that the values of modulus of elasticity $E_\perp$ are higher for choline plasmalogen BLM (the membranes are more rigid) as compared to those for POPC and OPPC BLM. This effect is in agreement with the reported data on lipid packing and mobility in monolayers. Choline plasmalogens exhibit a denser packing. On the other hand, the value of modulus of elasticity $E_\perp$ is smaller for diether-PC than those for choline plasmalogen, POPC or OPPC. This may be due to a greater

rotational freedom of the C–C bond adjacent to the oxygen atom in the vicinity of sn-1, as the result of the missing C=O group (as compared to POPC and OPPC) and the missing alkenyl group (as compared to choline plasmalogen). No significant differences in parameters $E_\perp$ and η could be observed between OPPC and POPC BLM.

Measurements of $E_\perp$ and η for BLM with various cholesterol contents clearly showed that mechanical parameters for choline plasmalogen BLM differ qualitatively from those for POPC BLM. For choline plasmalogen BLM, the values of parameters $E_\perp$ and η grow monotonically with the increasing cholesterol concentration, whereas a nonmonotonic dependence with a maximum at $c_k = 66$ mol% cholesterol is typical for POPC BLM. It has been shown previously that egg phosphatidylcholine (egg PC) BLM also show nonmonotonic dependences of $E_\perp$ and η, very similar to the respective patterns observed for POPC BLM, with the only difference being the value of c_k, which in this case is approximately 50 mol% (Hianik et al. 1984a). The nonmonotonic changes of mechanical parameters correlate well with the changes of microscopic parameters reported to occur in egg PC unilamellar vesicles containing various amounts of cholesterol, as measured with the use of spin probes (Hianik et al. 1986, Kusumi et al. 1986). Hianik et al. (1984a) analyzed the reasons of the nonmonotonic dependence of $E_\perp$ (c) for egg PC BLM, based on the idea that BLM is a two-dimensional non-mixing fluid. In line with this idea, POPC BLM may also be viewed as containing several regions with different relative areas and different values of modulus of elasticity $E_\perp$. Generally, it should be noted that for BLM containing e.g. two kinds of regions with the relative areas s and $1 - s$ and with the corresponding moduli of elasticity E_1 and E_2, the overall modulus of elasticity $E_\perp$ will be

$$1/E_\perp = s/E_1 + (1 - s)/E_2 \tag{5}$$

This means that any new region of altered structure arising within a BLM will affect the overall modulus of elasticity by both, its own modulus of elasticity and the area it takes. This is a typical peculiarity of the employed macroscopic method of measurement of $E_\perp$. In line with the representation of BLM as a two-dimensional heterogeneous fluid, there will be a single phase with modulus of elasticity E_L (phase 1) at membrane cholesterol concentration $c = 0$; at $c_k = 66$ mol%, there will be a phase with modulus of elasticity E_{LC} (phase 2); and at $c = 100$ mol% (pure cholesterol), there will be a phase with modulus of elasticity E_C (phase 3). At any other cholesterol concentration, the system will show equilibrium of two phases: at $c < c_k$, phase 1 and 2 will be in equilibrium, and at $c_k < c < 100$ mol%, there will be equilibrium between phases 2 and 3. As shown elsewhere (Hianik et al. 1984a), for the above cases the overall modulus of elasticity $E_\perp$ can be written as follows:

$$E_\perp = E_L/[1 - (c/c_k)(1 - E_L/E_{LC})] \qquad 0 \le c \le c_k \qquad (6)$$

$$E_\perp = E_C/\{1 - [(1 - c)/(1 - c_k)](1 - E_C/E_{LC})\} \qquad c_k \le c \le 100 \text{ mol\%} \qquad (7)$$

The dependence $E_\perp$ (c) calculated according to (6) and (7) is shown in Fig. 2*a* (curve 3). The calculated curve agrees well with the experimental data. The slight deviation of experimental from theoretical curve possibly reflects certain specificity in cholesterol-POPC interaction. On the other hand, cholesterol-egg PC mixtures apparently behave closer to the theory based on BLM representation as a non-mixing two dimensional fluid (Hianik et al. 1984a). Values of parameters E_L, E_{LC}, E_C as well as that of critical concentration c_k were estimated by the least squares method from experimental values of the dependence $1/E_\perp(c)$ illustrated by curve 1 in Fig. 2*a*: $E_L = 5.5 \times 10^6$ Pa, $E_{LC} = 3.6 \times 10^7$ Pa, $E_C = 1.4 \times 10^7$ Pa, $c_k = 60$ mol%.

The value of $E_C = 1.4 \times 10^7$ Pa required to fit the data is hypothetical since pure cholesterol does not allow the formation of stable BLM. On the other hand, experiments performed with oxidized cholesterol BLM supplied a value of $E_\perp \sim 4.5 \times 10^6$ Pa (Hianik et al. 1984b). Considering that lipid peroxidation decreases the elasticity modulus to about one half (Passechnik et al. 1984), values of E_C can be expected similar to those used in the above mentioned calculations.

