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A fluorescence decay study of parinaroyl-phosphatidylinositol incorporated into artificial and natural membranes

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Abstract. Phosphoinositide metabolism in the plasma membrane is linked to transmembrane signal transduction. In this study we have investigated some physical properties (e.g. molecular order and dynamics) of phosphatidylinositol (PI) in various membrane preparations by time-resolved fluorescence techniques, using a synthetic PI derivate with a *cis*-parinaroyl chain on the *sn*-2 position. Phospholipid vesicles, normal and denervated rat skeletal muscle sarcolemmal membranes, and acetylcholine receptor rich membranes from *Torpedo marmorata* were investigated both at 4 °C and 20 °C. For comparison we have also included 2-parinaroyl-phosphatidylcholine (PC) in this study. The fluorescent lipids were incorporated into the membrane preparations by way of specific phospholipid transfer proteins, to ensure an efficient and non-perturbing insertion of the lipid-probes. In the *Torpedo* membranes the order parameters measured for the parinaroyl derivatives of both PC and PI were higher than in phospholipid vesicles. For the *Torpedo* membrane preparations the acyl chain order for the PI was lower than that for PC, whereas the opposite was true for the vesicles. This inversion strongly suggests that PI has different interactions with certain membrane components as compared to PC. This is also suggested by the significantly higher rate of restricted rotation of PI as compared to PC. In contrast to the order parameters, the correlation times were almost identical for both probes and showed little difference between vesicles and the *Torpedo* membranes. In contrast to *Torpedo* membranes, the time-dependent fluorescence anisotropy of the two lipid probes in the sarcolemmal membranes showed, after an initial fast decay, a subsequent gradual increase. This phenomenon was satisfactorily analyzed by assuming two populations of probe lipids with distinct lifetimes, rotational correlation times and molecular order. The order parameter of the population with a short lifetime compared with that of phospholipid vesicles, whereas the population with a long lifetime agreed with that of the *Torpedo* membranes.

Introduction

Interactions between membrane lipids and integral membrane proteins are essential for the structural and dynamic properties of biological membranes. Lipid-protein interactions play an important role in the barrier function, and have been shown to affect the activity of several peripheral and integral membrane proteins.

Various spectroscopic techniques have been applied to the study of lipid-protein interactions (Devaux and Seigneuret 1985). Electron spin resonance (ESR) spectroscopy has been successful in revealing a motionally restricted, spin-labeled, lipid component, which was attributed to a population of lipid molecules interacting with integral membrane proteins. As for many specific proteins, such as (Na^+, K^+)ATPase, myelin proteolipid, acetylcholine receptor and rhodopsin, detailed studies have been performed on the extent and specificity of lipid-protein interactions (Marsh and Barrantes 1978; Watts et al. 1979; Marsh et al. 1981; Ellena et al. 1983; Brophy et al. 1984; Esmann et al. 1985; Esmann and Marsh 1985). In all these systems a motionally restricted lipid component was detected. While hardly any specificity was apparent in the case of rhodopsin (Watts et al. 1979), other proteins (e.g. (Na^+, K^+)ATPase, lypophilin, cytochrome oxidase)

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Abbreviations: PI, phosphatidylinositol; PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine, PnA, *cis*-parinaric acid: *cis,trans,trans,cis*-9,11,13,15-octadecatetraenoic acid: 2-PnA-PC, 1-acyl, 2-parinaroyl-PC; 2-PnA-PI, 1-acyl,2-parinaroyl-PI; DPH, diphenylhexatriene; POPOP, 1,4-di[2-(5-phenyloxazolyl)]-benzene; NMR, nuclear magnetic resonance; ESR, electron spin resonance; $I_{\parallel}$ parallel fluorescence intensity component; $I_{\perp}$ perpendicular fluorescence intensity component; SET-buffer, 0.25 *M* Sucrose, 1 m*M* EDTA, 10 m*M* *Tris*-HCl, pH 7.4

clearly favour negatively charged lipids with varying degrees of preference for a particular single lipid class (Brophy et al. 1984; Esmann et al. 1985; Esmann and Marsh 1985; Knowles et al. 1981). So far phosphatidylinositol (PI') has not been included in these studies. This omission may be due to the fact that incorporation of a labelled fatty acyl chain into this lipid requires a relatively complicated synthetic procedure (Somerharju and Wirtz 1982). On the other hand, the important role of PI metabolism in transmembrane signal transduction necessitates a better understanding of the behaviour of PI in membranes.

Other spectroscopic techniques such as ^{2}H-NMR and ^{31}P-NMR have been less successful in providing clear evidence for specific lipid-protein interactions as the motional time scales are not in the proper range of these techniques (Oldfield et al. 1978; Ellena et al. 1986).

Fluorescence anisotropy decay measurements provide information about reorientational motions in the 10^{-10} to 10^{-7} s time range, comparable to that of ESR. Wolber and Hudson (1981, 1982) have employed this technique to study the effects of membrane lipid composition and integral membrane proteins on the order and dynamics of parinaric acid, the fluorescent fatty acid used in the experiments. Parinaric acid closely resembles the acyl chain of phospholipids, and upon incorporation into the *sn*-2 position of a phospholipid molecule, its position in the bilayer is similar to that of the original membrane lipids (Sklar et al. 1975, 1977a, 1977b).

In the present study we have incorporated *cis*-parinaric acid into PI with the aim of investigating molecular order and dynamics of this phospholipid in biomembranes. In previous studies it was shown that PI was associated with the acetylcholine receptor (Lunt et al. 1971). Phospholipids have also been found to be essential for the activity of the receptor, and upon removal of the endogenous phospholipid, restoration of the activity was most readily achieved by the addition of PI (Chang and Bock 1979). For that reason we have selected biological membranes that contain different levels of the acetylcholine receptor. Phosphorescence spectroscopy measurements have indicated that the receptor is essentially immobile (rotational correlation time 10–26 μs (Bartholdi et al. 1981)), as compared to the mobility of phospholipids in the bilayer (rotational correlation times in the *ns*-range; Wolber and Hudson 1981, 1982). Spectroscopic studies using spin-labelled fatty-acids, phospholipids and steroids in acetylcholine receptor-rich membranes from *Torpedo marmorata* have revealed the occurrence of immobilized lipid components indicative of strong lipid-protein interactions (Marsh and Barrantes 1978; Marsh et al. 1981; Rousselet et al. 1979). However, contradictory results have been reported concerning the specificity of these effects. While one study has reported on the immobilization of fatty acids and steroids in membranes in the absence of an immobilized PC component (Rousselet et al. 1979), other studies have not provided evidence for this discrimination between lipid classes (Marsh and Barrantes 1978; Marsh et al. 1981). More recently, Ellena et al. (1983) have demonstrated the presence of an immobilized lipid component in a reconstituted system containing the acetylcholine receptor. While the mobility of all components tested was affected, the extent of immobilization decreased in the following order: androstane ≅ stearic-acid > phosphatidic acid (PA) > phosphatidylethanolamine (PE) ≅ PC ≅ phosphatidylserine (PS).

