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Molecular order and fluidity of the plasma membrane of human platelets from time-resolved fluorescence depolarization

C. R. Mateo, M. P. Lillo, J. González-Rodríguez, and A. U. Acuña

Instituto de Química-Física "Rocasolano", C.S.I.C., Serrano 119, E-28006 Madrid, Spain

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Abstract. The ability of seven fluorescence polarization probes (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) to report changes induced by temperature and Ca^{2+} in the plasma membrane of human platelets has been examined. The steady-state fluorescence anisotropy of the probes was compared after being incorporated into whole resting platelets, fragments of platelet plasma membrane and multilayers of lipids extracted from these membranes. In addition, we have investigated the molecular order and dynamics of the three preparations by time-resolved fluorescence depolarization of DPH and CE-DPH as a function of temperature and Ca^{2+} concentration. The high values of the order parameters found in intact platelets ($S_{DPH,\,36\,°C}=0.70$) were almost identical to those in membrane fragments and lipid vesicles, suggesting that lipid-lipid interactions and, therefore, the lipid composition are the main factors influencing the probe order parameter. Other lipid interactions such as those with membrane proteins and intracellular components have little effect on the S_{DPH} in platelets. These measurements also showed that the stationary fluorescence anisotropy of DPH and CE-DPH in platelets is largely determined (80%) by the structural order of the lipid bilayer. Therefore, the previous "microviscosity" values based on stationary anisotropy data reflect the alignment and packing rather than the mobility of the bilayer components. The dynamic component of the anisotropy decay of these probes was analyzed in terms of the wobbling-in-cone model, allowing an estimation of the apparent viscosity of platelet plasma membrane ($\eta_{DPH,\,36\,°C}=0.5$ P) that is similar to that of the erythrocyte membrane. This value decreased substantially in multilayers of native lipids, indicating a large effect of the lipid-protein interactions on the probe dynamics within the bilayer. When the temperature was raised from 25° to 36 °C a pronounced decrease was observed in the order parameter and apparent viscosity, followed by a tendency to level-off in the 36°–40 °C interval. This may be related to the end-point of the lipid phase separation reported by Gordon et al. (1983). Finally, the rigidifying (lipid ordering) effect of Ca^{2+} on the platelet plasma membrane could also be observed by the fluorescence anisotropy measurements, in the form of an increase (~2%) of the order parameter of CE-DPH for Ca^{2+} concentrations in the millimolar range.

Key words: Platelet membrane – Fluorescence anisotropy – Membrane probes

Introduction

Alteration of the fatty acid, phospholipid, cholesterol and protein composition in natural membranes leads to changes in the rate and amplitude of the motions of the bilayer components and, frequently, in the functional state of the cell (Benga and Holmes 1984; Stubbs and Smith 1984; Devaux and Signeuret 1985; Spector and Yorek 1985). In the case of the plasma membrane of blood platelets, there is a sustained interest in the identification of those chemical and physical factors that might perturb its crucial role in the haemostatic process. Several spectroscopic methods – optical absorption and emission, Raman and ESR – have been used to determine to what extent the structure and function of the platelet plasma membrane may be influenced by the lipid composition (Shattil and Cooper 1976, 1978; Berlin et al. 1984; Bevers et al. 1990; Feijge et al. 1990), temperature (Shattil and Cooper 1976; Aslanian et al. 1983; Gordon et al. 1983), Ca^{2+} ion concentration (Sauerheber et al. 1980), physio-

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-[(4-trimethyl-amino)phenyl]-6-phenyl-1,3,5-hexatriene; CE-DPH, (2-carboxyethyl)-1,6-diphenyl-1,3,5-hexatriene; 16AP, 16-(9-anthroyloxy)-palmitic acid; *c*-PnA, *CIS*-parinaric acid; *t*-PnA, *trans*-parinaric acid; PER, perylene; POPOP, *p-bis*[2(5-phenyl-oxazolyl)-benzene]; ESR, electron spin resonance

Offprint requests to: A. U. Acuña

logical agonists (Okhi et al. 1980; Nathan et al. 1979, 1980; Steiner and Lüscher 1984; Feijge et al. 1990), plasma proteins (Shattil et al. 1978; Kowalska and Cierniewski 1983), proteolytic enzymes (Simons et al. 1985) and drugs (Kitagawa et al. 1984, 1989; Nosal et al. 1985). These studies established clearly a correlation between platelet function and the physical and chemical properties of the plasma membrane, in spite of some apparent contradictions (e.g. Shattil and Cooper 1978; Berlin et al. 1984; Feijge et al. 1990). However, the causative details at the molecular level of the correlation remain obscure. Thus, for example, the relative contribution of the factors mentioned above to the modification of the order, dynamics and organization of the lipids in the bilayer is not known. Moreover, it is not even clear whether the recorded physical changes in the bilayer reflect a primary role for the membrane lipids in platelet function or, on the other hand, appear as secondary effects of protein-mediated processes.

The methods of steady-state fluorescence polarization, which are experimentally simple, provide the possibility of analyzing platelet function in native environments and may form the basis for diagnostic assays (Zubenko et al. 1987). On the other hand, the correct interpretation of these experiments requires a detailed characterization of the plasma membrane of resting platelets in terms of order and apparent viscosity. These two parameters, that can be estimated from the time-resolved fluorescence depolarization of suitable lipophilic probes, would facilitate the interpretation of the large amount of existing data obtained from steady-state polarization.

Thus, in the present work, we report on the comparative behavior in the platelet membrane of seven fluorescence polarization probes that differ in size, location in the bilayer, and polarity: DPH*, TMA-DPH, CE-DPH, *t*-PnA, *c*-PnA, 16AP and PER. Based on this study carried out in intact human platelets, fragments of the platelet plasma membrane, and vesicles made up from lipids of the platelet membrane, we determined the values of order parameters and apparent viscosities from the time-resolved fluorescence depolarization of DPH and CE-DPH in the temperature range from 25° to 43 °C. Those experiments also indicated that some sort of lipid segregation might take place in the membrane bilayer at temperatures close to the physiological range, as has been claimed before using ESR techniques (Gordon et al. 1983). This possibility has been studied in much more detail and is reported in a companion paper (Mateo et al. 1991), using the fluorescence kinetics of parinaric acid probes.

Materials and methods

Materials

DPH was purchased from Sigma Co., USA and perylene from Aldrich, Steinheim, W. Germany. TMA-DPH, CE-DPH, 16AP, *c*-PnA and *t*-PnA were from Molecular Probes, Oregon, USA. The purity of the probes was checked by absorption and emission spectroscopy, thin layer and gas chromatography (SE30 capilar column), HPLC (C18 reverse-phase column) and melting point. Purification, when needed, was carried out by crystallization in hexane.

