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Cholesterol effect on the physical state of lipid multibilayers from the platelet plasma membrane by time-resolved fluorescence

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Abstract

There are indications that the plasma membrane lipid composition and, in particular, the cholesterol/phospholipid (C/PL) ratio, affects platelet function. As a first approximation to the molecular characterization of the effect of cholesterol on the order, fluidity and lateral heterogeneity of the platelet plasma membrane, the steady-state and time-resolved fluorescence of 1,6-diphenyl-1,3,5-hexatriene (DPH) and *trans*-parinaric acid (tPnA) has been studied in multibilayer vesicles of phospholipids extracted from human platelet plasma membrane with different cholesterol/phospholipid molar ratios modified in vitro from 0.07 to 0.9. The DPH studies show that the increased presence of cholesterol has a stronger effect on the order than on the fluidity of the bilayer, as has been previously observed in other lipid membranes. On the other hand, from the analysis of the fluorescence kinetics of tPnA we conclude that a higher cholesterol content gives rise to an increase of the heterogeneity of the bilayer, due to a larger fraction of solid-like lipid domains. These domains contain a cholesterol concentration much higher than the macroscopic average value.

Keywords: Cholesterol; Time-resolved fluorescence; Fluorescence; Platelet lipid; Diphenylhexatriene; *trans*-Parinaric acid

1. Introduction

The platelet surface plays an essential role in the hemostatic process. The cellular response to platelet aggregating agents is triggered by the binding of the agonist to protein receptors, whose function can be influenced by the composition and physical state of the lipids in the cellular plasma membrane. It is known that membrane lipid fluidity can regulate membrane protein function in different ways, such as affecting ligand-induced redistribution of receptors [1], the expression of receptors [2,3], the modulation of enzymatic and transport activities [1] and the microaggregation of receptors that is essential for signal transduction of various hormones [4]. There are indications too that the plasma membrane lipid composition and, in particular, the cholesterol/phospholipid (C/PL) ratio, affects platelet function. Elevated serum cholesterol is one of the most consistent risk factors for atherosclerosis and related vasoocclusive disorders [5,6] presumably related with the increase of the membrane C/PL ratio. Platelets from patients with hypercholesterolemia have an increased sensitivity to ADP, epinephrine, and collagen [7–9] and form larger arterial thrombi in rabbits [10].

A series of in vitro studies has sought to establish the reasons for this hypersensitivity by using a model in which the C/PL ratio was elevated by incubation with cholesterol-rich liposomes. Different functional responses have been observed when the C/PL ratio was elevated by exposure to these liposomes: increased sensitivity to epinephrine and ADP [11], increased basal levels of adenylate cyclase, increased cAMP levels [12], increased aggregation in response to thrombin, with increases in the liberation of arachidonic acid [13,14] and in the levels of thromboxane A_2 secretion [15].

On the other hand, the effect of lipid composition on the physical and chemical properties of the plasma membrane has been studied ([16–20], González et al., unpublished data). Increasing the C/PL ratio of the platelet plasma membrane was seen to increase the apparent viscosity detected with DPH steady state anisotropy [16,21], but the molecular interactions associating the changes in membrane properties to changes in functional response have not yet been elucidated. Our laboratory has used

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time-resolved fluorescence depolarization of different lipid soluble fluorescent probes to study the relative contribution of Ca^{2+}, intracellular structures, membrane associated proteins and native lipid asymmetry to the order, dynamics and organization of the platelet plasma membrane [22,23]. The aim of those studies was, on the one hand, to select out of the seven fluorescence probes used (1,6-diphenyl-1,3,5-hexatriene, 1-((4-trimethylamino)phenyl)-6-phenyl-1,3,5-hexatriene, (2-carboxyethyl)-1,6-diphenyl-1,3,5-hexatriene, 16-(9-anthroyloxy)palmitic acid, *cis*-parinaric acid, *trans*-parinaric acid and perylene) those with higher reliability in this biologically oriented work. In addition, it sought to obtain molecular information on the structure and dynamics of the platelet membrane of resting cells. It was observed that not all the probes present the desired stability for working with platelet preparations of higher complexity or intact cells. The cationic and anionic derivatives of DPH, TMA-DPH and CE-DPH, together with tPnA, appear to be the most useful for platelet studies. The DPH derivatives have a better defined location in the membrane than DPH [24], close to the phospholipid polar head. However, DPH has the advantage over its derivatives that there is a large body of previous data obtained with this fluorophore in platelet preparations as well as in other related membranes (see, e.g., [22] and references therein). *trans*-Parinaric acid (tPnA), a 16 carbon fatty acid, has a well defined location in the membrane, parallel to the membrane fatty acids.