Here we have incorporated 2-parinaroyl-PC and 2-parinaroyl-PI into plasma membranes prepared from the electric organ of *Torpedo marmorata,* and rat skeletal muscle. Incorporation of the lipid probes was achieved without disturbing the membrane structure, by use of specific phospholipid transfer proteins carrying the fluorescent lipids (Rousselet et al. 1979). In this study the behaviour of the acidic 2-parinaroyl-PI in membranes was compared to that of the zwitterionic 2-parinaroyl-PC which may be considered as an internal reference probe. A preliminary account on this study was recently presented (Wirtz et al. 1987).

Materials and methods

Materials

Egg yolk PC and PA were purchased from Sigma (St. Louis, MO). Yeast-PI was prepared from autolyzed bakers' yeast (Somerharju and Wirtz 1982; Trevelyan 1966). 2-Parinaroyl-PC and 2-parinaroyl-PI were synthesized from *cis*-parinaric acid (Molecular Probes, Junction City Oregon) and egg yolk-PC or yeast-PI as described (Somerharju and Wirtz 1982; Somerharju et al. 1981, 1985).

PI-transfer protein was purified from bovine brain as described before (Van Paridon et al. 1987), and PC-transfer protein was isolated from bovine liver (Westerman et al. 1983). The lipid-transfer proteins were stored in 50% glycerol at −20 °C.

Preparation of membranes

Sarcolemmal membranes were prepared from normal and 6-day denervated rat skeletal muscle (Shute and Smith 1985). The membranes were stored in 0.25 *M* sucrose/1 m*M* EDTA/10 m*M* *Tris*-HCl (pH 7.6), at 4 °C. Acetylcholine receptor rich membranes from *Torpedo marmorata* (1.2 nmol α-bungarotoxin binding sites per mg protein) were kindly donated by Dr. Changeux, Institut Pasteur, Paris, France.

Incorporation of fluorescent lipids

2-Parinaroyl-PC and -PI were incorporated into the membrane fractions by incubating the membranes (approximately 400 nmoles phospholipid) with the PC- or PI-transfer protein (2–5 nmoles) carrying the fluorescent lipid. The transfer protein-fluorescent phospholipid complexes were prepared as described before (Van Paridon et al. 1987). Briefly, the transfer proteins (approximately 40 μg/ml) were incubated for 15 min at 25 °C with phospholipid vesicles, prepared according to the ethanol injection method (Batzri and Korn 1973). The vesicles consisted of either pure 2-parinaroyl-PI, or 2-parinaroyl-PC/egg-PA (60:40 mole%) (approximately 4 μ*M*) in 20 m*M* *Tris*/HCl/60 m*M* NaCl/5 m*M* $MgCl_2$ (pH 7.4). The vesicles were separated from the transfer protein by chromatography of the mixture on a small DEAE-cellulose (Whatman, DE 52) column (1 ml). This resulted in the binding of the negatively charged vesicles, while the transfer protein-fluorescent phospholipid complex was recovered in the eluent (yield above 90%). Subsequent incubation of these complexes with the membrane fractions resulted in the incorporation of the fluorescent lipids. After the incubation (15 min at 25 °C) the membranes were separated from the transfer protein by centrifugation (30 min at 100,000 *g*). The membrane pellets containing 2-parinaroyl-PI or 2-parinaroyl-PC were resuspended in 2 ml of SET-buffer [0.25 *M* Sucrose/1 m*M* EDTA/10 m*M* *Tris*-HCl (pH 7.4)] by way of a Dounce homogenizer, and used the same day for time-resolved fluorescence measurements.

Artificial phospholipid vesicles containing 2-parinaroyl-PI or 2-parinaroyl-PC (1 mol%) were prepared by a brief ultrasonic (1 min, 25 °C, in a bath-sonicator) of a phospholipid suspension (PC/PA 95:5 mole%, 210 nmole total) in SET-buffer. All manipulations with samples containing parinaroyl-phospholipid were carried out under an argon atmosphere in subdued light.

Time-resolved fluorescence

The fluorescence anisotropy decay measurements were performed at either 4° or 20 °C with the equipment described before (Van Hoek and Visser 1985). Samples were excited at 306 nm by a frequency doubled, synchronously pumped, dye laser (4 ps pulses). The emission was viewed through a Balzers K 45 band-pass filter; a HNP'B polaroid sheet was used in the set-up. The fluorescence and anisotropy decays were obtained via a repeated cycle (10 s) of measuring the parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) fluorescence intensity components. Ten cycles were sufficient for a good signal to noise ratio. Background correction was based on membrane samples which were carried through the entire incorporation procedure in the absence of the fluorescent lipids. The 1024 data points of each component were stored in subgroups of a multichannel analyzer (ND 66, Nuclear Data). The data were then transferred to a VAX computer of the Agricultural University, and analyzed.

Data analysis

Fluorescence and anisotropy decays were analyzed with a non-linear least-squares iterative reconvolution approach as described (Visser et al. 1985; Vos et al. 1987). The data analysis was performed in two steps. The two intensity components, $I_{\parallel}(t)$ and $I_{\perp}(t)$, were combined to the total fluorescence $S(t)$ according to Eq. (1)

$$S(t) = [I_{\parallel}(t) - I_{\parallel}^{B}(t)] + 2[I_{\perp}(t) - I_{\perp}^{B}(t)], \tag{1}$$

where then superscript B denotes the components arising from the blank. The total fluorescence $S(t)$ was fitted to a bi-exponential function of the form

$$S(t) = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2), \tag{2}$$

where α_1 and α_2 are pre-exponential factors belonging to the fluorescence lifetimes τ_1 and τ_2, respectively.