Preparation of platelets, membrane fragments and vesicles

Washed human platelets were prepared as described elsewhere (Barber and Jamienson 1970), usually from outdated platelet concentrates (72 h after blood collection in blood banks). Several control samples were prepared immediately after blood extraction from voluntary, healthy donors. The concentration of the cell suspensions used in the fluorescence polarization measurements was $1-4 \times 10^7$/ml. Fragments of platelet plasma membrane were isolated by glycerol lysis (Barber and Jamienson 1970) as described previously (Eirín et al. 1986). Multilamellar vesicles were prepared from platelet membrane lipids after extraction following Bligh and Dyer (1959).

Occasionally, unilamellar liposomes were prepared by controlled sonication. Plasma membrane fragments and lipid vesicles were suspended in 10^{-2} *M Tris*/HCl (pH 7.4); intact platelets were handled in the same buffer after addition of 0.15 *M* NaCl. The buffers used in the Ca^{2+} titration experiments were passed through a Chelex column (Bio-Rad, Richmond, CA, USA). Protein, phospholipid and cholesterol concentration in the samples were determined by the Folin technique (Lowry et al. 1957) and the methods of Bartlett (1959) and Courchaine et al. (1959), respectively.

Incorporation of the 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 5 ml of the biological preparation for 15–90 min in the dark. Since the parinaric acids are air sensitive, the mixing and incubation was carried out under inert gas. In all cases, the molar ratio of the fluorescent label to endogenous lipids was lower than 1 to 200. The kinetics of the incorporation of the fluorescent probes was followed by recording the total fluorescence intensity and anisotropy as a function of time. The polar fluorophores, TMA-DPH, CE-DPH, 16AP and parinaric acids, reached plateau values in 3–5 min, while the highly lipophilic probes DPH and PER needed 30–50 min. In lipid vesicles both fluorescence intensity and anisotropy remained constant for several hours. The same was also observed in membrane fragment suspensions, except that for *c*-PnA a monotonic increase in both parameters was found over this period. In suspensions of intact platelets, the fluorescence properties of DPH, PER, 16AP and *t*-PnA remained stable and reproducible. However the anisotropy of TMA-DPH and CE-DPH decreased with time (6% and 3% per hour at 36 °C, respectively), while that of *c*-PnA increased (8%/h). The absorption and emission spectra of the probes were virtually independent of the biological preparation.

Steady-state fluorescence anisotropy

Fluorescence spectra and steady-state anisotropy $\bar{r}$ were recorded with a SLM-8000D fluorimeter fitted with Glan-Thompson polarizers, using stirred cuvettes under an inert atmosphere. 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. In cell suspensions, the turbidity of the sample may severely influence the recorded anisotropy (Feijge et al. 1990). Thus, a turbidity correction was introduced, when necessary, by extrapolation to zero concentration in a plot of $\bar{r}$ vs. the apparent absorbance of the sample at the excitation wavelength. The absence of depolarization due to energy transfer was checked by measuring the anisotropy at several probe/lipid molar ratios. The G factor of the photodetection set-up, that accounts, in calculating the anisotropy as $\bar{r}=(i_{\parallel}-G\,i_{\perp})/(i_{\parallel}+2\,G\,i_{\perp})$, for the differential polarization sensitivity, was determined by measuring the polarized components of fluorescence of the probes with horizontally polarized excitation. The following wavelength combinations (excitation/emission, in nm) were used for the seven probes studied: DPH 360/425, TMA-DPH 362/450, CE-DPH 364/450, 16AP 364/435, PER 415/475, *t*-PnA 320/410, *c*-PnA 325/410 (slit widths exc. 1 nm; em. 2–8 nm).

The intrinsic fluorescence anisotropy of CE-DPH ($r_0=0.390\pm0.002$) was determined using a $10^{-6}\,M$ solution in glycerol at $-5\,^{\circ}C$, taking advantage of the much higher photochemical stability of this dye in polar solvents than that of DPH. The corresponding values for the remaining probes were taken from the literature.

Time-resolved fluorescence

The decay of the observed parallel $I_{\parallel}(t)$ and perpendicular $I_{\perp}(t)$ components and that of the total intensity $I_{55}(t)$ of the fluorescence elicited by vertically polarized excitation were recorded in a improved version of a time-correlated single-photon counting spectrometer (Acuña et al. 1987) built using ORTEC 583 and 473A discriminators and a 457 time-to-amplitude converter. The samples were excited with a thyratron-gated nanosecond flash lamp (Edinburgh Instr. EI 199), filled with N_2 or H_2 fitted with a 10 cm monochromator. The emission was passed through a combination of band-pass (Schott KV) or interference filters (Oriel) and detected with a Philips XP2020Q photomultiplier. Polaroid HNP'B sheets were used in both excitation and emission channels.

Data analysis

The kinetic parameters (lifetimes and amplitudes) of the decay of the fluorescence intensity were determined by iterative reconvolution of the $I_{55}(t)$ signal. Our fitting routines were developed based on the non-linear least-squares convolution technique (Knight and Selinger 1971; Grinvald and Steinberg 1974), using standard weighting factors (Bevington 1969; Wahl 1977). The experimental $I_{55}(t)$ was usually fitted to a bi-exponential function $I(t)=\alpha_1\exp(-t/\tau_1)+\alpha_2\exp(-t/\tau_2)$ convolved with the instrumental function $L(t)$:

$$I_{55}(t)=\{\alpha_1\exp(-t/\tau_1)+\alpha_2\exp(-t/\tau_2)\}*L(t) \qquad (1)$$

The wavelength-dependent timing effects in the photodetection (Lewis et al. 1973), although very small with the XP2020Q photomultiplier (Van Hoek and Visser 1985), can be corrected to some extent by a time-shift of the instrument response function (Van der Zegel et al. 1986) or can be suppressed by the "reference" methods introduced by Wahl and coworkers (1974). Here the first technique was used routinely (time shifts of 0.02 ± 0.02 ns). Occasionally, for checking purposes, the reference method was also applied (Zuker et al. 1985), using dimethyl-POPOP as the reference compound, $\tau_F=1.294\pm0.002$ ns (Visser et al. 1987).

