Biochimica et Biophysica Acta, 642 (1981) 106—118

BBA 79156

THE RELATIONSHIP BETWEEN PLASMA MEMBRANE LIPID COMPOSITION AND PHYSICAL-CHEMICAL PROPERTIES

I. FLUORESCENCE POLARIZATION STUDIES OF FATTY ACID-ALTERED EL4 TUMOR CELL MEMBRANES

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(Received August 27th, 1980)
(Revised manuscript received November 11th, 1980)

Key words: Lipid composition; Phospholipid; Fluorescence polarization; Fatty acid; (Tumor cell membrane)

Summary

EL4 cells were cultured with exogenous fatty acids under conditions that resulted in their incorporation into membrane phospholipids. The behavior of the fluorescent lipid probes diphenylhexatriene and perylene was monitored in intact EL4 cells and in isolated EL4 plasma membranes. In whole cells substituted with unsaturated fatty acids, there was always a marked decrease in the *P* value of both probes compared to the *P* value of the probes in unsubstituted cells. In whole cells substituted with saturated fatty acids, on the other hand, *P* values for both probes were unchanged compared to unsubstituted cells. In plasma membrane isolated from EL4 cells, no difference in *P* values for either probe was observed among membranes from unsubstituted, saturated fatty acid substituted or unsaturated fatty acid substituted cells, even when the degree of fatty acid substitution was quite substantial. Most of the fluorescent signal for both probes in whole cells appeared to come from cytoplasmic lipid droplets. The value of techniques such as fluorescent polarization for monitoring physical properties of membranes (such as 'fluidity') is discussed.

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Introduction

The influence of plasma membrane lipid composition and physical properties on plasma membrane-associated cell functions has not been well studied. In recent experiments, we have shown that modifying the fatty acid compositon of the membrane phospholipids of murine cytotoxic effector T lymphocytes has profound effects on effector cell function [1,2]. Knowledge of the relationship between such fatty acid alterations and alterations in the physical state of the plasma membrane could be useful in interpreting the observed changes in biological function. Because effector cell populations are heterogeneous with respect to cell composition, physical studies carried out directly on such populations or on membranes derived from them, would be ambiguous. We have, therefore, turned to a homogeneous murine T cell tumor line, EL4, to study the relationship between plasma membrane lipid composition and physical properties as detected by small lipophilic probes. In this paper we show that extensive changes in the fatty acid composition of EL4 plasma membranes do not lead to physical changes that can be detected by alterations in behavior of the fluorescence probes diphenylhexatriene or perylene. In a subsequent paper, we will show that such fatty acid alterations do cause dramatic changes in the function of plasma membrane-associated enzyme systems. Possible explanations for the failure to observe physical alterations under conditions that induce profound biological and biochemical changes are discussed.

Materials and Methods

Isolation and culture of cells. The leukemic murine T cell line, EL4, was maintained in ascites form by serial passage in the peritoneal cavity of C57BL/6 retired breeders. To prepare cells for culture, the peritoneal cavity of a carrier mouse was rinsed several times with cold, sterile phosphate-buffered saline. The ascites cells were subsequently washed twice with cold phosphate-buffered saline and suspended to $1.5 \cdot 10^6$ cells/ml in an appropriate culture medium. When necessary, red blood cells were removed by suspension in isotonic NH_4Cl prior to washing the ascites cells with saline.

EL4 cells were in some cases cultured in 100-mm plastic culture dishes ($1.5 \cdot 10^6$/ml) in Dulbecco's modified Eagle's medium (GIBCO No. H-21, Grand Island, NY) containing $5 \cdot 10^{-5}$ M 2-mercaptoethanol plus 5% fetal calf serum. For fatty acid substitution, cultures were grown at the same concentration in the above medium supplemented with the desired fatty acid dissolved in C_2H_5OH. C_2H_5OH alone was added to control cultures. The final concentration of C_2H_5OH in the cultures was never more than 1%.

Spinner cultures of 3—7 l were also used to culture EL4 cells when large amounts of cells were required. A 5 or 9 l bottle with suspended magnetic stirrer and aeration funnel was used with essentially the same medium as described above, except that calf serum was substituted for fetal calf serum. Starting cell concentration was $1.5 \cdot 10^6$ cells/ml. Spinner cultures and plates were incubated at 37°C in a 5% CO_2 humidified atmosphere. Lipid-substituted cells were harvested after a 24 h exposure to fatty acid. Control cells were

cultured 24 h in the absence of exogenous fatty acid. Only cultures in which at least one cell number doubling occurred during 24 h were used for further experiments.