According to the above idea, the increase of modulus of elasticity at $0 < c < c_k$ is due to areas of highly ordered mixed LC type clusters with moduli of elasticity $E_{LC} > E_L$. BLM is maximally ordered at point c_k, where $E_\perp(c)$ reaches a maximum. At this point, the ratio is 1 phospholipid molecule per 2 cholesterol molecules. The decrease of $E_\perp$ at $c_k < c < 100$ mol% may be due to the area growth of clusters containing cholesterol molecules. This idea agrees with previous suggestions (Barton 1976) showing that if the cholesterol-phospholipid ratio exceeds 0.5, cholesterol can form separate clusters in sterol-phosphatidylcholine mixtures. As shown by McInthosh et al. (1989), the area occupied by dipalmitoylphosphatidylcholine (DPPC) heads starts extending in the presence of approx. 50 mol% cholesterol. Nevertheless, this phenomenon alone cannot account for the $E_\perp$ decrease. As a matter of fact, the modulus of elasticity of pure cholesterol clusters E_C is smaller than E_{LC}. This has been suggested by our recent experiments (Hianik and Haburčák 1993) which indeed showed smaller values of E_C for BLM prepared, according to Montal and Mueller (1972), from two monolayers of pure cholesterol than E_{LC} for BLM prepared in the same way from DMPC.

It is interesting that, when measuring two-dimensional modulus of elasticity $E_{||}$ of 1-stearoyl-2- oleoylphosphatidylcholine (SOPC) vesicles as a function of cholesterol concentration, a maximum value was obtained at about 60 mol% cholesterol with practically no decrease at higher cholesterol contents up to 89 mol% (Needham and Nunn 1990). The value of $E_{||}$ increased from 0.193 N/m (withouth cholesterol) up to 1.19 N/m (at $c = 89$ mol% cholesterol). Using LUVs Evans and Kwok (1982)

found an $E_{\|}$ of 0.14 N/m. This value is considerably higher than that derived from our experiments by multiplying $E_{\perp}$ by membrane thickness (for example, $E_{\|}$ for POPC BLM would be $E_{\|} \sim 4 \times 10^6$ N/m^2 $\times$ 4.6×10^{-9}m=1.84×10^{-2} N/m). The striking differences between elasticity moduli derived from our experiments and from those mentioned above can probably be ascribed to the presence of hydrocarbons in the membranes used in our study, and by anisotropy of membrane mechanical properties. In fact, we have shown previously that solvent-free BLMs are less compressible (by a factor of approximately 7) than solvent-containing membranes. This increase in $E_{\perp}$ in solvent-free membranes is practically independent of the structure of the constituent lipids (Passechnik and Hianik 1991). Taking into account this difference a value of 3.5×10^7 Pa can be assumed for $E_{\perp}$ of a solvent-free POPC BLM. The corresponding value for $E_{\|}$ will then be approximately 9.1×10^{-2} N/m. For this calculation we used a value of $h = 2.6 \times 10^{-9}$ m for the thickness of a solvent-free phosphatidylcholine BLM (Benz et al. 1975). $E_{\|}$ thus calculated is about 1.5 times less than $E_{\|}$ reported by Evans and Kwok (1982) for solvent-free PC LUVs. This difference may be explained by the fact that lipid bilayers exhibit a considerable mechanical anisotropy. BLM of the same composition showed $E_{\|} > E_{\perp}$ when measured at a similar frequency of deformation (Passechnik et al. 1978). $E_{\perp}$ reflects the compressibility of the hydrocarbon region of a lipid bilayer while $E_{\|}$ measures the expansion of the area of a lipid bilayer which is mainly determined by the phospholipid head group.

In contrast to POPC BLM, the dependences $E_{\perp}(c)$ and $\eta(c)$ for choline plasmalogen BLM present entirely different patterns (Fig. 2, curves 2). Both parameters grow monotonically with the increasing cholesterol concentration. At $c < 7$ mol%, choline plasmalogen BLM have higher values of $E_{\perp}$ than do POPC BLM. This agrees with a previous report (Smaby et al. 1983) which showed that in POPC monolayers the molecules are ordered more loosely than in choline plasmalogen monolayers. The low ordering density has been explained by the presence of the 1-ester carboxylic group which fills in the space between the lipids. In choline plasmalogen, this group is replaced by an alkenyl group. At cholesterol concentrations ranging between 30-66 mol%, choline plasmalogen BLM have smaller values of $E_{\perp}$ than do POPC BLM. Within this range cholesterol is probably distributed homogeneously over the entire BLM area and does not induce the formation of compact clusters. At around 80 mol% cholesterol, choline plasmalogen BLM again have higher values of $E_{\perp}$ than do POPC BLM. This suggests that cholesterol is unable, at least within the concentration range studied, to form separate clusters in choline plasmalogen bilayers.

The inability of cholesterol to form clusters in membranes containing choline plasmalogen may suggest a significant role played by choline plasmalogen as a structural membrane-protective element. The presence of choline plasmalogen may

prevent the formation of clusters and thus act to protect against cholesterol crystallization in the membranes.

As shown in Fig. 3, BLM composed of a mixture of POPC and choline plasmalogen also typically show nonmonotonic dependence of modulus of elasticity $E_\perp$ on the choline plasmalogen contents. $E_\perp$ reaches a maximum at 10–15 mol% choline plasmalogen, i.e. when a ratio of 10 POPC molecules per 1–2 choline plasmalogen molecules is reached. Upon further increasing the choline plasmalogen concentration, $E_\perp$ decreases. In agreement with what has been said above, this may mean that choline plasmalogen clusters can form at relatively low concentrations of the lipid. Based on our results we may speculate that, in biomembranes, choline plasmalogens can aggregate at even lower concentrations.

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