In the previous work from this laboratory mentioned above [22] the fluorescence polarization experiments carried out with DPH and CE-DPH revealed that the structural order and apparent viscosity ($\simeq$ 0.5 P) of the human platelet plasma membrane were very similar to those of the erythrocyte membrane. Interestingly, it was found that the high value of the platelet membrane order is due almost exclusively to its lipid composition, with little contribution (if any) from interactions with membrane proteins or intracellular components.

DPH and tPnA, the two probes selected for the present study, differ in their ability to provide information about heterogeneous micro-environments with different densities. DPH has occasionally been used to investigate the presence of solid-like domains in bilayers (e.g., [25,26]), although it is not ideally suited for that purpose. Its fluorescence parameters are only weakly sensitive to the packing density of its micro-environment [27], and the partition coefficient between the fluid and gel-like regions is close to unity [28]. Therefore, if the fraction of lipids with gel-like properties is small, the feeble effect on the photophysics of DPH would render it difficult to separate experimentally from that due to the dominant, fluid fraction. In contrast, tPnA dissolves preferentially in lipid environments with gel-like properties [29–32] and, in addition, some of its photophysical properties (absorption spectrum and fluorescence lifetimes) are sensitive to local packing density [30,33,34]. The advantage of tPnA to study lipid heterogeneity derives from the assignment of the multiple components of its fluorescence decay to distinct fractions of the bilayer. Thus, there is ample evidence that probe molecules in the gel phase show a lifetime that can be an order of magnitude higher that of those molecules located in a more fluid environment [31–38]. Based on these properties, it is frequently possible to obtain an image of the bilayer consistent not only with the complex set of fluorescence lifetimes and amplitudes observed in natural membranes but also with the decay of the probe fluorescence anisotropy.

Taking advantage of these properties of tPnA we reported recently [23] that an important fraction of the platelet plasma membrane lipids are in the form of solid-like microdomains, at temperatures below 35° C (20% at 20° C). On the other hand, this heterogeneity in the lipid packing density almost disappears at 40° C, where the platelet aggregation rate becomes maximal. These changes in the platelet plasma membrane are similar to those observed previously by Gordon et al. [39] using EPR lipid probes, which were interpreted [39] as due to the formation of cholesterol-rich patches in the membrane. Therefore, we decided to carry out the present work to study the effect of cholesterol on the order, fluidity and lateral heterogeneity of the platelet plasma membrane from the steady-state and time-resolved fluorescence of DPH and tPnA in multibilayers of phospholipids extracted from human platelet plasma membrane, with different cholesterol/phospholipid molar ratios. The DPH studies show that the presence of cholesterol has a stronger effect on the order than on the fluidity of the bilayer, as has been previously observed in other lipid bilayers [40–42]. On the other hand, from the analysis of the fluorescence lifetimes of tPnA we conclude that the increased cholesterol contents gives rise to a higher proportion of solid-like regions within the bilayer.

2. Materials and methods

2.1. Materials

DPH, phospholipid standards and cholesterol were purchased from Sigma (St. Louis, MO, USA) while tPnA was from Molecular Probes (Oregon, USA). Silica gel LK5 plates (250 μm thick) with a preabsorbent area were from Whatman (Whatman, Clifton, NJ, USA).