Membrane fractionation. Membrane fractions were prepared from normal (cultured) EL4 cells, and from EL4 cells supplemented with fatty acids. The cells were washed twice with cold saline and resuspended to 1% the original volume in cold 10 mM Tris-HCl, pH 7.4/0.2 mM $CaCl_2$ and kept on ice for 30 min. Cell homogenates were prepared by nitrogen cavitation, during which the cells were subjected to 125 lb/inch^2 N_2 for 10 min in a pressure vessel. The plasma membrane fraction was obtained by using the fractionation scheme described by Crumpton and Snary [3]. Briefly, the nuclei, mitochondria, and unbroken cells were removed by centrifugation at 6000 $\times g$. A microsomal pellet was then formed by centrifugation of the supernate at 160 000 $\times g$. The microsomal pellet was resuspended in 10 mM Tris-HCl buffer, pH 7.5, and placed atop a step gradient (36% sucrose in 10 mM Tris-HCl buffer, pH 7.5, overlaid with 25% sucrose in 10 mM Tris-HCl buffer, pH 7.5) and centrifuged at 86 000 $\times g$ in a Beckman SW-40 rotor for 18 h. Membrane fractions were recovered as described by Crumpton and Snary [3]. The protein in various fractions was determined by the method of Lowry et al. [30].

Fatty acid analysis. Analyses were performed on either whole cell membranes or on isolated plasma membrane fractions. Cells grown under the conditions described above for cell culture were washed twice with cold saline and counted with trypan blue. A minimum of $(1—3) \cdot 10^7$ cells were extracted per experiment. For plasma membrane, 0.5—1 mg protein equivalent of the plasma membrane preparation was extracted per sample. The lipids of the whole cells or isolated plasma membrane were extracted with 2 : 1 $CHCl_3$/CH_3OH as described by Bligh and Dyer [4]. After 10 min of extraction at 4°C, an equal volume of 1 : 1 cold $CHCl_3$ and water was added, with vortexing, and the sample was kept at 4°C for at least 10 min. The organic layer was removed, evaporated to dryness under nitrogen, and taken up in $CHCl_3$/CH_3OH, 2 : 1, for thin-layer chromatography. Phospholipids were resolved on thin-layer chromatography plates predeveloped with running solvent ($CHCl_3$/CH_3OH/NH_4OH, 65 : 35 : 10, as described by Wisnieski et al. [5]). Experimental samples were always run concurrently with phospholipid standards. The phospholipid standards were visualized by exposing them to I_2 vapors. Areas corresponding to phosphatidylcholine and phosphatidylethanolamine from the experimental samples were scraped from the dried thin-layer chromatography plates either separately or together, eluted with $CHCl_3$/CH_3OH, 2 : 1, and passed over glass wool to remove the silica. After evaporation under N_2, the phospholipids were transmethylated with boron trifluoride 14% in CH_3OH [5]. The methylated fatty acids were analyzed by gas chromatography, using either 6 ft $\times$ 1/8 inch columns of 10% SP-2340 at constant temperature (220°C), or using temperature programming from 175 to 230°C, with columns of 1% OV-275 on 100/120 mesh gas chrom Q (Supelco, Inc.). The fatty acids were identified by comparison with methylated standards. Peak areas and retention times were measured on a Hewlett-Packard Model No. 5830A gas chromatograph and integrator.

Sample preparation for fluorescence polarization. Samples of either whole

cells or plasma membrane preparations were prepared for fluorescence polarization measurements by the addition of perylene or 1,6-diphenyl-1,3,5-hexatriene. Perylene in C_2H_5OH was added to a final concentration of $1 \cdot 10^{-6}$ M to whole cells that had been washed 2 times with saline and resuspended at $2 \cdot 10^6$ cells/ml. Under these conditions the mole ratio of cell membrane lipid to perylene in the suspension was greater than 500, which according to Yguerabide and co-workers [6] eliminates the complicating effects of depolarization by excitation energy transfer between perylene molecules. The samples were allowed to incubate with gentle shaking for 60 min, which was determined to be sufficient for maximal fluorescence intensity. Measurements of the intensity of polarized fluorescence were then immediately made and corrected for machine background where necessary.