In order to remove any wavelength dependency in the response of the detection system, the data were deconvoluted against a reference compound with a single exponential fluorescence decay measured under the same conditions as the sample. The reference compound used was POPOP in ethanol yielding a fluorescence lifetime of 1.3 ns both at 4° and 20 °C (Zuker et al. 1985; Vos et al. 1987).

As a first approximation the anisotropy decay $r(t)$ was analyzed using the following equation:

$$r(t) = (r_0 - r_\infty) \exp(-t/\phi) + r_\infty, \tag{3}$$

where r_∞ is the residual anisotropy at the end of the time scale of the fluorescence experiment (about 60 ns), r_0 is the initial anisotropy and ϕ the effective relaxation time for the diffusion in a cone model (Kinosita et al. 1977; Lipari and Szabo 1980). As a second approximation the anisotropy decay was fitted according to a bi-exponential function and a constant term:

$$r(t) = \sum_{j=1}^{2} \beta_j \exp(-t/\phi_j) + t_\infty. \tag{4}$$

This decay function must be considered as a purely mathematical model (Van der Meer et al. 1984; Ameloot et al. 1984). The parameters r_∞, β_j and ϕ_j were optimized by fitting $I_{\parallel}(t)$ and $I_{\perp}(t)$ simultaneously to the following equations:

$$I_{\parallel}(t) = \frac{S(t)}{3}[1 + 2r(t)], \tag{5a}$$

$$I_{\perp}(t) = \frac{S(t)}{3}[1 - r(t)]. \tag{5b}$$

Details of this procedure and its advantage over other methods (i.e. preservation of Poissonian statistics and usual fitting criteria) have been summarized by Vos et al. (1987).

In addition, order parameters and diffusion constants were determined. The order parameter, S, was derived from Eq. (6)

$$S^2 = r_\infty / r_0 . \tag{6}$$

The cone angle, θ_c, which describes the angle between the emission transition moment and the membrane normal, and which reflects the amplitude of acyl chain motion in the cone model, was derived from Eq. (7)

$$(1/2)\cos\theta_c(1+\cos\theta_c) = S . \tag{7}$$

It has been shown by Lipari and Szabo (1980) that in the cone model the wobbling diffusion constant, D_w, is part of the following equation:

$$D_w \phi \left(1 - \frac{r_\infty}{r_0}\right) = f(\cos\theta_c) \tag{8}$$

in which:

$$\begin{aligned} f(\cos\theta_c) \equiv f(x_c) = &- x_c^2(1+x_c)^2 \{\ln[(1+x_c)/2] \\ &+ (1-x_c)/2\}/[2(1-x_c)] \\ &+ (1-x_c)(6+8x_c - x_c^2 - 12x_c^3 - 7x_c^4)/24 . \end{aligned} \tag{9}$$

Thus if θ_c is known from r_∞/r_0, the constant D_w can be evaluated using Eqs. (3) and (8). It should be noted that $\phi(1 - r_\infty/r_0)$ is exactly the area under $(r(t) - r_\infty)/r_0$. Therefore, if the anisotropy decay cannot be represented by a single exponential as in Eq. (3), one can obtain D_w by measuring the area under $(r(t) - r_\infty)/r_0$ (Lipari and Szabo 1980).

In recent years the rotational diffusion model has been generally applied (Zannoni et al. 1983; Van der Meer et al. 1984; Szabo 1984). It has been recognized that the rotational diffusion coefficient, $D_\perp$, for reorienting the long probe axis is model-independent. $D_\perp$ can be estimated from the initial slope of $r(t)$ (Ameloot et al. 1984):

$$D_\perp = -\frac{1}{6r_0}\left.\frac{\delta r}{\delta t}\right|_{t=0} . \tag{10}$$

If the anisotropy is mathematically described by Eq. (4), evaluation of $D_\perp$ leads to the following equation:

$$D_\perp = \frac{1}{6r_0}\sum_{j=1}^{2}\frac{\beta_j}{\phi_j} . \tag{11}$$

From Eq. (11) it is clear that $D_\perp$ depends upon the value of r_0. Initially, rather than setting a fixed value for r_0 the data were analyzed with r_0 as an unconstrained parameter in the fitting procedure. From measurements on the various membrane preparations it turned out that, upon excitation at 306 nm, the average value of r_0 is 0.32. This initial anisotropy is identical to the value obtained from anisotropy decay experiments on 2-parinaroyl-PI and 2-parinaroyl-PC bound to phospholipid transfer proteins (Van Paridon et al. 1987). The data were subsequently reanalyzed with the constraint that $r_0 = 0.32$, yielding the order parameters and diffusion constants.

In the sarcolemmal membranes the anisotropy shows an initial rapid decay followed by a subsequent gradual increase. In this instance, the anisotropy decay can be approximated by assuming two probe populations A and B which are characterized by lifetimes, correlation times and order (the associative model):

$$r(t) = [d_A(t) + d_B(t)]/[s_A(t) + s_B(t)] . \tag{12}$$

In this equation d_A and s_A are defined as follows:

$$s_A(t) = \exp(-t/\tau_A) , \tag{13a}$$

$$\begin{aligned} d_A(t) &= \mathrm{I}_\parallel^A(t) - \mathrm{I}_\perp^A(t) \\ &= s_A(t)\,[(r_0 - r_\infty)\exp(-t/\phi) + r_\infty]_A , \end{aligned} \tag{13b}$$

where the subscript A under the square brackets denotes the parameters r_0, r_∞ and ϕ belonging to population A. In applying Eqs. (5a) and (5b) to the model described by Eq. (12), an important constraint is that the observed final anisotropy must equal $r_\infty^A + r_\infty^B$ with positive values for r_∞^A and r_∞^B. A further valid approximation is that $r_0^A = r_0^B$.