The fluorescence anisotropy decay functions were determined by fitting the measured intensities $I_{\parallel}(t)$ and $I_{\perp}(t)$ simultaneously to the expressions:

$$I_{\parallel}(t)=\{i(t)\,[1+2\,r(t)]/3\}*L(t) \qquad (2a)$$

$$I_{\perp}(t)=\Gamma\{i(t)\,[1-r(t)]/3\}*L(t)\,, \qquad (2b)$$

where the form of the anisotropy function $r(t)$ depends on the a priori selected model (*vide infra*). The fitting was accomplished using a non-linear least-squares global analysis method developed from published descriptions (Knutson et al. 1983; Cross and Fleming 1984; Löfroth 1985; Vos et al. 1987). The time shift and lifetimes determined in the analysis of $I_{55}(t)$ were used in the anisotropy fitting routine as fixed input parameters. The quality of the fits was checked with standard statistical parameters (Van der Zegel et al. 1986; Ameloot and Hendrickx 1982). The analysis of the polarized fluorescence intensities requires the determination of a normalization factor, Γ, that accounts for small fluctuations in the number and intensity of the excitation pulses. This factor was obtained by comparing the steady-state anisotropy $\bar{r}$ of the samples with that computed from the time-resolved experiment (Dale 1980). The average value of Γ which also contains the G factor of the nanosecond spectrometer, was 1.01 ± 0.02. Although no time-dependence is expected for G or Γ, the measurement of $\Gamma(t)$ is a useful check of instabilities and systematic errors. The data presented in Fig. 1 show the degree of coincidence of the instrumental response to vertically and horizontally polarized components of emission elicited by horizontally polarized excitation.

The simplest model of the restricted motion of some specific probes in a biomembrane, based on the Brownian diffusion of the label in a cone with a wobbling diffusion constant D_w, leads to the following, single exponential approximation (Kinosita et al. 1977):

$$r_w(t)=(r_0-r_\infty)\exp(-t/\langle\Phi\rangle)+r_\infty\,, \qquad (3)$$

where $\langle\Phi\rangle$ is an average relaxation time for return to the initial equilibrium distribution. The fitting of the experimental data described here to (3) was very poor. Therefore, the decaying part of $r(t)$ was approximated as

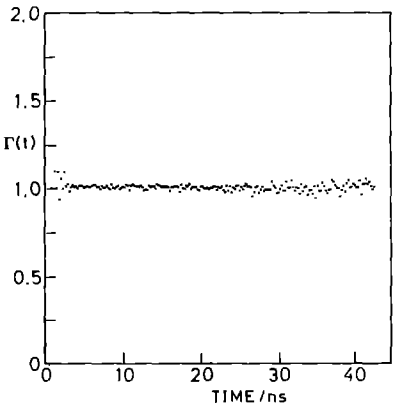

Fig. 1. Time profile of the Γ factor. This was determined by computing the ratio of the vertically to the horizontally polarized intensity components, relative to the scattering plane, of the polarized fluorescence of $\sim 10^{-6}$ M DPH in viscous paraffin oil for horizontally polarized exciting light

a biexponential function (Dale et al. 1977):

$$r(t) = (r_0 - r_\infty)\left[\sum_{i=1}^{2} b_i \exp(-t/\Phi_i)\right] + r_\infty \quad (4)$$

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

When a third exponential term is introduced, as demanded by some of the more detailed models (see, e.g., Ameloot et al. 1984), the fitting did not improve significantly. The advantages of the numerical two-exponential approximation to the anisotropy decay of DPH in lipid bilayers have been discussed recently (Heyn 1989). It should be noted that the residual anisotropy values r_∞ reported here were almost independent of the number of exponentials (1 to 3) used in the fitting.

The average relaxation time $\langle\Phi\rangle$ of the wobbling-in-cone model was determined from the area under the double exponential decay curve (Kinosita et al. 1984; Heyn 1989) as follows:

$$\langle\Phi\rangle = \sum_{i=1}^{2} b_i \Phi_i . \quad (5)$$

A value of $r_0 = 0.385$ was used in this work for DPH (Ameloot et al. 1984; Best et al. 1987). Occasionally the fixed r_0 value in (4) was replaced by the unconstrained variable $r(0)$. In this case by similar reasoning as above the average relaxation time is given by:

$$\langle\Phi\rangle = \frac{r(0) - r_\infty}{r_0 - r_\infty} \sum_{i=1}^{2} b_i \Phi_i . \quad (6)$$

The mean relaxation times given by (5) and (6) were very similar to those obtained in the direct, but statistically unacceptable, fitting to the single-exponential function of (3). The wobbling diffusion constant was estimated from the expression $D_w = \sigma/\langle\Phi\rangle$ (Kinosita et al. 1977), where the numerical values of σ were computed by the approximation of Yguerabide and Yguerabide (1984):

$$\sigma = (1/4)(1 - r_\infty/r_0) \quad (7)$$

The values of D_w calculated in this way were also very close to those obtained from the area under $[r(t) - r_\infty]/[r_0 - r_\infty]$ (Kinosita et al. 1977; Lipari and Szabo 1980), that is, independent of the number of exponentials in $r(t)$. The coefficient D_w can also be estimated from the initial slope of the normalized $r(t)$, in a model-independent analysis (Kinosita et al. 1977, 1984). However, in the case of cell suspensions, scattering artifacts may make this approach less reliable than that using the entire decay function (5).

Another way of expressing the kinetic part of the anisotropy represented by $\langle\Phi\rangle$ is by the apparent "viscosity in the cone", η, (Kawato et al. 1977). For DPH, it was estimated from the value of D_w and the classical relationship (Kinosita et al. 1984) between the rotational diffusion constant and the hydrodynamic parameters. CE-DPH was approximated by an ellipsoid with dimensions of 17.5×1.4 Å (Acuña et al. to be published). More elaborate alternatives, considering the probe as a prolate ellipsoid rotating under "soft" orienting potentials, yielded viscosity values for the platelet membrane that did not differ significantly ($\sim 30\%$) from those computed as detailed above (Mateo CR, Doctoral dissertation, 1989).

The average order parameter S of the probes DPH and CE-DPH in the platelet membrane preparations was computed by solving:

$$r_\infty = r_0 S^2 [P_2(\cos\theta)]^2 , \quad (8)$$

where $P_2(\cos\theta)$ is the second Legendre polynomial for the angle θ between the transition moments and the unique long inertial axis of the probe ($\sim 14°$). The expression in Eq. (8) applies to a probe molecule with cylindrical symmetry (emission and absorption transition moments assumed parallel) oriented in such a way that the equilibrium angular distribution function is preserved when the molecules are pumped to the emitting state (Naqvi 1980, 1981).