Plasma membrane preparations were treated differently. Approximately 100 μg protein equivalents of the plasma membrane preparation were brought to a final concentration of 2 μM perylene in a total volume of 100 μl. Assuming a 1 : 1 w/w protein to lipid ratio in the plasma membrane, the mole ratio of lipid to perylene in the suspension was greater than 500. The sample was incubated for 60 min as described with whole cells. However, the 100 μl was diluted to 2.5 ml at the end of the incubation time to give a final free background perylene concentration of less than $8 \cdot 10^{-8}$ M and 4 μg/ml labeled plasma membrane sample. Controls of free probe and unlabeled sample were also examined in all cases.

The preparation of diphenylhexatriene-labeled samples differed for whole cells from the above descriptions in the following way. A stock solution of $2 \cdot 10^{-3}$ M diphenylhexatriene in tetrahydrofuran was diluted 1/1000 in a vigorously stirred solution of saline. This was stirred for 30 min at room temperature to produce a stable diphenylhexatriene suspension, $2 \cdot 10^{-6}$ M diphenylhexatriene in saline. The solution was then bubbled with N_2 for 15—20 min to remove tetrahydrofuran which can be toxic to whole cells. The cells, suspended at $5 \cdot 10^6$/ml in saline, were then diluted 1 : 1 with diphenylhexatriene/saline and incubated as described above, giving a final diphenylhexatriene concentration of 1 μM in whole cell preparations.

Fluorescence measurements and instrumentation. Measurements of intensity of polarized fluorescence were made with a sensitive spectrofluorimeter designed by Juan Yguerabide (see Ref. 7). In order to avoid variations in P values caused by concentration-dependent differences in light scattering, samples were always analyzed at the same concentrations (cells/ml or mg membrane preparation/ml). The absolute amount of light scattering was reduced with the use of emission cutoff filters (Corning No. 3-72 filter for perylene and No. 3-70 filter for diphenylhexatriene). To reduce stray light, an interference filter was interposed between the sample and the excitation monochromometer (403 nm filter for perylene excited at 410 nm, and a 365 nm filter for diphenylhexatriene excited at 365 nm). The exciting light was polarized in a vertical direction and the emission was detected through a polarizer rotated 0° from the vertical ($I_\parallel$) or 90° from the vertical ($I_\perp$). The recording system gave digital readings of the values $I_\parallel$, $I_\perp$ and $R = I_\parallel/I_\perp$ as well as a chart tracing. A circulating waterbath controlled the temperature of the sample jacket and a thermoprobe correlated the temperature with the chart

tracing for $I_{\parallel}$ and $I_{\perp}$ for temperature gradient scans.

The degree of fluorescence polarization (P) was calculated according to the following equation:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the emission intensities measured through a polarizer oriented parallel and perpendicular, respectively, to the direction of polarization of the excitation light.

Fluorescence lifetime measurements were made at 37°C with an Ortec 9200 single-photon-counting ns spectrometer equipped with a high pressure hydrogen-filled flash lamp [8]. The excitation light was selected with a 405 nm interference filter (10 nm bandpass) for perylene, and a Corning 7-37 bandpass filter (360 nm peak) for diphenylhexatriene, and was vertically polarized. Fluorescence was detected through a Corning 3-72 cutoff filter in tandem with a polarizer rotated 55° from the vertical. Average fluorescence lifetimes were determined by fitting the raw data to a single exponential decay using the method of moments. In addition, the method of moments was used for deconvolution of the observed fluorescence decay fit to two exponentials [8]. The analysis was greatly improved by shifting the lamp profile to obtain a minimum chi-square for the convoluted fit. The shift was made using a PDP 11/45 computer or with a variable length decay line inserted between the photomultiplier anode and the time-to-amplitude converter. Mean deconvoluted lifetimes ($<\tau_D>$) were calculated according to the following equation:

$$\langle \tau_D \rangle = \frac{A_1\tau_1 + A_2\tau_2}{A_1 + A_2}$$

where the deconvoluted total fluorescence intensity decays as

$$S(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}$$

Results

Fluorescence polarization determinations on whole cells. EL4 cells were cultured overnight in the presence of exogenously added fatty acid. A control with no exogenous fatty acid added was run with each experiment. 24 h later the cells were harvested by centrifugation, washed with saline and the growth and viability determined by trypan blue exclusion. All experiments showed cell growth and the cells were usually 90—99% viable. The washed cells were labeled with the appropriate probe for 60 min and steady-state fluorescence polarization (P) measurements made immediately thereafter. Seven saturated fatty acids and seven unsaturated fatty acids (in both the *cis* and *trans* configuration) were tested for their effect on the fluorescence polarization of the two probes in fatty acid-substituted cells, as compared to the control cells.