Results

2-Parinaroyl-PI and 2-parinaroyl-PC were incorporated into vesicles, into plasma membranes from the electric organ of *Torpedo marmorata*, and into sarcolemmal membranes from normal and denervated rat skeletal muscle as described in the "Materials and methods". The fluorescence decay curve at 4 °C for 2-parinaroyl-PI incorporated into the *Torpedo* membranes is shown in Fig. 1. As judged from the fitting criteria (Visser et al. 1985; Vos et al. 1987) the experimental data points could be well represented by a minimal decay model consisting of a sum of two exponential terms. Very similar curves were obtained for 2-parinaroyl-PC in *Torpedo* membranes, and for 2-parinaroyl-PC and 2-parinaroyl-PI incorporated into phospholipid vesicles and sarcolemmal membranes prepared from rat skeletal muscle both at 4° and 20 °C. The two fluorescence lifetimes derived from the curves are given in Table 1. The fluorescence decay at 4 °C is described by a short lifetime (in the range of 5–11 ns) and a long lifetime component (in the range of 17–23 ns). In each of the four membrane preparations 2-parinaroyl-PI and 2-parinaroyl-PC displayed a similar fluorescence decay behaviour. The shortest lifetime component (5 ns) was observed in vesicles

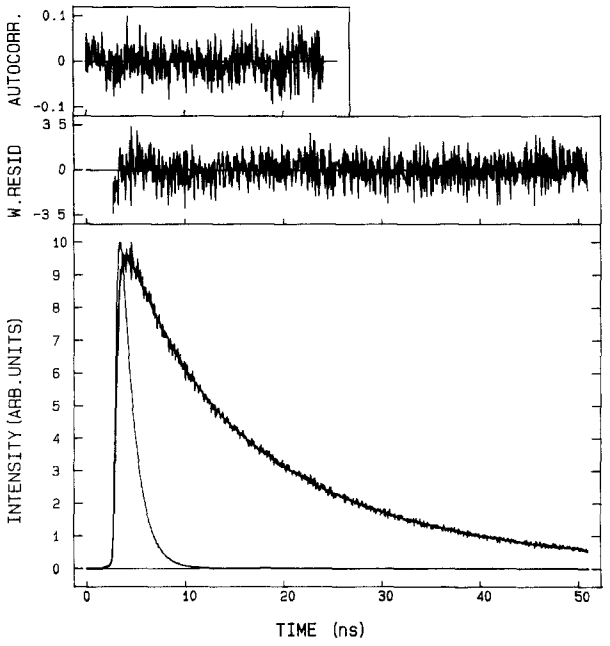

Fig. 1. Fluorescence decay analysis of 2-parinaroyl-PI incorporated into acetylcholine receptor rich membranes from *Torpedo m.*, as prepared according to "Materials and methods". Both the fluorescence response of POPOP and the experimental and calculated fluorescence patterns (1024 channels, time equivalence 0.050 ns per channel) of the fluorescent lipid are shown. Background correction was performed with identical samples only lacking the fluorescent probe. The quality of the data analysis is given by the weighted residual and the autocorrelation of the residuals, depicted on top of the figure. The fluorescence decay parameters as measured at 4 °C are: $\alpha_1 = 0.38 \pm 0.01$, $\tau_1 = 6.5 \pm 0{,}2$ ns, $\alpha_2 = 0.62 \pm 0.01$ and $\tau = 19.1 \pm 0.2$ ns. For this particular fit: $\chi^2 = 1.07$; Durbin-Watson parameter (Vos et al. 1987) $DW = 2.04$, and the number of zero passages in the autocorrelation function $ZP = 237$. Analysis was performed from channels 55 until 1024, POPOP has its maximum response in channel 70

Table 1. Fluorescence decay parameters of 2-parinaroyl-PC (PnA-PC) and 2-parinaroyl-PI (PnA-PI) in various membrane preparations

Sample	Temperatur [°C]	Probe	α_1 [a]	τ_1 [b] [ns]	α_2 [c]	τ_2 [d] [ns]	$\langle\tau\rangle$ [e] [ns]
Vesicles [f]	4	PnA-PC	0.20	4.9	0.80	17.3	16.5
		PnA-PI	0.25	4.8	0.75	16.6	15.6
Torpedo m.	4	PnA-PC	0.32	6.8	0.68	22.7	20.7
		PnA-PI	0.38	6.5	0.62	19.1	17.0
Sarcolemma	4	PnA-PC	0.56	9.7	0.44	23.0	18.4
		PnA-PI	0.56	9.3	0.44	21.4	17.2
Denervated Sarcolemma	4	PnA-PC	0.57	9.2	0.43	21.9	17.3
		PnA-PI	0.60	11.4	0.40	21.5	17.0
Vesicles	20	PnA-PC	0.21	3.9	0.79	9.5	8.9
		PnA-PI	0.25	3.9	0.75	9.2	8.6
Torpedo m.	20	PnA-PC	0.44	5.7	0.65	13.9	11.9
		PnA-PI	0.52	5.5	0.48	12.4	10.1
Sarcolemma	20	PnA-PC	0.73	7.1	0.27	16.0	11.1
		PnA-PI	0.72	6.8	0.28	14.3	10.1
Denervated Sarcolemma	20	PnA-PC	0.69	6.6	0.31	14.4	10.5
		PnA-PI	0.59	6.5	0.41	11.9	9.5

[a,c] Normalized pre-exponential factors α_1 and α_2 $(=1-\alpha_1)$ (± 0.01), corresponding to the two lifetime components
[b,d] τ_1 and τ_2 $(\pm 0.2$ ns), respectively
[e] Average fluorescence lifetime $\{(\alpha_1\tau_1^2 + \alpha_2\tau_2^2)/(\alpha_1\tau_1 + \alpha_2\tau_2)\}$ $(\pm 0.2$ ns)
[f] (PC/PA, 95.5 mole%)

followed by a lifetime of 6.6 ns in *Torpedo* membranes, and a 9–11 ns lifetime in the sarcolemmal membranes. The long lifetime component in the four membrane preparations did not show as much variation. This was also true for the average lifetimes ($\langle\tau\rangle$) which were virtually independent of the membrane preparation with values between 16 and 21 ns. As for the measurements performed at 20 °C the curves were equally well represented by a two exponential fit, but with a considerably faster fluorescence decay. The average lifetimes varied between 9 and 12 ns (see Table 1).