Results

Steady-state fluorescence anisotropy in platelet preparations

The temperature dependence (25°–43 °C) of the steady-state fluorescence anisotropy of the seven probes incorporated in resting intact platelets, platelet plasma membrane vesicles and multilamellar vesicles made of lipids from the platelet plasma membrane fraction is shown in Fig. 2. These data represent average values from several measurements of at least two independent human platelet batches. Since the anisotropy of TMA-DPH and *c*-PnA in intact platelets at temperatures higher than 30 °C varied slowly with time (see Methods), the corresponding values are not shown. The observed value of the anisotropy of the probes tended to decrease on going from platelets to isolated plasma membranes and from here to lipid multilayers and, for any particular preparation, always did so from low to high temperatures. Strikingly, all the probes except *c*-PnA in vesicles showed a more or less noticeable change in the slope of the plots in the 36°–40 °C range (Fig. 2). This behaviour was fully reversible in the three preparations. The anisotropy of

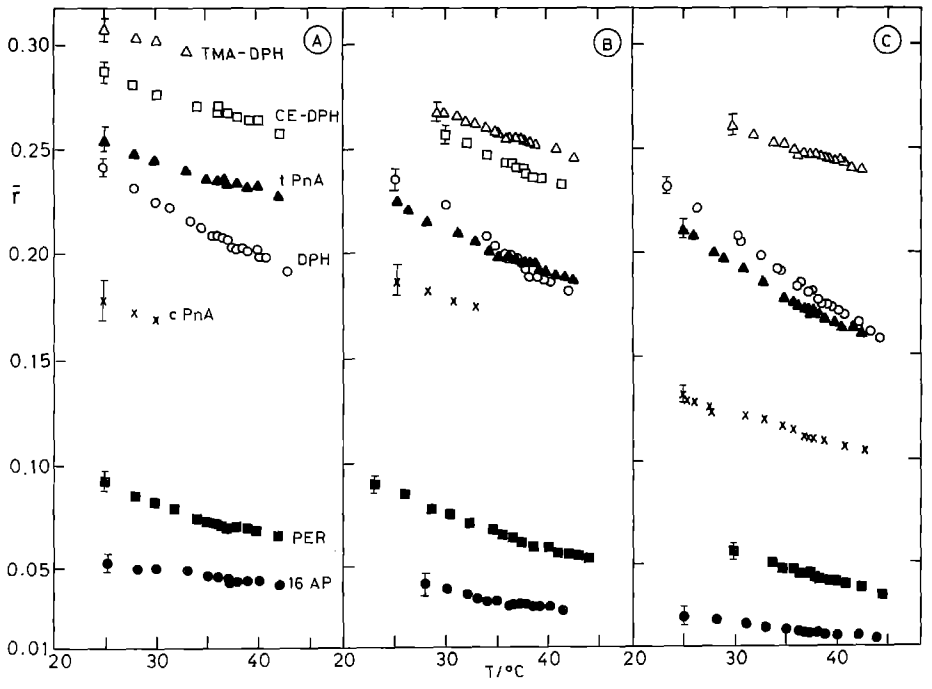

Fig. 2A–C. Temperature dependence of the stationary fluorescence anisotropy of seven lipid probes incorporated into intact human platelets (**A**), fragments of platelet plasma membrane (**B**) and multilamellar vesicles (**C**) made up from lipids of the platelet membrane. Each plot is the average of 3 to 6 temperature scans using for each one a new preparation (see Fig. 8 for abbreviations)

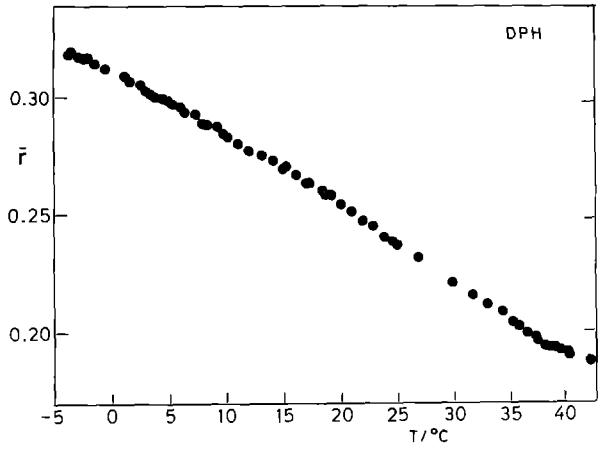

Fig. 3. Temperature dependence of the stationary fluorescence anisotropy of DPH in fragments of platelet membrane in an extended range. To prevent freezing, a small amount of glycerol was added to the samples being measured below 0 °C

DPH was determined several times over a much extended temperature range, from −3° to 43 °C (Fig. 3), but no additional discontinuities were superimposed on the broad curvature observed between 0° and 37 °C. The changes associated with the storage time of the platelet-rich plasma, from 0 to 4 days and determined by monitoring the $\bar{r}$ value of *t*-PnA, were within the quoted error bars.

The addition of Ca^{2+} at physiological extracellular levels to the three preparations elicited a fast increase (1–5%) in the anisotropy of the polar probes. This effect, which is reversible after EDTA addition, was larger at higher temperatures (Fig. 4) and with anionic probes such as CE-DPH and *t*-PnA, while with the very lipophilic dyes DPH and PER it was very small. That these changes reflect the interaction of the Ca^{2+} ions with the membrane and not with the probe was determined by observing that the $\bar{r}$ values of the polar probes in very dilute

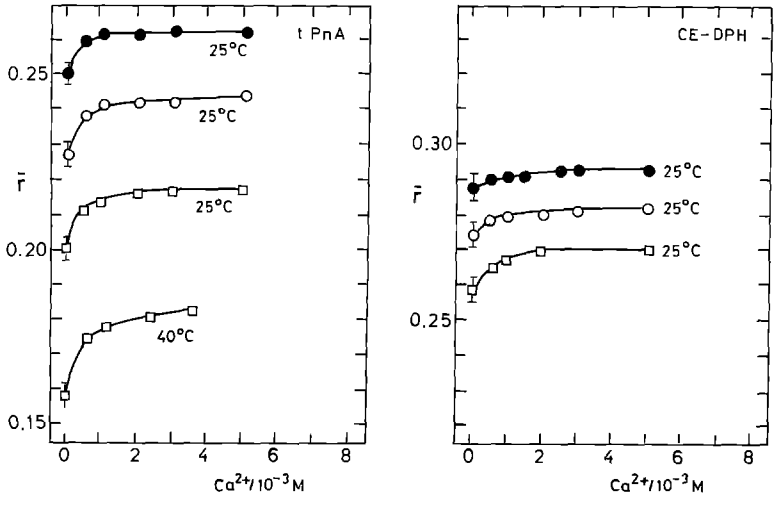

Fig. 4. The influence of Ca^{2+} concentration on the stationary fluorescence anisotropy of tPnA and CE-DPH incorporated into platelets (●), fragments of plasma membrane (○) and vesicles of platelet lipids (□). These plots are averages of at least two different preparations

aqueous solution and incorporated into micelles of non-ionic detergents was independent of the Ca^{2+} concentration.