Table I shows the results of these experiments. Saturated fatty acids sub-

TABLE I

WHOLE CELL FLUORESCENCE POLARIZATION

EL4 cells were cultured with the indicated fatty acid for 24 h at concentrations that had no grossly observable adverse effects on the cells. Cultured cells were harvested, washed, and exposed either to diphenylhexatriene or to perylene, as described in Materials and Methods. Fatty acid, fatty acid added to EL4 cultures. Percent in control, laboratory average for determinations of the representation of the indicated fatty acid in EL4 cell total membranes cultured 24 h in the absence of exogenous fatty acid. *n*, number of samples. Percent in sample, representation of the indicated fatty acid above the level found in control cells. P_E, polarization of probe in EL4 cells substituted with the indicated fatty acid. P_C, polarization of probe in unsubstituted EL4 cells.

Fatty acid	Melting point (°C)	Percent in control	*n*	Percent in sample	Perylene		Diphenylhexatriene	
					P_E	P_E/P_C	P_E	P_E/P_C
None		—	9	—	0.06 ± 0.004	1.0	0.188 ± 0.022	1.0
15 : 0	52	0	4	5	0.049 ± 0.004	0.82 ± 0.071	0.163 ± 0.027	0.86 ± 0.140
16 : 0	63	29	2	0	0.050	0.84	0.164	0.87
17 : 0	61	0	3	41	0.055 ± 0.004	0.91 ± 0.062	0.169 ± 0.081	0.90 ± 0.171
18 : 0	70	28	5	9	0.055 ± 0.007	0.92 ± 0.120	0.148 ± 0.024	0.79 ± 0.125
19 : 0	69	0	5	36	0.056 ± 0.007	0.94 ± 0.124	0.176 ± 0.010	0.94 ± 0.052
20 : 0	73	0	4	21	0.057 ± 0.002	0.96 ± 0.034	0.179 ± 0.015	0.95 ± 0.007
22 : 0	80	1	4	41	0.060 ± 0.003	0.99 ± 0.051	0.175 ± 0.011	0.93 ± 0.062
16 : 1_{cis}	0.5	0	4	16	0.043 ± 0.004	0.71 ± 0.069	0.107 ± 0.007	0.57 ± 0.034
16 : 1_{trans}	30	0	2	8	0.048	0.79	0.134	0.72
18 : 1_{cis}	16	31	4	19	0.045 ± 0.004	0.74 ± 0.071	0.111 ± 0.007	0.59 ± 0.036
18 : 1_{trans}	44	0	3	53	0.037 ± 0.007	0.62 ± 0.115	0.117 ± 0.012	0.62 ± 0.034
18 : 2_{cis}	−6	4	2	31	0.039	0.65	0.112	0.60
18 : 2_{trans}	29	0	4	52	0.042 ± 0.003	0.71 ± 0.048	0.101 ± 0.009	0.54 ± 0.048
18 : 3_{cis}	−12	0	4	17	0.043 ± 0.007	0.71 ± 0.122	0.105 ± 0.010	0.56 ± 0.054

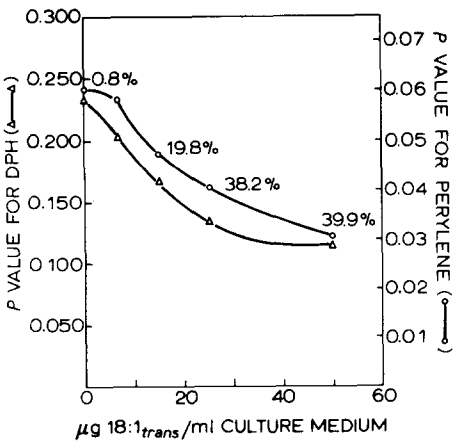
stituted into the cells do not seem to alter the P values significantly from the control for either probe, regardless of the chain length of the substituting fatty acid or the degree of fatty acid incorporation over control phospholipid fatty acid content. For instance, 17 : 0, ordinarily not found in phospholipids of control (unsubstituted) EL4 membranes, can be substituted into the whole cell membranes so as to represent about 40% of the total phospholipid fatty acids. Nevertheless, the P_E (polarization of probe in experimental cells) remains 90—92% of the P_C (control P values). The saturated fatty acid 16 : 0 for some reason is not substituted into whole cell membranes, above the level found in control cells, to any discernable degree; it also produced a P_E value which is 83—87% of the P_C. $P_E : P_C$ ratios in general were between 0.9 and 1.0. It would appear, therefore, that saturated fatty acid substitution does not significantly alter the fluorescence polarization of probe in fatty acid-altered cells, compared to unsubstituted cell values, regardless of the degree of substitution.