The fluorescence anisotropy decay curves of 2-parinaroyl-PI in vesicles and *Torpedo* membranes at 4 °C are shown in Fig. 2. The curves demonstrate a fast initial decay to a constant anisotropy value. The fast initial decay was taken as a measure of the mobility of

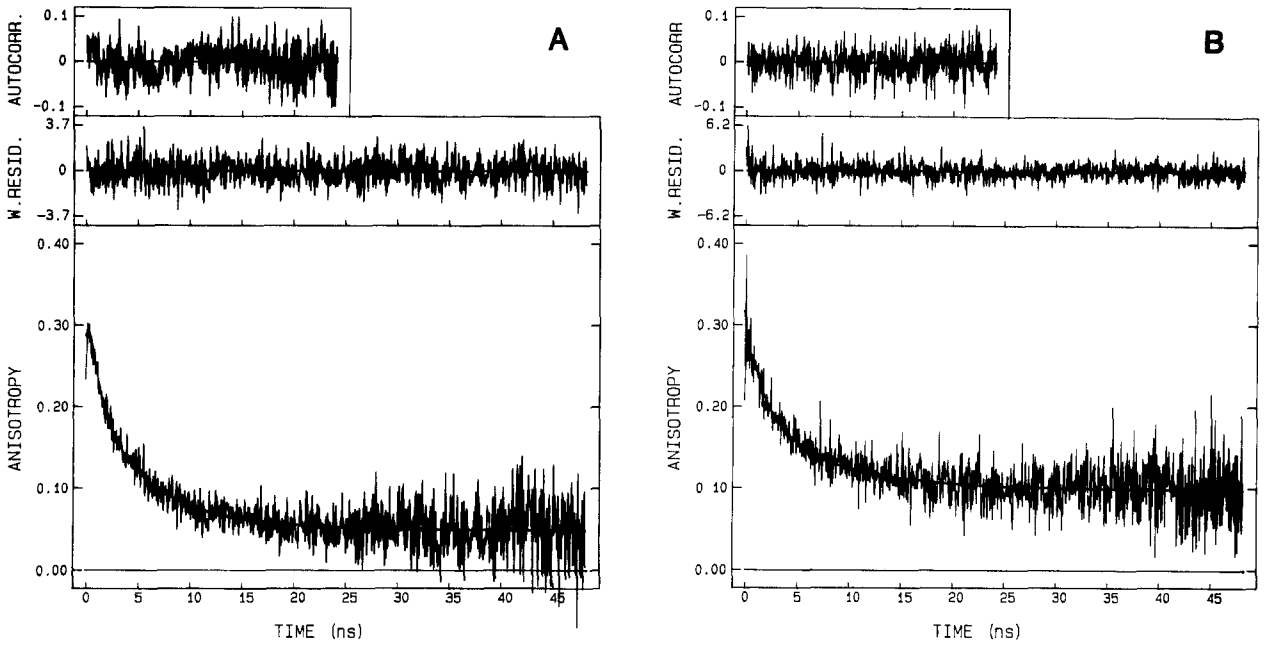

Fig. 2 A and B. Fluorescence anisotropy decay analysis for 2-parinaroyl-PI in artificial lipid vesicles and in membranes from *Torpedo* m. Samples containing approximately 1 mol% fluorescent lipid, were prepared as described in the "Materials and methods". The measurements were performed at 4 °C. **A** and **B** represent 2-parinaroyl-PI present in phospholipid vesicles (PC : PI, 95 : 5 mol%) and incorporated into acetylcholine receptor rich membranes from *Torpedo m.*, respectively. The experimental anisotropy is indicated by the noisy curve, while the smooth line shows the fitted data function. Only the first part of the anisotropy decay is shown. The analysis was performed between channels 55 and 1024 of the decay profiles of $I_{\parallel}$ and $I_{\perp}$. The weighted residuals and the autocorrelation of the residuals are shown on top of the curves. All the parameters from the data analysis are given in Table 2. For vesicles (**A**) the fitting criteria were: $\chi^2 = 1.09$, $DW = 1.85$, $ZP(I_{\parallel}) = 215$ and $ZP(I_{\perp}) = 211$ (see Fig. 1 for the abbreviations). For *Torpedo m.* membranes (**B**) the fitting criteria were: $\chi^2 = 1.04$, $DW = 2.04$, $ZP(I_{\parallel}) = 254$ and $ZP(I_{\perp}) = 247$. Note that, because of convolution, the apparent initial anisotropy is lower than the true one (Papenhuijzen and Visser 1983)

the parinaroyl chain, while the ratio of the limiting to the initial anisotropy values is simply related to the order parameter 5 (see Methods). 2-Parinaroyl-PI clearly shows a much lower residual anisotropy in phospholipid vesicles (Fig. 2 A) than in the *Torpedo* membranes (Fig. 2 B). Similar observations were made for 2-parinaroyl-PC in these membrane systems. When the data were analyzed according to Eq. (3) it turned out that the derived value for r_0 was lower than the experimental one, resulting in poor a fit of the initial decay data. Apparently the anisotropy decay cannot be represented by a single exponential. Hence, the data were analyzed according to a combination of two exponentials and a non-zero residual anisotropy, r_∞ (see Eq. (4)). Order parameters (i.e. S and θ_c) and diffusion constants (i.e. D_w and $D_\perp$) were derived from r_0, r_∞, β_j and ϕ_j, as described in "Methods". This set of parameters determined for 2-parinaroyl-PI and 2-parinaroyl-PC in vesicles and *Torpedo* membranes both at 4° and 20 °C is presented in Table 2.

The correlation times for 2-parinaroyl-PI both in vesicles and in *Torpedo* membranes are similar to those measured for 2-parinaroyl-PC. Moreover, no significant differences were observed between vesicles and *Torpedo* membranes. In all cases the correlation times were found to decrease with increasing temperature. From the diffusion constants (D_w and $D_\perp$) it appears that at 4 °C the rates of diffusion are similar for 2-parinaroyl-PI and -PC in both vesicles and *Torpedo* membranes. At 20 °C the diffusion constants for both probe lipids have increased. It is noteworthy that in *Torpedo* membranes, in contrast to what is observed in vesicles, the rate of diffusion of 2-parinaroyl-PI is two times higher than that of 2-parinaroyl-PC. As for the limiting anisotropy (r_∞), and the measures of order (S and θ_c), significant differences were observed for 2-parinaroyl-PI and 2-parinaroyl-PC in vesicles and in *Torpedo* membranes both at 4 °C and at 20 °C. In general, the degree of order for both probes was higher in the *Torpedo* membranes than in the vesicles, most likely reflecting the effect of other membrane components (e.g. proteins) on the order of the probe lipids. In the vesicles, PI displayed a higher degree of order than PC. In view of the low probe content (1 mole%), we assume that this difference in order parameter is an intrinsic property of the two probe lipids. Conversely, in the *Torpedo* membranes PI displays less order than PC at both temperatures.