2.2. Sample preparation

Washed human platelets were prepared as described elsewhere [43], usually from outdated platelet concentrates (72 h after blood collection in blood banks). Fragments of platelet plasma membrane were isolated by glycerol lysis as described previously [44]. Multilamellar vesicles were prepared from platelet membrane lipids after extraction following Bligh and Dyer [45]. Lipid vesicles were sus-

pended in 10^{-2} M Tris-HCl (pH 7.4). The phospholipid and cholesterol concentration in the samples was determined by the methods of Bartlett and Courchaine et al. [45]. The fluorescence experiments were carried out with the lipid extract obtained from a pool of about 100 platelet concentrates.

Cholesterol was removed from the platelet plasma membrane lipid mixture by precipitating the phospholipids in cold acetone [45]. The phospholipid composition was analyzed by TLC [46]. The cold acetone extraction only affected the cholesterol content, whereas the relative concentration of the different phospholipid species remained unchanged; 90% of the cholesterol present in the native lipids was removed by this procedure, lowering the C/PL molar ratio from 0.7 (native lipids) to 0.07 in the cholesterol-depleted sample. Pure cholesterol was added to part of the phospholipid fraction to raise its C/PL molar ratio to 0.9.

2.3. Incorporation of fluorescent probes

A few microliters of a stock solution of the probe (10^{-3}–10^{-4} M) in *N,N'*-dimethylformamide or acetone were incubated at 40° C with 1 ml (40 μg of phospholipid) of the biological preparation in the dark. Since the parinaric acid is air sensitive, the mixing and incubation was carried out under inert gas. The molar ratio of the fluorescent label to the lipids was lower than 1 to 200 in all cases.

2.4. Steady-state fluorescence anisotropy

The steady-state fluorescence anisotropy $\langle r \rangle$ was recorded with a SLM-8000D Fluorimeter fitted with Glan-Thompson polarizers. The vertical and horizontally polarized emission intensities ($i_{\parallel}$,$i_{\perp}$) elicited by vertically polarized excitation were corrected for background scattering (< 2%) by subtracting the corresponding polarized intensities of a blank containing the unlabelled preparation. The G factor of the photodetection set-up, that accounts, in calculating the anisotropy as $\langle r \rangle = (i_{\parallel} - Gi_{\perp})/(i_{\parallel} + 2Gi_{\perp})$, for the differential polarization sensitivity, was determined by measuring the polarized components of the probes with horizontally polarized excitation. The following wavelength combinations (excitation/emission, in nm) were used: DPH 365/425, tPnA 320/410.

2.5. Time-resolved fluorescence

Fluorescence experiments were performed using the time-correlated single-photon counting spectrometer described previously [22,23]. The emission of DPH and tPnA was excited with a N_2-filled flashlamp (Edinburgh Instruments, EI 199) and detected through a combination of long-pass cut-off KV filters (Schott Glaswerk, Germany). Polaroid HNP'B sheets were placed in both channels. Lifetime experiments were carried out with the emission polarizer oriented at the magic angle (54.7°) relative to the (vertical) transmission axis of the excitation polarizer. The fluorescence collected in this way was analysed by iterative convolution using a local version of the non-linear least-squares method [22,23] and fitted to a multiexponential function:

$$I(t) = \sum_i \alpha_i \cdot \exp(-t/\tau_i) \quad (1)$$

The decay of the fluorescence anisotropy of DPH was analysed by fitting the two polarized components of the emission intensity to the $r(t)$ function described below, by means of a non-linear least-squares 'global' technique described elsewhere [22,23]. The rotational motions of DPH in a lipid bilayer can be approximated by the 'wobbling-in-cone' model [46] in terms of two physical parameters: an average rotational correlation time $\langle \phi \rangle$ and a residual anisotropy r_∞. The rotational time was determined [22,47,48] from the area under the $r(t)$ curve:

$$\langle \phi \rangle = (r_0 - r_\infty)^{-1} \int_0^\infty (r(t) - r_\infty) \mathrm{d}t \quad (2)$$

where r_0 is the intrinsic anisotropy of DPH: r_0(DPH) = 0.385 [49,50]. The value of $\langle \phi \rangle$ obtained from Eq. (2) is independent of the number of exponential terms used to fit the $r(t)$ decay. With the samples studied here it was found that the following biexponential function provided an accurate description of the experimental decays:

$$r(t) = (r_0 - r_\infty)\left[\sum_{i=1}^{2} b_i \cdot \exp(-t/\phi_i)\right] + r_\infty \quad (3)$$

with

$$\sum_{i=1}^{2} b_i = 1$$

Thus, the values of $\langle \phi \rangle$ and r_∞ for DPH in the platelet lipid bilayers were obtained from the fitting of the decay of the fluorescence anisotropy to Eq. (3). Due to the finite time resolution of the nanosecond spectrometer, the values of the fastest rotational time (ϕ_1) and r_0 are strongly correlated, that is, the fitted value of ϕ_1 depends to some extent on the specific r_0 value introduced in the fit. The influence of this on the average rotational time $\langle \phi \rangle$ can be estimated by fitting the experimental data to a modified form of Eq. (3), where the fixed parameter r_0 is replaced by the unconstrained variable $r(0)$. The new $\langle \phi \rangle$ values obtained in this way can be compared with $\langle \phi \rangle$ if they are first multiplied by the factor $(r(0) - r_\infty)/(r_0 - r_\infty)$; this factor is obtained from Eq. (2) by considering that the area under the $r(t)$ curve is quite similar in the two kinds of fits. In the samples studied here $r(0)$ values were in the 0.30–0.36 range and did not introduce changes larger than 30% on the average rotational times.

3. Results

3.1. DPH fluorescence in platelet lipids

Steady state fluorescence anisotropy

The temperature dependence of the steady-state fluorescence anisotropy of DPH in the three samples is shown in Fig. 1. Removal of 90% of the cholesterol from the plasma membrane platelet lipid extract (C/PL = 0.07) produces a 35% decrease in the $\langle r \rangle$ value relative to that of the native lipids. Furthermore, the shape of the temperature dependence of the anisotropy is also different. There is a stronger temperature dependence of $\langle r \rangle$ (higher slope) in the temperature range from 20° C to 30° C that decreases significantly starting at about 30° C.

The vesicles enriched in cholesterol (C/PL = 0.9) show a 28% increase in $\langle r \rangle$ with respect to the native lipids and a weaker temperature dependent variation of $\langle r \rangle$ that does not show any discontinuity in the temperature range studied (25–45° C).

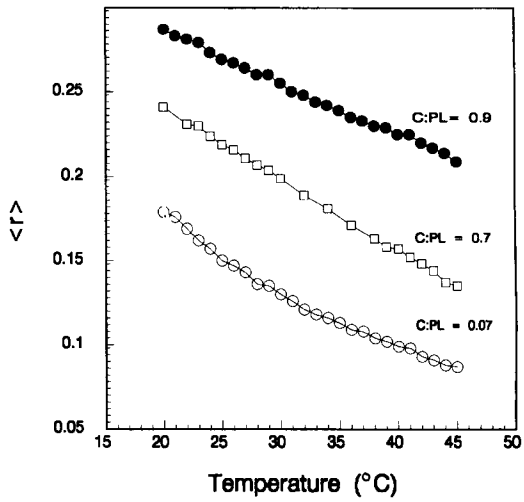

Fig. 1. Temperature dependence of the steady-state anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) in lipid bilayers made up from (1) cholesterol-depleted platelet plasma membrane phospholipids (C/PL = 0.07; ○), (2) native lipids (C/PL = 0.7; □), (3) cholesterol-enriched lipids (C/PL = 0.9; ●).

Static and dynamic components of the anisotropy

The dynamic and the static components of the anisotropy were separated by measuring at different temperatures the time-resolved fluorescence intensity and anisotropy of DPH in multibilayers of lipids depleted and enriched in cholesterol. Fig. 2 shows a representative set of experimental data of the time dependent fluorescence and anisotropy of the two kinds of sample studied. The decay of the fluorescence of DPH is biexponential, with a major contribution (80–90%) of a long lifetime component (9–11 ns) and a small fraction of a short component (2–3 ns), similar to what has been observed before in the unmodified native lipids [22,23]. The larger cholesterol concentration slightly increases the fraction of the long lifetime component, without affecting its temperature dependence.