Unsaturated fatty acids, however, substantially decrease the P values of both probes from those found in unsubstituted cells. The $P_E : P_C$ values for most unsaturated fatty acid-substituted cells were between 0.6—0.7 for diphenylhexatriene, and between 0.5—0.6 for perylene. The fatty acid 16 : 1_{trans} could only be substituted into whole cell membranes until it represented 8—10% of the total phospholipid fatty acids. This low representation of 16 : 1_{trans} in the substituted cells seems to be reflected in the highest P_E/P_C value for the unsaturated fatty acids studied. Other unsaturated fatty acids that can be substituted into membrane phospholipids to a higher level cause much lower P_E/P_C values. There thus seemed to be a correlation between the amount of unsaturated fatty acid substituted into the cell, and the drop in the fluorescence polarization value as compared to that of the control.

To test this further, a dose kinetics study was made on the dependence of 18 : 1_{trans} fatty acid substitution on the fluorescence polarization using both diphenylhexatriene and perylene as probes. The results are shown in Fig. 1. Each point on the curve represents a cell suspension grown with varying amounts of exogenous 18 : 1_{trans} added to the culture media. As the percentage of 18 : 1_{trans} increases compared to the total phospholipid fatty acid profile analyzed by gas chromatography, the P value decreases. This effect was consistent with either diphenylhexatriene or perylene as the probe. The percentage value shown by each point on the graph reflects the percentage of 18 : 1_{trans} phospholipid fatty acid as compared to the total phospholipid fatty acid in that experiment, and ranges from 0.8% to about 40%.

Surprisingly, the values for substituted unsaturated fatty acids in Table I do not seem to show a difference between *cis* and *trans* unsaturated bond configurations, despite the fact that *cis* and *trans* fatty acids have vastly different melting properties in synthetic membranes. Furthermore, if there are any subtle differences due to carbon chain length, they are difficult to discern due to the difficulty in substituting the cells to the same degree with the various fatty acids studied.

Fluorescent visualization of probe-labeled cells. In order to monitor directly the uptake of probe by EL4 cells, visualization of fluorescent probe-labeled cells was carried out using a Universal Zeiss microscope equipped with an ultraviolet light source and ultrasensitive silicon intensified video camera



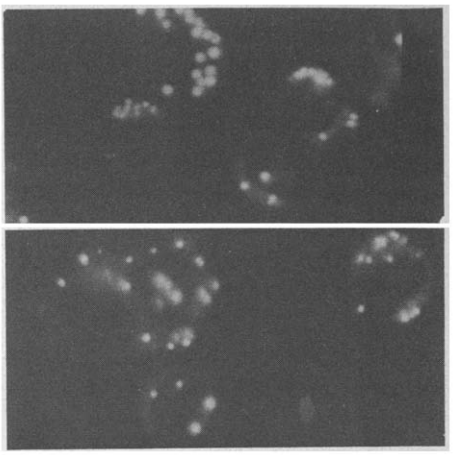


Fig. 1. The fluorescence polarization of the probes diphenylhexatriene (△——△) and perylene (○——○) as a function of the amount of 18 : 1_{trans} added to the EL4 whole cell culture medium. The actual uptake of exogenous 18 : 1_{trans} fatty acid incorporated into the phospholipids of the membrane was analyzed by GLC and is represented as the percent value written by each data point.

Fig. 2. Fluorescence micrograph of normal EL4 cells incubated with diphenylhexatriene as described in Materials and Methods. Identical figures are seen when the cells are incubated with perylene.

and recorder. Perylene- and diphenylhexatriene-labeled cells were prepared as described in Materials and Methods. Fresh cells taken directly from the peritoneal cavity of the carrier mouse, as well as cultured fatty acid-substituted and unsubstituted cells, were mixed with probe on a microscope slide and recorded on video tape so that interaction of the cells with the probe could be monitored as a function of time. It was found that both perylene and diphenylhexatriene internalized into all cells rapidly. The process of label incubation and preparation of the cell samples for fluorescence polarization measurements as described in the preceeding section required minimally 1.5 h. Visualization of the dynamics of the internalization process showed that both probes had clearly internalized during this time. In fact some internalization could be seen within 2 min for perylene and 5 min for diphenylhexatriene.