In membrane preparations of normal and denervated sarcolemma, most notably at higher temperature, a remarkable phenomenon concerning the

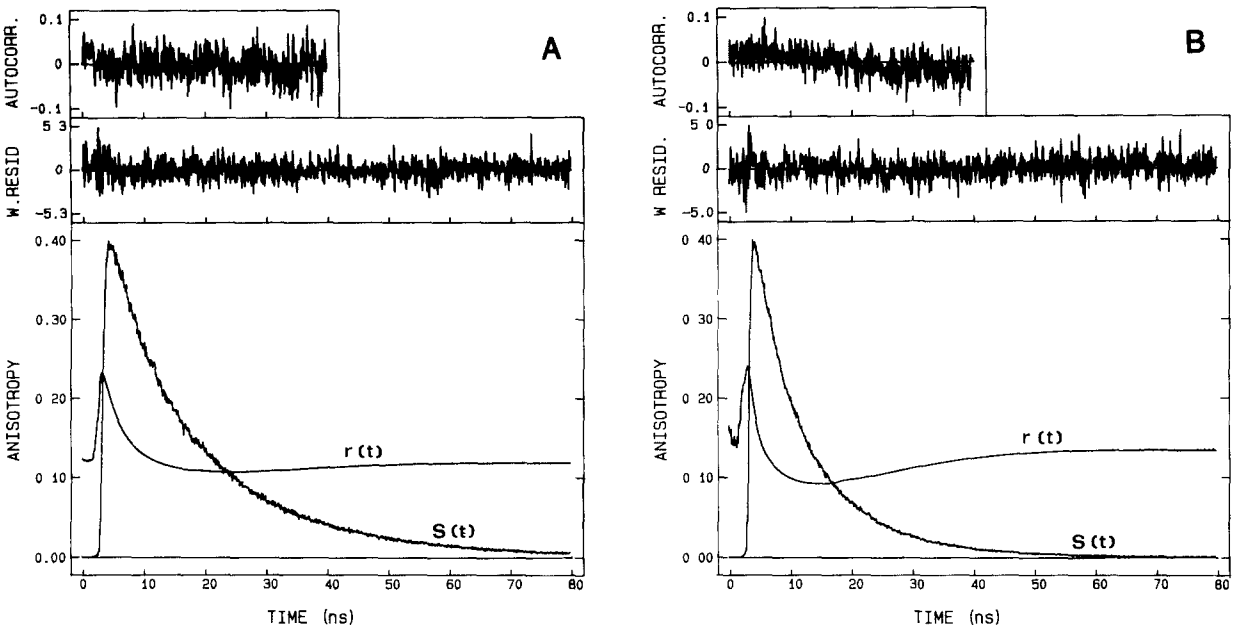

Fig. 3 A and B. Fluorescence anisotropy decay of 2-parinaroyl-PI in rat skeletal muscle sarcolemmal membranes. The experimental details are as described in "Materials and methods". Only the fitted decay function, represented by the smooth line, is shown. The fluorescence anisotropy clearly does not decay to a constant value, but rises again after having reached a minimum. The experimental data were fitted according to the associative model. The quality of the fit is represented by the weighted residuals between the experimental and calculated anisotropies and by the autocorrelation function of the residuals. The time equivalence was 0.087 ns per channel and 920 channels out of 1024 are represented. The fit was carried out over the whole decay profiles of $I_{\parallel}$ and $I_{\perp}$. For comparison the normalized, experimental fluorescence decay profile is shown. The results of data analysis are given in Table 3. **A** Experiment at 4 °C. The fitting criteria (cf. Figs. 1 and 2) were: $\chi^2 = 1.5$, $DW = 1.73$, $ZP(I_{\parallel}) = 222$ and $ZP(I_{\perp}) = 213$. **B** Experiment at 20 °C. The fitting criteria were: $\chi^2 = 1.7$, $DW = 1.79$, $ZP(I_{\parallel}) = 246$ and $ZP(I_{\perp}) = 207$

Table 2. Fluorescence anisotropy decay parameters of 2-parinaroyl-PC (PnA-PC) and 2-parinaroyl-PI (PnA-PI) in phospholipid vesicles and in *Torpedo* membranes

Sample	Temp. [°C]	Probe	β_1	ϕ_1 [ns]	β_2	ϕ_2 [ns]	r_∞	r_0	S[a]	θ_c[a] [°]	D_w[a] [ns^{-1}]	$D_\perp$[a] [ns^{-1}]
Vesicles[b]	4	PnA-PC	0.13±0.02[c]	1.4 ±0.3	0.14±0.02	5.4±0.6	0.041±0.001	0.305	0.36	61	0.041	0.077
		PnA-PI	0.14±0.01	0.9 ±0.1	0.16±0.01	5.6±0.4	0.050±0.001	0.343	0.40	59	0.034	0.066
Torpedo m.	4	PnA-PC	0.10±0.02	1.8 ±0.5	0.08±0.02	7.9±1.8	0.117±0.002	0.288	0.60	45	0.020	0.058
		PnA-PI	0.09±0.02	1.3 ±0.4	0.11±0.02	6.1±0.9	0.102±0.001	0.302	0.56	48	0.028	0.071
Vesicles	20	PnA-PC	0.13±0.02	0.3 ±0.1	0.18±0.07	2.7±0.1	0.023±0.001	0.334	0.27	67	0.051	0.161
		PnA-PI	0.16±0.01	0.8 ±0.1	0.13±0.01	3.7±0.4	0.029±0.001	0.321	0.30	65	0.063	0.127
Torpedo m.	20	PnA-PC	0.18±0.11	0.11±0.07	0.11±0.04	3.1±0.6	0.092±0.001	0.383	0.54	50	0.040	0.155
			(0.13±0.03)	(0.48±0.03)	(0.10±0.03)	(3.6±0.1)	(0.092±0.001)	(0.321)[d]				
		PnA-PI	0.07±0.02	0.4 ±0.2	0.14±0.01	2.9±0.2	0.081±0.001	0.289	0.50	52	0.080	0.287

[a] Order parameters and diffusion constants were determined using the average r_0 value of 0.321
[b] PC/PI, 95:5 mole%
[c] Standard errors derived from the fit of the data
[d] Data in parenthesis were analyzed with the constraint that $r_0 = 0.321$

anisotropy decay was observed. After a minimal anisotropy value was reached, a subsequent rise in anisotropy occurred (see Figs. 3 A and B). A similar increase in fluorescence anisotropy after an initial decrease was also observed by Wolber and Hudson (1982) for parinaric acid in vesicles with reconstituted M 13 bacteriophage coat protein. We have carried out the analysis of the anisotropy data for both parinaroyl probes in normal and in denervated sarcolemmal membranes at 4 °C and 20 °C, applying the simple "wobbling-in-cone" model (i.e. the non-associative model). From statistical criteria, notably the autocorrelation function, we could conclude that this model did not adequately describe the experimental data.