The fluorescence anisotropy of DPH in the platelet lipid bilayers, considering a unique rotational species, decays in a few nanoseconds to a residual anisotropy value r_∞. The fit parameters for the cholesterol-depleted and cholesterol-enriched multibilayers at different temperatures are presented in Table 1. The residual anisotropy values r_∞ (or better r_∞/r_0) are related to the orientational order imposed on the fluorophore by the microenvironment, and are not strongly dependent on the accuracy of fit, as has been discussed previously [22,23]. The cholesterol depleted sample (C/PL = 0.07) shows an r_∞ value that is much lower (60–80%, depending on the temperature) than the one observed for the native lipid preparation (C/PL = 0.7) [22,23]. It also shows a discontinuity in its temperature

Table 1
Fluorescence anisotropy parameters of DPH in the cholesterol-enriched and the cholesterol-depleted lipid vesicles as a function of temperature

Sample	T (° C)	$\langle \phi \rangle$	r_∞ [a]	$\langle r \rangle$ [b]
Cholesterol-enriched (C/PL = 0.9)	25	1.1	0.214	0.266
	30	1.1	0.206	0.252
	35	0.7	0.190	0.235
	37	0.7	0.188	0.230
	40	0.7	0.173	0.221
	45	0.6	0.156	0.205
Cholesterol-depleted (C/PL = 0.07)	25	1.6	0.084	0.146
	30	1.3	0.056	0.124
	35	1.2	0.040	0.109
	37	1.0	0.037	0.105
	40	0.9	0.032	0.095
	45	0.8	0.026	0.083

Average rotational relaxation time $\langle \phi \rangle$ in ns.
[a] Residual anisotropy (±0.01).
[b] Steady-state anisotropy (±0.004).

Table 2
Fluorescence decay parameters of *trans*-parinaric acid in the cholesterol-enriched and the cholesterol-depleted vesicles as a function of temperature

Sample	T (° C)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)
Cholesterol-enriched (C/PL = 0.9)	25	0.1	1	0.3	6.7	0.6	16.7
	35	0.1	1.2	0.4	6.3	0.5	12.0
	45	0.1	1.4	0.6	4.7	0.3	8.7
Cholesterol-depleted (C/PL = 0.07)	25	0.14	1.3	0.72	6.2	0.14	28.1
	30	0.28	1	0.68	4.5	0.04	21.0
	35	0.33	1.6	0.64	4.1	0.03	19.0
	45	0.32	1	0.66	3.0	0.02	15.0

Lifetimes ±0.5 ns; fractional amplitudes ±0.02.

dependence at 30° C that is analogous to the one observed in $\langle r \rangle$. The sample enriched in cholesterol (C/PL = 0.9) presents higher r_∞ values ($\cong$ 10%, depending on the temperature) than the native lipid preparation and shows no indication of a break on its temperature dependence.