Static and dynamic components of the anisotropy

It is well known that the stationary fluorescence anisotropy $\bar{r}$ is made up from a structural (r_∞) and a dynamic ($r_0 - r_\infty$) contribution, that can be separated (to some extent) by time-resolved techniques (Kinosita et al. 1977; Jähnig 1979). This was carried out here for DPH in intact platelets, isolated plasma membranes and lipid multilayers, as a function of temperature, and for CE-DPH in the same preparations as a function of Ca^{2+} ion concentration. The parameters of the fluorescence decay analysis of DPH for three out of the eleven temperature values studied here are shown in Table 1. The kinetics of the fluorescence of DPH in the three preparations were satisfactorily described by a major contribution (75–85%, constant for each sample) with a long lifetime between at least 9.5 and 11 ns (decreasing with temperature) and a short component of 3–5 ns, which was fairly independent of temperature within the 25°–43 °C interval. The contribution of the short lifetime increased after prolonged irradiation, suggesting a photochemical source (Shinitzky and Barenholz 1974; Duportail and Weinreb 1983; Lentz 1989). However, experiments carried out under controlled illumination showed that at least a significant fraction of the short lifetime component must have an alternative source, possibly related to the heterogeneity of the probe distribution in the platelet membrane.

Table 1. Decay parameters of the total emission of DPH in intact platelets, fragments of plasma membrane and multilamellar vesicles of platelet membrane lipids. The error levels include the variations between different batches of platelet concentrates

Sample	Temperature [°C]	α_1	τ_1 [a] [ns]	α_2	τ_2 [a] [ns]	$\langle\tau\rangle$ [b] [ns]
Platelets	25	0.24	4	0.76	10.2	9.5
	36	0.24	3.5	0.76	9.8	9.2
	40	0.24	3.5	0.76	9.5	8.9
Membrane fragments	25	0.20	3.6	0.80	10.5	10.0
	36	0.20	3.6	0.80	10.2	9.7
	40	0.20	3.9	0.80	10.0	9.5
Lipid vesicles	25	0.15	3.7	0.85	10.9	10.5
	36	0.15	3.4	0.85	10.5	10.1
	40	0.15	3.5	0.85	10.3	9.9

[a] fluorescence lifetimes (± 0.1 ns)
[b] average lifetime $\langle\tau\rangle = (\alpha_1 \tau_1^2 + \alpha_2 \tau_2^2)/(\alpha_1 \tau_1 + \alpha_2 \tau_2)$

The fluorescence anisotropy of DPH in the platelet preparations decays in a few nanoseconds to a residual anisotropy value. A representative set of the experimental and fitted decays for intact platelets and plasma membrane fragments at 36 °C is shown in Fig. 5. The fit parameters for platelets, plasma membrane fragments, and vesicles of membrane lipids at three selected temperature values, out of the eleven measured, are included in Table 2. The residual anisotropy values r_∞ (or better r_∞/r_0) are directly related to the degree of order imposed on the fluorophore by its microenvironment, and are not very sensitive to the accuracy of fit to the decaying part of $r(t)$, as was mentioned before. In Fig. 6 is shown the temperature dependence of r_∞ for DPH in intact platelets, membrane fragments and lipid multilayers in the 25°–43 °C range. The discontinuity observed in the $\bar{r}$ plots of Fig. 2 in the $\approx$36°–40 °C interval is also visible here. The r_∞ values were used to calculate, from Eq. (8), the average order parameter (S) of the probe in these preparations (Table 2). At the lower temperature the S values are relatively high, do not differ too much in the three platelet preparations, and decrease quite sharply with the increase in temperature. It should be noted that the order parameters determined here from the time-resolved data are very close to those estimated directly from the steady-state anisotropy $\bar{r}$ by the semi-empirical expressions of Van der Meer et al. (1986).

Table 2. Fluorescence anisotropy decay parameters of DPH in intact platelets, fragments of plasma membranes and multilamellar vesicles of platelets lipids. The error levels include the variation between different batches of platelet concentrates

Sample	Temperature [°C]	b_1	Φ_1 [a] [ns]	b_2	Φ_2 [a] [ns]	$\langle\Phi\rangle$ [b] [ns]	r_∞ [c]	$\bar{r}$ [d]	S [b]	η [b] [P]
Platelets	25	0.57	0.8	0.43	3.9	2.0	0.21	0.240	0.76	0.8
	36	0.74	0.8	0.26	4.0	1.6	0.18	0.207	0.70	0.5
	40	0.75	0.8	0.25	3.5	1.3	0.17	0.196	0.68	0.4
Membrane fragments	25	0.65	0.8	0.35	5.3	2.3	0.20	0.233	0.74	0.8
	36	0.66	0.6	0.34	4.1	1.6	0.17	0.198	0.68	0.5
	40	0.73	0.7	0.27	4.3	1.5	0.16	0.187	0.66	0.5
Lipid vesicles	25	0.70	0.6	0.30	4.0	1.7	0.20	0.225	0.74	0.6
	36	0.79	0.7	0.21	3.4	1.1	0.16	0.183	0.66	0.3
	40	0.83	0.8	0.18	3.1	1.0	0.15	0.171	0.64	0.3

[a] Standard error in $\Phi_1 \pm 0.3$ ns
[b] Average correlation time $\langle\Phi\rangle$, order parameter (S) and viscosity in the cone (η) computed as described in Methods.
[c] Residual anisotropy (± 0.01)
[d] Steady-state anisotropy (± 0.004)

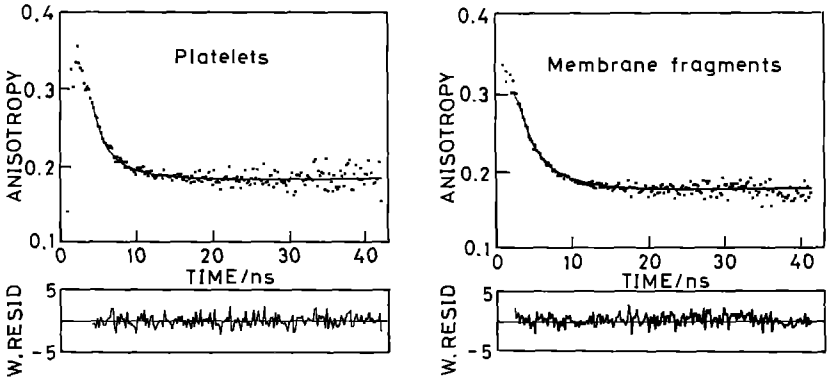

Fig. 5. Fluorescence anisotropy decay of DPH in platelets and fragments of platelet plasma membrane at 37 °C. For platelets the fitting parameters of the overlayed function shown were $\tau_1 = 4.0$, $\tau_2 = 9.9$, $\Phi_1 = 0.9$, $\Phi_2 = 4.4$ (in ns), $r(0) = 0.37$, $r_\infty = 0.178$, reduced $\chi^2 = 1.17$. For membrane fragment the fitting parameters were $\tau_1 = 3.7$, $\tau_2 = 10.3$, $\Phi_1 = 0.7$, $\Phi_2 = 4.1$ (in ns), $r(0) = 0.36$, $r_\infty = 0.170$, reduced $\chi^2 = 1.04$. A set of decay parameters averaged over different platelet concentrates is given in Tables 1–2