Table 3. Fluorescence anisotropy decay parameters of 2-parinaroyl-PC (PnA-PC) and 2-parinaroyl-PI (PnA-PI) in normal and denervated sarcolemma membranes calculated according to the "associative model"

Sample	Temp.	Probe	r_0	Lifetime 1 (short)[a]			Lifetime 2 (long)[a]		
				ϕ	r_∞[b]	S	ϕ	r_∞[c]	S
	[°C]			[ns]			[ns]		
Sarcolemma	4	PnA-PC	0.29	0.8±0.2	0.041	0.38	5.3±0.3	0.097	0.58
		PnA-PI	0.25	2.5±0.7	0.024	0.31	7.3±1.0	0.094	0.62
Denervated sarcolemma	4	PnA-PC	0.28	0.9±0.2	0.034	0.35	5.8±0.3	0.090	0.57
		PnA-PI	0.24	3.0±1.4	0.040	0.41	6.9±0.9	0.061	0.51
Sarcolemma	20	PnA-PC	0.27	1.3±0.3	0.051	0.43	3.9±0.6	0.090	0.58
		PnA-PI	0.26	0.7±0.2	0.064	0.50	3.3±0.3	0.070	0.52
Denervated sarcolemma	20	PnA-PC	0.27	1.2±0.2	0.016	0.24	4.2±0.4	0.079	0.54
		PnA-PI	0.24	1.2±0.5	0.021	0.30	3.5±0.4	0.060	0.50

[a] Experimental fluorescence lifetimes (see Table 1)
[b] Residual anisotropy (±0.02)
[c] Residual anisotropy (±0.001)

Application of the associative model (see Methods) resulted in a superior fit. Examples are given in Fig. 3 A and B for 2-parinaroyl-PI in sarcolemmal membranes at 4 °C and 20 °C. In the associative model the two experimental fluorescence lifetimes as presented in Table 1, were taken to represent two different populations of fluorescent lipids. In the analysis it was assumed that the fluorescence anisotropy decay of each population could be described by the "wobbling in cone" model. From the analysis over the whole time range of the experiment (Fig. 3), it is clear that the initial anisotropy (r_0) is smaller than the expected one ($r_0 = 0.32$). The effect is more pronounced for the sarcolemmal membranes than for the vesicles and *Torpedo* membranes (see Fig. 2). This may be related to the longer time span of the experiment (i.e. 80 ns as compared to 50 ns) chosen to emphasize the 'growing-in' phenomenon. In Table 3 we have indicated the correlation times and order parameters for the two populations of probe lipids in normal and denervated sarcolemmal membranes at 4 °C and 20 °C. The results indicate that the short fluorescence lifetime was connected to a more mobile population ($\phi \cong 1-3$ ns) of membrane lipids with less order ($S \cong 0.3-0.5$). The longer fluorescence lifetime was coupled to a more rigid population ($\phi \cong 3-7$ ns) of more ordered lipids ($S \cong 0.5-0.6$). In the analyses, the optimized parameters, especially the time constants, were highly correlated. They depended to some extent on the initial estimates in the calculation. The standard deviations of the correlation times were rather large. The results in Table 3 have been obtained with the same initial estimates using the constraints summarized in "Methods". The order parameters for the long lifetime components show the same trends as described for the *Torpedo* membranes. It is evident that, in general, 2-parinaroyl-PI has a lower order parameter than 2-parinaroyl-PC. As for the short lifetime components, the order parameters are in the same range as those observed in vesicles (see Table 2).

One should realize that the associative model used in the above analysis is almost certainly an oversimplification. It should be considered as certainly no more than a first approximation, since even in simple vesicle systems the fluorescence decay already required description in terms of at least two fluorescence lifetimes.

Discussion

Fluorescently labelled phospholipids can be efficiently incorporated into biological membranes by way of phospholipid transfer proteins without perturbing the bilayer. The two transfer proteins used in this study, i.e. the PC-transfer protein from bovine liver and the PI-transfer protein from bovine brain, form a one to one complex with a lipid molecule (Berkhout et al. 1984; Van Paridon et al. 1987). We have prepared the transfer protein-fluorescent lipid complex, and by incubating the complex with the membranes, have incorporated this lipid into the membrane fractions. In this incubation the transfer protein-bound lipid is exchanged for a membrane lipid molecule from the outer leaflet of the bilayer without grossly altering the membrane composition or perturbing the bilayer structure. We have opted for this method of probe incorporation instead of the usual procedures, where liposomes are used as probe donors. Rousselet et al. (1979) made use of a similar scheme to incorporate spin-labelled PC into *Torpedo marmorata* membranes. In this way we avoid the risk of membrane perturbation caused by fusion, or possible sticking of the liposomes. After the introduction of the fluorescent PC and PI into the outer leaflet of the membrane preparation, the probe

molecules will rapidly diffuse in the outer monolayer, while translocation to the inner leaflet may also be a fast process, as has been shown for PI in sarcolemmal membranes (Shute, unpublished observation). Hence the time between the incorporation of the lipid probes and the fluorescence measurements (2–4 h) should be sufficient for the complete redistribution of the probes in the membrane.

The fluorescence decay of 2-parinaroyl-PI and 2-parinaroyl-PC in the different membrane preparations was adequately described by a function consisting of the sum of two exponential terms (see Table 1). The short lifetime component was found to vary considerably between the various membrane preparations, but no significant difference was observed between the two probe lipids. At 4 °C the shortest lifetime was found in vesicles (4.8–4.9 ns), followed by *Torpedo* membranes (6.6 ns) and by normal and denervated sarcolemmal membranes (9.2–11.4 ns). Less variation was detected in the long lifetime component. A similar trend was observed for the lifetimes at 20 °C. The average lifetimes both at 4 °C and at 20 °C were virtually independent of the membrane preparation. In a study of the effect of M 13 coat protein on the order and dynamics of parinaric acid in a reconstituted membrane system, Wolber and Hudson (1982) also observed that the average lifetimes were independent of the presence of protein.