The average relaxation time $\langle \phi \rangle$ of DPH in the multibi-

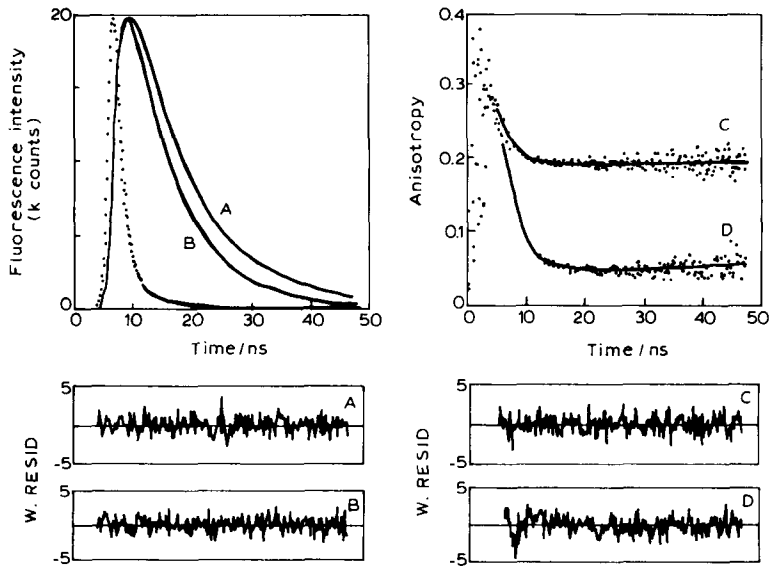

Fig. 2. The decay of the fluorescence intensity and fluorescence anisotropy of DPH at 35° C in cholesterol-enriched (A,C) and cholesterol-depleted (B,D) lipid vesicles. The lower panel shows the weighted residuals distribution.

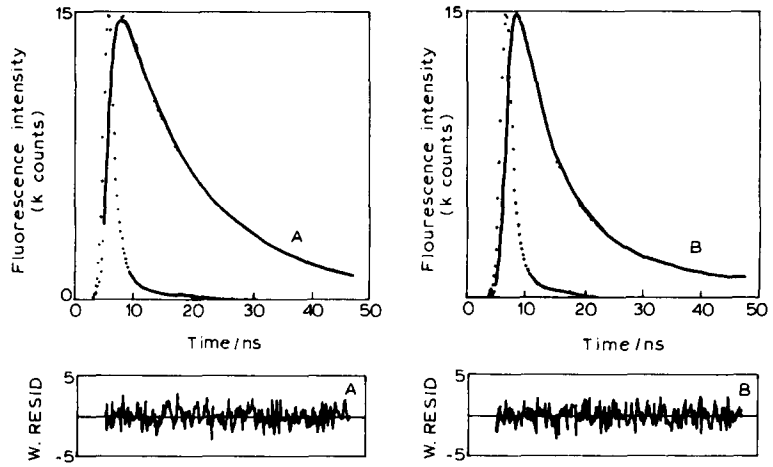

Fig. 3. Fluorescence intensity decay of tPnA at 35° C in cholesterol-enriched (A) and cholesterol-depleted (B) platelet lipid vesicles. The lower panel shows the weighted residuals distribution.

layer vesicles is also included in Table 1. It shows a slight increase with the cholesterol content of the samples. According to the 'cone' physical model [40], the value of $\langle\phi\rangle$ depends on the rotational coefficient of the probe, $D_{\perp}$, and on the probe order parameter, which is related with the r_{∞} value. Then, the observed increase in $\langle\phi\rangle$ may reflect the corresponding change in the residual anisotropy of the cholesterol-rich vesicles.

3.2. tPnA fluorescence in platelet lipids

Time-resolved fluorescence studies

The time-resolved fluorescence intensity of tPnA for the cholesterol-depleted and the cholesterol-enriched samples at 35° C is shown in Fig 3. The parameters of the analysis of the lifetime components of tPnA in the temperature interval 25° C to 45° C are shown in Table 2. The best description of the emission (reduced $\chi^2 < 1.3$) was obtained by fitting the fluorescence to a decay function with three lifetimes. In all three preparations with different cholesterol content (C/PL = 0.07, 0.7 and 0.9) the long lifetime of tPnA shows a strong temperature dependence, decreasing both the lifetime value and its amplitude with increasing temperatures, while the intermediate and short lifetimes show smaller changes. The cholesterol content in the membrane affects the amplitudes of the different lifetime components as well as their values, being the long lifetime component again the more sensitive to these changes. The higher the cholesterol content, the higher the amplitude of the long lifetime component, with a corresponding decrease in the amplitude of the two shorter components. In the vesicles with the lowest cholesterol composition (C/PL = 0.07) the fractional contribution of the long lifetimes in the 30–45° C range was very small (Table 2) and, therefore, both its absolute value (τ_3) and pre-exponential term (α_3) were determined with a much higher uncertainty than the rest of the values.