More significant, however, was the fact that all the EL4 cells tested, whether fresh or cultured, substituted or not, displayed intense areas of label immediately under the plasma membrane (Fig. 2). These areas were round, droplet-like concentrations of probe which in some cases were so intense that they clearly contributed a major portion of fluorescent signal from that individual cell, and would presumably also contribute a major portion of the signal from a labeled cell suspension. No quantitative comparisons between normal and fatty acid-substituted cells were possible; both, however, had extensive visible droplets. The actual identification of substance or properties of these droplets was not made; they could be free or lysosome-associated oil or fat droplets.

It thus became important to ask whether the differential effect on *P* values seen when the cells were substituted with saturated versus unsaturated fatty acids is in fact related to changes in plasma membrane properties. We have

addressed this question by measuring fluorescence polarization in isolated plasma membrane preparations.

Fluorescent polarization measurements of plasma membrane-enriched preparations. A study of the effects of exogenous fatty acid substitution on the polarization of diphenylhexatriene and perylene in membranes was made using plasma membrane-enriched preparations from normal and fatty acid-altered EL4 cells. The study included control, seven saturated and seven unsaturated fatty acid cell membrane preparations, identical to those screened in the whole cell study.

Plasma membrane enriched preparations were made according to the procedure in Materials and Methods. Phospholipids purified by thin-layer chromatography were analyzed by gas chromatography to determine the extent of fatty acid substitution and the phospholipid fatty acid profile. Plasma membrane preparations could be stored at −70°C and remain stable over long periods of time (repeatable results are still obtainable from frozen preparations which are over 1 year old). A 100 μg (protein weight) sample of each preparation was used for both fluorescent polarization measurement and fluorescence lifetime (τ) measurements. This quantity produced an adequately strong signal.

Fluorescence polarization values of probes in the plasma membrane preparations are shown in Table II. The differential effect seen with saturated vs. unsaturated fatty acid in the whole cell study was completely lost when isolated plasma membranes were analyzed. No significant difference can be seen between the fluorescence polarization values of the control and that of any of the substituted membranes.

Fluorescence lifetime measurements of normal and fatty acid-modified EL4 plasma membrane. The average excited-state lifetimes ($<\tau>$) of perylene

TABLE II

FLUORESCENCE POLARIZATION MEASUREMENTS OF PLASMA MEMBRANE PREPARATIONS

Procedure and definitions are the same as for Table I, except that plasma membrane was isolated from control and substituted EL4 cells, and all measurements were carried out on the purified plasma membrane preparations as described in Materials and Methods.

Fatty acid	Percent in control	n	Percent in sample	Perylene		Diphenylhexatriene	
				P_E	P_E/P_C	P_E	P_E/P_C
Control	—	6	—	0.098 ± 0.003	1.0	0.255 ± 0.008	1.0
14 : 0	2.6	2	8.6	0.099	1.01	0.246	0.97
15 : 0	0	2	17.4	0.098	0.99	0.232	0.91
17 : 0	0	3	31.5	0.098 ± 0.002	1.00 ± 0.017	0.266 ± 0.011	1.04 ± 0.08
18 : 0	30.6	2	6.7	0.104	1.06	0.236	0.93
19 : 0	0	8	16.5	0.097 ± 0.012	0.99 ± 0.11	0.255 ± 0.009	1.00 ± 0.034
20 : 0	0	2	14	0.105	1.07	0.277	1.09
22 : 0	0	2	21.5	0.099	1.01	0.274	1.08
16 : 1*cis*	0	2	11.4	0.092	0.94	0.256	1.00
18 : 1*cis*	25	3	22	0.096 ± 0.002	0.98 ± 0.015	0.214 ± 0.031	0.85 ± 0.12
18 : 1*trans*	0	3	40.3	0.095 ± 0.014	0.97 ± 0.14	0.247 ± 0.022	0.97 ± 0.10
18 : 2*cis*	10.4	6	17.5	0.101 ± 0.010	1.03 ± 0.10	0.261 ± 0.018	1.02 ± 0.07
18 : 2*trans*	0	6	36.5	0.104 ± 0.014	1.06 ± 0.015	0.259 ± 0.026	1.02 ± 0.10
18 : 3*cis*	0	1	—	0.121	1.23	0.275	1.08