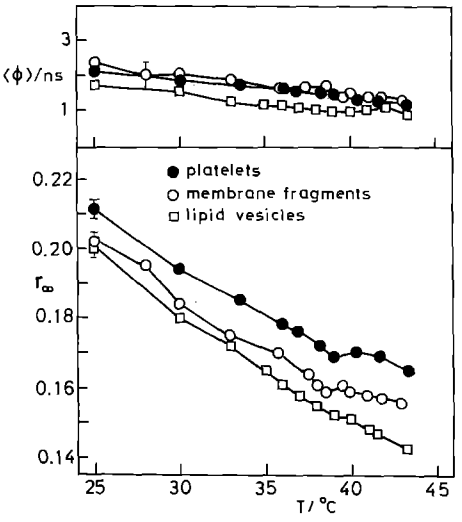

Fig. 6. The temperature dependence of the average relaxation time $\langle \Phi \rangle$ and the residual anisotropy r_∞ of the DPH fluorescence in platelets (●), fragments of plasma membranes (○) and vesicles of platelet lipids (□)

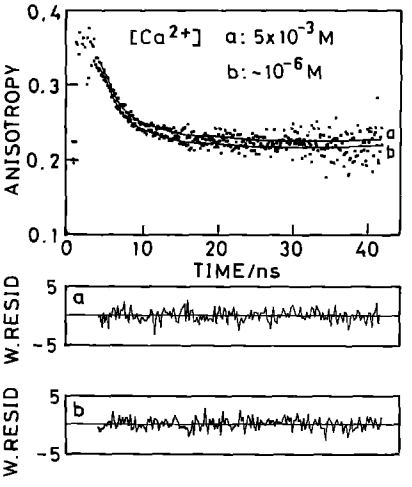

Fig. 7. The influence of Ca^{2+} concentration on the fluorescence anisotropy decay of CE-DPH in fragments of platelet membranes at 37 °C. The fitting parameters in the calcium free samples (**a**) were $\tau_1 = 2.7$, $\tau_2 = 7.2$, $\Phi_1 = 0.6$, $\Phi_2 = 6.0$ (in ns), $r(0) = 0.37$, $r_\infty = 0.209$, reduced $\chi^2 = 1.26$. The fitting parameters of the samples in 5×10^{-3} M Ca^{2+} (**b**) were $\tau_1 = 2.7$, $\tau_2 = 7.1$, $\Phi_1 = 0.6$, $\Phi_2 = 7.1$ (in ns), $r(0) = 0.38$, $r_\infty = 0.216$, reduced $\chi^2 = 1.28$

The average relaxation time $\langle \Phi \rangle$ of DPH in the three preparations, computed as described above from the two experimental relaxation times (Table 2), is also shown in Fig. 6 as a function of temperature in the 25°–43 °C range. It is shown that the temperature-dependent phenomena that induce a change in the slope behavior of the r_∞ in the 36°–40 °C interval also affects to some extent the relaxation dynamics of the probe. The $\langle \Phi \rangle$ values depend on the frictional resistance to probe rotation, although the precise relationship with a fluidity parameter is model dependent (see e.g. Lipari and Szabo 1980). Here, the apparent viscosity of the platelet preparations, η, was estimated according to the "wobbling in the cone" model (see Methods), taking into account the fact that a large number of synthetic and natural membranes have already been characterized in that way. The data in Table 2 indicate that intact platelets and membrane fragments have essentially the same viscosity for a given temperature, while that of the lipid multilayers is much lower (0.5 P and 0.3 P respectively, at 36 °C). The viscosity of the three preparations decreased exponentially between 25°–35 °C (data not shown) with an average "activation energy" of ~7 kcal/mol.

The static and dynamic components of the small Ca^{2+}-dependent changes in $\bar{r}$ described above (Fig. 4) were investigated with CE-DPH in the 25°–40 °C temperature range, in platelet membrane fragments and in multilamellar vesicles of platelet lipids. Intact platelets were studied only at 25 °C, due to the drift of the anisotropy of CE-DPH at higher temperatures (see Methods). The decay of the total fluorescence intensity was not changed for Ca^{2+} concentrations in the 10^{-6}–5×10^{-3} M range. In Table 3 it is shown that the fluorescence kinetics can be described by a two-exponential function, with a major component (7.2 ns, 75%) that decays faster than in DPH and a shorter component similar to that of the parent chromophore. The decay of the CE-DPH anisotropy as a function of Ca^{2+} concentration was analyzed as in DPH and the fitted parameters are shown in Table 3, for $[Ca^{2+}] \approx 10^{-6} M$. The relaxation times were virtually independent of the Ca^{2+} level. However, a small (1–2%) but consistent increase in the residual anisotropy was observed (Fig. 7) at the higher Ca^{2+} levels ($5 \times 10^{-3} M$). The absolute values of r_∞ (and consequently the order parameters) were higher than those of DPH, as would be predicted given the expected localization of the anionic probe in the membrane (Fig. 8).

Table 3. Total fluorescence intensity and anisotropy decay parameters of CE-DPH in intact platelet, fragments of the plasma membrane and multilamellar vesicles of platelet membrane lipids. The error levels include the variation between different platelet concentrates

Sample	Temperature [°C]	α_1	τ_1[a] [ns]	α^2	τ_2[a] [ns]	$\langle\tau\rangle$[b] [ns]	$\langle\Phi\rangle$[c] [ns]	r_∞[d]	$\bar{r}$[e]	S[c]	η[c] [P]
Platelets	25	0.36	3.0	0.64	6.8	6.1	5.4	0.23	0.286	0.79	0.9
Membrane fragments	25	0.26	3.4	0.74	7.4	6.8	3.1	0.24	0.270	0.80	0.8
	37	0.25	2.6	0.75	7.2	6.7	2.2	0.21	0.245	0.76	0.6
	40	0.26	2.7	0.74	7.2	6.7	2.2	0.20	0.239	0.74	0.5
Lipid vesicles	25	0.24	3.4	0.76	7.4	6.9	2.5	0.23	0.260	0.79	0.6
	37	0.21	4.0	0.79	7.8	7.3	1.5	0.21	0.236	0.76	0.4
	40	0.20	3.2	0.80	7.3	6.9	2.0	0.20	0.233	0.74	0.4