Fluorescence anisotropy decay measurements on 2-parinaroyl-PI and 2-parinaroyl-PC in vesicles indicated a similar mobility of either probe at 4 °C and 20 °C (see the rotational diffusion constants, Table 2). This was also observed for *Torpedo* membranes at 4 °C. Yet it appears that at 20 °C 2-parinaroyl-PI has a significant higher rate of diffusion than 2-parinaroyl-PC. The order parameters indicated a higher degree of acyl chain order for both probes in the *Torpedo* membranes at 4° and 20 °C. This is in agreement with previous publications on other membrane systems (Wolber and Hudson 1982; Kinosita et al. 1981). In the vesicles 2-parinaroyl-PI has a higher order parameter than 2-parinaroyl-PC, presumably reflecting inherent probe characteristics. In the *Torpedo* membrane the situation was reversed. This strongly suggests that compared to PC, PI in *Torpedo* membranes must be located in an environment characterized by a lower degree of order.

The normal and denervated sarcolemmal membranes gave rise to a completely different time-resolved anisotropy behaviour (Fig. 3). After an initial decay to a minimal anisotropy value, the anisotropy subsequently increased. With a total lipid extract from normal sarcolemmal membranes a normal decay to a limiting anisotropy was observed for both probes (Visser et al. 1987). This anomalous behaviour may result from the fact that in contrast to the *Torpedo* membranes, where the acetylcholine receptor is the main protein constituent, the sarcolemmal membranes contain a larger variety of membrane proteins. Because of this heterogeneity, the probe lipids may have distributed over different membrane environments. Wolber and Hudson (1982) have simulated increasing anisotropy patterns by proposing two populations of probe molecules characterized by different fluorescence lifetimes and residual anisotropies. Dale et al. (1977) were the first to delineate the two possible models that can be postulated when the fluorescence decay is heterogeneous. The fluorescence lifetimes can all be linked to the same rotation (i.e. the non-associative model), or each lifetime component can be associated with its own environment, experiencing a particular rotation (i.e. the associative model). Here, in line with the studies mentioned, an adequate fit of the experimental data was achieved only by assuming the presence of two populations of probe molecules. Each population is characterized by its own lifetime (i.e. one of the two experimental fluorescence lifetimes), and a distinct restricted rotational motion. The resulting parameters (Table 3) indicate that the short lifetime is connected to a population of probe lipid molecules with a high mobility, and a low degree of order. The long lifetime component is coupled to a population with a reduced mobility, and a higher degree of order. It is to be noted that the short lifetime component resembles the probe lipids in vesicles, while the long lifetime component more resembles the situation in *Torpedo* membranes (see Table 2). Except for sarcolemmal membranes at 4 °C, the order parameter for the short lifetime component of the PI-probe is significantly higher than that of the PC-probe. In contrast, for the long lifetimes component the order parameter is lower for PI than for PC.

The various membrane preparations under investigation are characterized by a large variation in the acetylcholine receptor content. *Torpedo* membranes contain the highest level of receptor (i.e. approximately 15% of the membrane protein), while the receptor content of denervated and normal sarcolemma is approximately two and three orders of magnitude lower, respectively (Conti-Tronconi and Raftery 1982). The order parameter measured for PI in *Torpedo* membranes and for the long lifetime component of PI in normal sarcolemma is identical. Apparently the thousand-fold difference in the acetylcholine receptor content of these two membranes is not reflected in this parameter. These results demonstrate that even if the receptor has a specific affinity for PI, this cannot be detected by this technique. In the evaluation of the results one has to take into account that even in the *Torpedo* membranes, the receptor protein only represents 15% of the total membrane protein. Therefore,

any effect of the receptor on the fluorescent properties of the lipid probes, will be masked by a large fraction of the probe being at other locations.

Kinosita et al. (1981) have proposed a model to explain similar experiments with diphenylhexatriene and cytochrome C oxidase in model membranes. In this model they suggest that the protein acts as a rigid structure in the bilayer, without disturbing the lipids in the bilayer region. At the protein surface the amplitude of probe motion is restricted, while the average direction of the acyl chains may vary considerably owing to the irregular protein surface. ESR and fluorescence decay techniques in the ns time range are sensitive to the restriction in amplitude near the protein surface as reflected in a smaller cone angle (θ_c) and a higher order (S). The rate of motion (D_w, $D_\perp$), however, may not be very different from a pure lipid bilayer. With NMR, which operates on a much slower time-scale (μs), the contribution of exchange between the possible orientations at the protein surface and the bilayer lipids becomes important. This is the reason that NMR records a decrease in order upon introduction of membrane proteins (Oldfield et al. 1978; Ellena et al. 1986).

In general, our experiments are in agreement with the model of lipid-protein interactions described above. In the *Torpedo* membranes as compared to phospholipid vesicles, the order is increased (S is higher, θ_c is lower), while the rate of motion (D_w, $D_\perp$) is not very much affected with the possible exception of 2-parinaroyl-PI at 20 °C (Table 2). As one can see, in this case the rate of motion of PI is significant larger than that of PC. Differences between PC and PI in order parameters and diffusion constants, could indicate a preferential interaction with membrane proteins. Such preferential interactions with phospholipid classes must be dictated by the unique features of the polar headgroup. It is apparent from Tables 2 and 3 that these interactions do not hinder the mobility of the acyl chains to a great extent. This is in striking contrast to the interactions of the probe lipids with the phospholipid transfer proteins, in which the acyl chains are completely immobilized (Van Paridon et al. 1987; Berkhout et al. 1984).

In this study we have demonstrated that 2-parinaroyl-PI and 2-parinaroyl-PC in biological membrane preparations experience a higher degree of order than in phospholipid vesicles. Small differences in the degree of order and diffusion constants were interpreted to indicate that PI and PC are not equally distributed in the membrane. An inherent problem connected to the use of biological membranes in these fluorescence studies is the fact that any specific lipid-protein interactions (e.g. with the acetylcholine receptor) may be masked by the large fraction of unbound lipids. However, part of the problem is most likely resolved when time-resolved fluorescence spectroscopy is applied to better defined reconstituted systems.

This is the first report on the application of a fluorescent PI probe in the study of structure and dynamics of lipids in biological membranes.

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