TABLE III

AVERAGE FLUORESCENCE LIFETIMES OF PERYLENE AND DIPHENYLHEXATRIENE IN EL4 PLASMA MEMBRANE PREPARATIONS AT 37°C

Fatty acid	Lifetime measurements (ns) *			
	Perylene		Diphenyl hexatriene	
	$\langle\tau_D\rangle$	$\langle\tau_R\rangle$	$\langle\tau_D\rangle$	$\langle\tau_R\rangle$
None	5.7	7.6	8.4	10.0
17 : 0	5.0	7.4	7.4	9.6
18 : 0	5.2	7.5	7.8	9.6
19 : 0	5.4	7.5	7.6	9.4
11 : 0	5.8	7.8	8.4	10.0
16 : 1	5.0	7.3	8.5	10.3
16 : 1	5.7	7.8	8.3	10.1
18 : 1	5.5	7.7	7.8	9.8
18 : 1	5.7	7.8	8.0	9.8
18 : 2	5.6	7.7	8.2	9.9

* $\langle\tau_D\rangle$, deconvoluted values; $\langle\tau_R\rangle$, raw values.

and diphenylhexatriene probes embedded in normal and fatty acid-enriched plasma membrane preparations were measured at 37°C as described in Materials and Methods. As shown in Table III, no large changes in $<\tau>$ were observed for either probe. In fact, the majority of fatty acid-substituted membrane samples had $<\tau>$ values within 0.4 ns of the normal (unsubstituted) preparations. As expected, deconvoluted lifetimes ($<\tau_D>$) were consistently lower than raw values ($<\tau_R>$).

Discussion

This study was undertaken to gain insight into how alteration of plasma membrane phospholipid fatty acid composition affects cell membrane physical properties. We have shown in other studies that such alterations can have profound effects on the biological function of cytotoxic effector T cells [1,2]. In a subsequent paper, we will show that fatty acid alterations also affect the biochemical properties of plasma membrane-associated enzymes. However, we conclude from the work presented in this paper that such alterations, even when quite extensive, do not significantly affect the properties or behavior of fluorescent lipid probes embedded in isolated plasma membranes. In parallel with these studies, we have found that these types of alterations also fail to alter the behavior of spin-labeled lipid probes detectable by electron spin resonance spectroscopy (Poon, R. and Clark, W., unpublished results). This does not mean that the physical properties of the membranes are not altered; only that, using the kinds of probes and physical techniques described in this paper, on isolated, highly purified EL4 plasma membrane, we cannot detect any alteration.

On the other hand, we did observe striking alterations in the behavior of probes added to fatty acid-altered whole cells. Probe polarization in cells cultured in the presence of saturated fatty acid showed a slight trend toward increasing P values for both diphenylhexatriene and perylene with

increasing chain length of the substituting fatty acid. Probe polarization in whole cells substituted with unsaturated fatty acids was uniformly lower, regardless of chain length, degree or configuration of double bonds, or physical properties of the fatty acid in the free or phospholipid-associated form. Why the effect of fatty acids on probe behavior should fall into these two categories is unclear. What is clear, however, is that the effects of fatty acid on probe behavior seen in whole cells are not due to alterations in the properties of the cell plasma membrane, since the effects entirely disappear when plasma membrane is studied in isolation. We feel that the effects seen in whole cells are most likely due to probe sequestered in the intracellular droplets shown in Fig. 2.

Various other recent studies have pointed to the problems inherent in using fluorescent probes in studies with whole cells, if the object of the analysis is the physical state of the plasma membrane. Van Hoeven et al. [9] and Van Blitterswijk et al. [10] have compared fluorescence polarization of diphenylhexatriene in whole cells and their isolated plasma membranes. They found, as we do, that P values for diphenylhexatriene in plasma membrane are substantially higher than for diphenylhexatriene in the corresponding whole cell. They concluded that fluorescence polarization measurements made on whole cells do not give reliable information about the physical state of the plasma membrane, most likely because the signal measured represents an average of the behavior of the probe in many different cell compartments, including intracellular lipid droplets. It has been shown that at least diphenylhexatriene is capable of penetrating into internal cell compartments [11], and our visual observations of cells incubated with either diphenylhexatriene or perylene suggest that a major portion of the signal in both cases may be contributed by probe concentrated in cytoplasmic fat droplets. Esko et al. [12] and Pessin et al. [13] also found conflicting results when measuring P values with diphenylhexatriene in whole cells vs. that in plasma membrane. Careful analysis showed that in intact cells the probe was localizing in cytoplasmic fat droplets containing triacylglycerides and alkyldiacylglycerides. In our studies, a comparison of the P values obtained for diphenylhexatriene and perylene in intact EL4 cells substituted with various fatty acids suggests that the P values correlate at least roughly with the melting points of the fatty acids in the free form (Table I). We thus also feel that fluorescence polarization measurements made on whole cells using diphenylhexatriene and perylene do not give accurate information about the physical state of the plasma membrane.