4. Discussion

Previous work from this laboratory [22,23] has shown that the platelet plasma membrane, when probed by the rotational depolarization of DPH, displays the properties of a densely packed bilayer. The measured high values of the residual anisotropy, which were attributed to the specific lipid composition and high cholesterol content of the bilayer, are influenced only to a very small extent, if at all, by the intracellular structures, membrane-associated proteins and the native lipid asymmetry. The temperature dependence of both DPH order parameter and bilayer apparent viscosity showed that a change in membrane properties should take place in the narrow range of 36–40° C. Time-resolved fluorescence studies with the transparinaric probe confirmed the presence of a thermotropic lipid phase separation, at temperatures of 37° C and lower, proposed by Gordon et al. [39], and also demonstrated that those authors were correct in suggesting that the observed effects were due to the lipid component of the membrane only.

By changing the cholesterol content of the platelet plasma membrane lipids we were able to address some of the questions that arose from that work: the contribution of cholesterol to the high order parameter measured by DPH, the effect of the cholesterol on the change of membrane properties detected by the DPH in the range from 36–40° C, and, by using the tPnA as fluorescent probe, explore the influence of cholesterol on the temperature-dependent solid-like domains detected by this probe.

Steady-state measurements with DPH (Fig. 1) show that increasing the cholesterol of the membrane lipids increases the measured DPH anisotropy value, as has been observed before [16,21] and in agreement with the well documented rigidifying effect of cholesterol on fluid lipid bilayers [40,42,47]. We do not observe significant changes in the fluorescence kinetic components of the probe in the different preparations, indicating that the cholesterol content is not affecting the DPH position in the membrane.

Time-resolved fluorescence studies of DPH showed that the residual anisotropy r_{∞} was more sensitive to the changes in cholesterol than the average relaxation time $\langle\phi\rangle$, in agreement with previous experience [47,51,52]. The large decrease of the r_{∞} value in the cholesterol-depleted samples (Table 1) indicates that the cholesterol content is the most important factor determining the order (as detected by DPH fluorescence) in multilayers of the isolated lipids of the platelet plasma membrane. Moreover, since we showed before [22] that the DPH order parameter of the plasma membrane of intact platelets is very similar to that of multilayers of membrane lipids, it could be anticipated that this large effect of the cholesterol concentration on the lipid order would also take place on the native system. The temperature dependence of the DPH r_{∞} in the cholesterol-poor bilayers (Table 1) shows that the platelet phospholipids reach a very fluid, highly disordered state above 35° C that remains relatively unchanged with further temperature increases. Therefore, in the platelet cell at physiological temperatures, the phospholipid part of the plasma membrane presents a liquid-like matrix that would be very sensitive to structure-modulating agents as cholesterol.

In the analysis of the fluorescence kinetics and depolarization of tPnA, the association of the long lifetime (τ_3) with dye molecules located in a more dense environment (A) provides a consistent interpretation of the temperature dependence of the complex lifetime data [22,23,38]. To explain the fluorescence properties of tPnA in the membrane it is necessary to assume that two distinct lipid regions coexist, characterized by a large difference in packing density. The fraction of probe in each of them

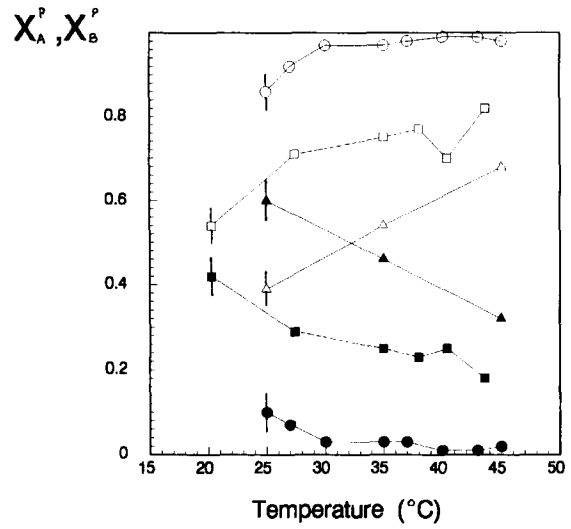

Fig. 4. Fraction of tPnA in the solid-like X_A^P (closed symbols) and fluid-like X_B^P (open symbols) regions of the lipid bilayers as a function of temperature. (1) cholesterol-depleted lipids (C/PL = 0.07; ●, ○); (2) native platelet lipids (C/PL = 0.7; ■, □) (from [22,23]), (3) cholesterol-enriched lipids (C/PL = 0.9; ▲, △).