[a] Fluorescence lifetimes (± 0.1 ns)
[b] Average lifetimes $\langle\tau\rangle = (\alpha_1 \tau_1^2 + \alpha_2 \tau_2^2)/(\alpha_1 \tau_1 + \alpha_2 \tau_2)$
[c] Average correlation time $\langle\Phi\rangle$, order parameter (S) and viscosity in the cone (η) computed as described in Methods
[d] Residual anisotropy (± 0.01)
[e] Steady-state anisotropy (± 0.004)

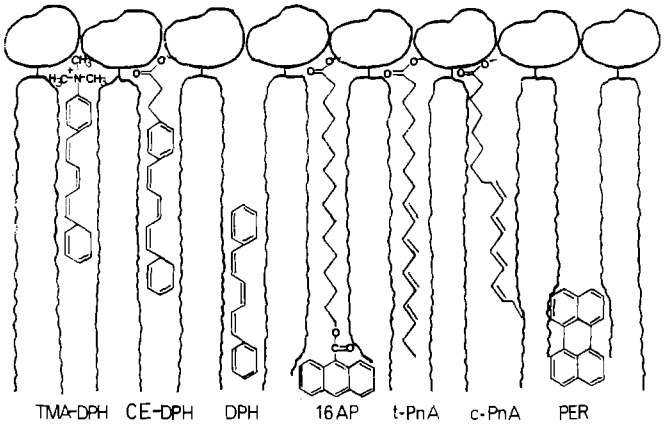

Fig. 8. A pictorial scheme of the fluorescence probes in the lipid bilayer. This static picture shows approximate average locations based on chemical intuition and does not indicated either the locational or orientational distributions of the labels

Discussion

Probe selection for stationary anisotropy studies of platelets

Since the polarization of the fluorescence of lipophilic probes introduced into the plasma membrane of human platelets is very sensitive to changes in the activity and molecular properties of the membrane (see Introduction), one of the aims of this work was to select those polarization probes that can be used with higher reliability in such biologically oriented work. The other aim was to obtain molecular information on the structure and dynamics of the platelet membrane of inactivated cells from these experiments. The stationary fluorescence anisotropy experiments reported here show that the seven fluorescence probes that we used (Fig. 8) are suitable for studies of multilamellar vesicles made up of the platelet membrane lipids. However, this is not true for all these probes when incorporated into platelet preparations of higher complexity. In membrane fragments the anisotropy of *c*-PnA increased with time, although that of the other probes was quite stable. The use of these probes in intact cells is even more restricted. Thus, of the two isomeric parinaric acids, *c*-PnA shows the same lack of stability as in membrane fragments, in contrast with *t*-PnA which remains very stable. The complex fluorescence kinetics of the latter in platelet preparations is highly temperature-dependent. This is probably due to the heterogeneous distribution of the label between membrane regions of different packing density and is discussed in detail in a companion paper (Mateo et al. 1991). The fluorescence anisotropy of perylene and 16AP is stable in intact cells, but the utility of these probes is limited by the uncertain location within the membrane of the first fluorophore and the low absolute value of $\bar{r}$ in platelets and complex photophysics of the second. The cationic probe TMA-DPH shows a very high initial value of $\bar{r}$, indicating a location of the probe close to the phospholipid polar heads. However, the anisotropy decreases appreciably with time, as has been noted before (Donner and Stoltz 1985; Verhalen et al. 1989). The anionic derivative CE-DPH and the parent fluorophore DPH display high values of $\bar{r}$ in platelets. Moreover, the anisotropy of CE-DPH was shown here to report the known changes in membrane rigidity measured by spin-label ESR (Sauerheber et al. 1980) induced by Ca^{2+}. Therefore these two probes appear, together with *t*-PnA, to be the most useful for platelet studies compared with the other labels investigated in this work, although the slow change (3%/h) of the CE-DPH anisotropy in intact cells may be inconvenient at temperatures higher than 30 °C. An advantage of DPH over the other two is the large body of previous data obtained with this fluorophore in platelet preparations as well as in other related biomembranes. In this regard, the remarkable batch-to-batch precision of the DPH steady-state anisotropy values in intact human platelets, not only within the same laboratory (Table 3) but also among different laboratories should be noted: $\bar{r}$ (DPH, 37 °C) = 0.197 ± 0.002 (Donner and Stoltz 1985); 0.197 ± 0.006 (Zubenko et al. 1987); 0.204 ± 0.004 (this work).

There is some controversy on the specific location of DPH in platelets. The early suggestion (Shattil and Cooper 1976) that the probe "gains entry into internal membranes" of the platelet was dismissed in later work from quenching experiments (Boudet et al. 1985), where it was concluded that DPH remains localized within the plasma membrane of the platelet. This conclusion is also consistent with our own data (*vide infra*) that show that the DPH anisotropy parameters in whole platelets remain stable for hours and are very similar to those of plasma membrane fragments.

The transverse location of DPH in the platelet plasma membrane should be similar to that determined in egg phosphatidylcholine vesicles from energy transfer experiments (Davenport et al. 1985), that is, close to the central, disordered region of the lipid bilayer. As will be discussed later, our own data conforms with that expectation. On the other hand, the polar, anionic head of CE-DPH would tend to place the chromophore close to the external boundary of the bilayer (Fig. 8), as is reflected in the larger residual anisotropy and average correlation time of this probe compared with those of DPH.

Order and dynamics of the platelet plasma membrane

The decay of the fluorescence anisotropy of DPH and CE-DPH in the various platelet preparations was analysed assuming a homogeneous location of the probe in the membrane (a unique rotating molecular species) with discrete fluorescence decay times. Since these preparations are essentially multicomponent systems, the probe fluorescence kinetics might be physically better described by one or more continuous distributions of lifetime values (Fiorini et al. 1987). However, for the purposes of the present work we found that the discrete biexponential analysis is accurate enough. Furthermore, we note that the two-exponential description of the fluorescence decay of DPH in erythrocyte membranes (Fiorini et al. 1988) is a fairly good approximation to the bimodal distribution of lifetimes of that probe. The data from the lifetime analysis (Tables 1 and 3) show that the contribution of the short lifetime is higher in intact platelets than in vesicles containing only the membrane lipids. This might be due to heterogeneity of label environments. Nevertheless, the modest changes in the relative contribution of the two lifetimes suggests that the probe depolarization is sampling preferentially the lipid fraction of the membrane as is also indicated by the similar order parameters in membrane fragments and lipid multilayers.