A number of papers have been written about the influence of phospholipid fatty acyl groups on the fluidity of eukaryote cell membranes. Gilmore et al. [14] added exogenous $18 : 2_{cis}$ to LM cells in culture, and showed that the microviscosity of the plasma membrane decreased in comparison to cells substituted with choline or ethanolamine. However, it is difficult to compare our study with theirs since they showed no data for plasma membrane from unsubstituted cells. A series of studies in *Tetrahymena* [15—17] have shown that changes in growth temperature induce changes in phospholipid fatty acid profiles, presumably to maintain an appropriate membrane fluidity at the new growth temperature. The presumed fluidity alteration induced by temper-

ature shifts, and its restoration to 'normal' by endogenous fatty acid alterations, were assessed by the distribution of intramembranous particles seen in freeze-fracture electron micrographs. The spectroscopic behavior of lipid probes was not studied in this system. Temperature-induced alterations in fatty acid composition have also been related to membrane fluidity in fish [18,19] amphibians [20], and even mammals [21]. However, in none of these cases is it clear that there is a direct correlation between membrane fluidity, at least as measured by techniques such as fluorescence polarization, and phospholipid fatty acid composition. Either fluorescence polarization (or ESR) measurements were not made, or changes in membrane lipid composition other than fatty acids, that could influence fluidity, were not assessed. Fatty acid-induced alterations in headgroup composition [12], and cholesterol : phospholipid ratio [22], for example, could both be expected to affect membrane physical properties. Temperature-induced alterations in fatty acid composition might be necessary to maintain critical microenvironments around membrane proteins, but it is not obvious that they would affect bulk membrane properties as measured with small lipid probes.

Thus it is not entirely clear that fatty acid alterations of phospholipids can be expected to alter those properties of real cell membranes detected by diphenylhexatriene and perylene. Axelrod et al. [23] showed that introduction of substantial amounts of 18 : 1 into cell membranes had no measurable effect on the diffusion coefficient of a membrane integral protein. The distribution of the same exogenous fatty acid into different phospholipids, and different positions within different phospholipids, will have different impacts on the thermotropic properties of the membrane [24,25]. Introduction of unnatural amounts of a given exogenous fatty acid into the membrane may trigger compensating changes in endogenous lipid classes such that the overall properties of the membrane as measured by these probes remain constant, much as has been postulated for the temperature-induced compositional changes described above.

It is also possible that diphenylhexatriene and perylene localize preferentially in defined lipid regions of the membrane, such as phase-separated lipid domains or protein-associated annular lipids, that are not markedly changed by the kinds of fatty acid alterations induced in the present study. Foster and Yguerabide [26] showed that perylene distributes equally between phase-separated domains in vesicles composed of mixtures of dipalmitoyl phosphatidylcholine and distearoyl phosphatidylethanolamine. Nevertheless, the structural complexity of biological membranes is considerably greater, and the partitioning behavior of lipid probes may also be more complex. Lentz et al. [24] showed that even in defined vesicle systems, unsaturated fatty acids in particular tend to phase-separate fairly readily. In addition, they tend to lower the activation energy of microviscosity at temperatures above their phase transition, which would have the effect of modulating theoretically inducible changes in fluidity over a wide range of temperatures. Hildenbrand and Nicolau [27] on the basis of a very careful study of ns fluorescence anisotropy decay of diphenylhexatriene in a variety of natural and synthetic membranes, concluded that when differences in steady-state anisotropy values are observed, they represent changes in static motion of the probe rather than

changes in diffusion. Mely-Goubert et al. [28] similarly concluded that diphenylhexatriene does not measure fluidity per se, but rather restrictions in the motion of the probe caused by interactions with hydrophobic regions of the bilayer, including proteins and their annular lipids.

In summary, we find that extensive alterations in the fatty acyl composition of EL4 plasma membrane phospholipids, while significantly perturbing the biological and biochemical properties of plasma membrane-associated activities, do not affect the polarization or lifetime of the fluorescent probes diphenylhexatriene and perylene. We feel that great caution should be employed in utilizing such probes in an attempt to assess the physical properties of complex biological membranes, and especially in correlating biological and biochemical properties of membranes with inferred physical properties such as 'fluidity'.

(During the processing of this manuscript, an article appeared by Stubbs et al. [29] that reaches conclusions essentially identical to those presented here.)

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