(X_A^P, X_B^P) can be estimated from the fractional fluorescence amplitudes:

$$\left(A_i = \alpha_i / \sum_{i=1}^{3} \alpha_i\right) : X_A^P = A_3; X_B^P = A_1 + A_2, \quad (4)$$

provided that the probe radiative rate constants are the same [29]. The fluorescence kinetics of tPnA in the cholesterol-poor vesicles is dominated by the short lifetime components (τ_1 and τ_2), whereas the fluorescence kinetics in the cholesterol rich sample is dominated by the long lifetime component (Table 2). As was mentioned in the Introduction, the relative amplitudes of the three lifetime components may be associated to the heterogeneous distribution of the probe in bilayer regions of different density. The low amplitude of the long lifetime component (τ_3) of tPnA in the cholesterol-depleted samples indicates that, in these conditions, only a very small fraction of lipids are forming solid-like domains. The fact that τ_3 is longer than in the cholesterol-rich bilayers (Table 2 and Ref. [23]) is probably due to the different lipid/cholesterol composition of these more densely-packed regions in the two cases. Fig. 4 shows the thermal dependence of the probe fraction in the samples with different cholesterol content. Removing 90% of the cholesterol present in the native lipids reduces the amount of tPnA in the solid like region by 70% at 25° C. Increasing the temperature further lowers the amount of tPnA in solid like domains down to about 1% at 45° C. On the contrary, a 20% increase in the amount of cholesterol present in the membrane doubles the amount of tPnA in dense regions at 25° C. These results show that the presence of solid-like domains in the membrane is directly related to the amount of cholesterol present.

The relative amount of lipids in the two regions with different lateral packing densities can be estimated from the tPnA fractional fluorescence amplitudes as determined with this associative model if the partition coefficient of the probe in the two environments is known. The probe partition coefficient $K_P^{A/B}$ for the solid/fluid distribution in synthetic lipid bilayers [31,32] and in rat liver plasma membranes [35] is known to take values in the range of 3 to 5. If this range of values is also appropriate for the platelet lipid bilayers, then, taking the partition coefficient to be 3, the percentage of solid-like domains in the vesicles enriched with cholesterol varies from 20% to 10%, and in the cholesterol-depleted samples from to 3% to less than 1%, in the temperature range of 25 to 45° C.

5. Conclusions

The work of Gordon et al. with EPR methods [39] and our previous observations with fluorescence lipid probes [23] provided strong indications that regions of distinct densities coexist in the platelet plasma membrane and multibilayers made up from the membrane lipids. We now present evidence that this lateral heterogeneity (which is not due to the protein composition of the membrane) is dependent on the amount of cholesterol present in the lipid mixture.

Changing the cholesterol content of the platelet plasma membrane lipid extract modifies the overall order of the fatty acid chains, as detected by DPH fluorescence, and affects the amount of solid-like and fluid-like domains present in the membrane, as detected by tPnA. These results are compatible with a membrane model with regions where cholesterol concentration is much higher than the macroscopic average value. These cholesterol domains, distributed either laterally or across the lipid bilayer, may differ from the rest of the membrane in the lipid exchange rate [51–54] and/or in their lateral packing densities. It is interesting to note that a protein-dependent lipid lateral heterogeneity has also been described [55]. In that case, lipid domains with unequal enrichment of different phospholipid were detected using fluorescence microscopy with digital image processing. The mechanisms by which membrane cholesterol content affects protein function is far from being understood, and is probably dependent upon the type of protein involved. However, more detailed information on the cholesterol organization in the membrane helps visualize indirect mechanisms through which protein-cholesterol interactions could take place.

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