The structural properties of the bilayer detected with this technique appear to depend very little on interactions with cytoplasmic structures and membrane proteins, the order parameter being practically unchanged from intact cells to lipid multilayers. On the other hand, by comparing the residual anisotropy values of DPH and CE-DPH with the steady-state anisotropy obtained in the same conditions (Tables 2 and 3), it is observed that more than 80% of $\bar{r}$ is contributed by r_∞, as is usually found for DPH in synthetic and natural membranes (see, e.g., Hildebrand and Nicolau 1979; Van Blitterswijk et al. 1981; Heyn 1989). Therefore, the changes in the platelet membrane induced by external effectors that were monitored with the $\bar{r}$ value of DPH (see Introduction) reflect mainly alterations of the extent of alignment and packing density of the lipid bilayer.

The dynamic component of the anisotropy, analyzed here using the "wobbling-in-cone" approximation, in spite of the difficulties associated with a detailed physical interpretation, provides a semi-quantitative way of comparing (Kinosita et al. 1981) the fluidity of the different platelet preparations, both between themselves and with that of other biomembranes studied under similar experimental conditions. The data from Tables 2 and 3 show that the apparent viscosity of the platelet lipid bilayer is increased by the presence of membrane proteins, but is not affected by the interactions of the membrane with cytoplasmic structures. Existing data have been interpreted in terms of a "microviscosity" of the platelet membrane ranging from 2.4 to 4.7 P (Crawford 1985; Tandon et al. 1988) but these values were derived from steady-state anisotropy values without separating the static and dynamic contributions, assuming only the latter contributed. Since $\bar{r}$ (DPH) in these samples depends almost exclusively on r_∞, the derived "microviscosity" values are predominantly determined by the angular amplitude of the probe motion, and not the rate of reorientation. When the two contributions are differentiated from each other, the apparent viscosity of the platelet membrane is reduced by almost an order of magnitude, and approaches that of the erythrocyte membrane (Table 4), which is of similar (but not identical) composition. Interestingly, the small differences between

Table 4. Fluorescence anisotropy parameters of DPH and molar ratio of cholesterol and sphingomyelin to phospholipids in the plasma membrane of human platelets and erythrocytes

Sample	Temperature [°C]	$\frac{\text{Chol}}{\text{PL}}$	$\frac{\text{Sphing}}{\text{PL}}$	$\bar{r}$	r_∞	$\langle\phi\rangle$ [ns]	S	η [P]	$S(T_\parallel)$[d]
Platelets	36	0.74[a]	0.27[a]	0.207	0.18	1.6	0.70	0.5	0.69
Erythrocytes	35	0.85[b]	0.2[b]	0.219[c]	0.201[c]	1.1[c]	0.74	0.42	0.75

[a] From the compilation of Crawford (1985)
[b] Van Blitterswijk et al. (1981)
[c] Kinosita et al. (1981)
[d] Order parameter from ESR spectra of 5-nitroxide stearate-labelled cells at 30 °C (Sauerheber et al. 1980)

the DPH order parameters and viscosities in the two membranes are in the directions expected, from their different cholesterol/phospholipid ratios (Chapman 1983; Kinosita et al. 1984). In Table 4 the order parameters for the two biomembranes derived from ESR spectroscopy (Sauerheber et al. 1980) are also included. The striking agreement with those determined from polarized fluorescence suggest that the two probe techniques are monitoring essentially the same intrinsic property of the membranes.

Temperature effects on platelet anisotropy parameters

The data presented here show an overall decrease in the value of $\bar{r}$ when the temperature is raised from 25° to 43 °C (Fig. 2) with a small but noticeable change in the slope at ~36 °C, more easily detected with probes like CE-DPH and *t*-PnA. When the anisotropy is time-resolved into the two parameters r_∞ and $\langle\Phi\rangle$, it is observed that both are affected by temperature and decrease gradually (Fig. 6) until, in the reduced temperature range of 37° to 43 °C, this thermal dependence is attenuated. This suggests that, precisely in the physiological temperature range, the composition of the membrane is such as to prevent large changes on its cohesion and fluidity. This interesting behaviour cannot be observed in the early DPH-labelled platelet data of Shattil and Cooper (1976), in which a linear variation of the logarithm of the "microviscosity" with $1/T$ for a temperature interval of 12° to 44.5 °C was reported. In contrast to this, a very similar change in the thermal properties of the membrane to that observed here is presented (without comment) in a more recent study of the "microviscosity" of the platelet plasma membrane (Menashi et al. 1981), carried out in the same way as that of Shattil and Cooper (1976).

A simple explanation for these changes in the temperature-dependent properties of the platelet membrane might be that, when the membrane is cooled below ~36 °C, a lateral phase segregation takes place, giving rise to a mixture of domains differing in packing and viscosity. At physiological temperatures this membrane lateral heterogeneity is minimized, and the structure and fluidity of the membrane is fully adapted to support its function(s), unperturbed by the thermal variations of the environment. In fact it is known (Gear 1982) that platelet aggregation velocity remains essentially maximal over the narrow temperature range of 35° to 40 °C. A thermotropic phase separation at 37 °C in platelet membranes has been reported by Gordon et al. (1983), based on ESR measurements. These authors also demonstrated that the acid phosphatase activity in platelet membranes exhibits biphasic behaviour with a break at 36 °C. There are also additional indications, from Raman spectral measurements (Aslanian et al. 1983), of thermotropic transitions in the platelet membrane at 5 °C and 17 °C. Further evidence of microdomain segregation in the platelet membrane was obtained from the fluorescence kinetic of probes with a preferential solubility in the most densely packed regions of the lipid bilayer and are presented in a companion paper (Mateo et al. 1991).

Conclusions

The platelet plasma membrane, when probed by the rotational depolarization of DPH and CE-DPH, displays the properties of a densely packed bilayer, with an order parameter $S_{DPH,25\,°C} \sim 0.8$, somewhat lower than that of the erythrocyte membrane. The high *S*-value arises from the specific lipid composition and high cholesterol content of the bilayer and is influenced only to a very small extent, if at all, by intracellular structures, membrane-associated proteins, and the native lipid asymmetry. The apparent viscosity of the platelet membrane (~0.8 P at 25 °C), which is the same as that of erythrocyte membrane, decreases significantly when the probes are incorporated into multilayers containing exclusively the randomized mixture of native lipids. Thus, the rate of motion of the probe is sensitive to lipid-protein interactions while the order parameter is not. The temperature dependence of both order and viscosity shows that a change in membrane properties should take place in the narrow range of 36°–40 °C. This may be related to the end-point of the lipid lateral phase segregation reported by Gordon et al. (1983). Finally, the rigidification of the platelet plasma membrane resulting from Ca^{2+} addition at physiological extra-platelet concentrations is evidenced in the fluorescence anisotropy measurements as an increase in the order parameter of CE-DPH reflecting changes brought about near the phospholipid head groups but not transmitted deep into the hydrophobic interior of the bilayer.

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