Biochimica et Biophysica Acta, 689 (1982) 230–240
Elsevier Biomedical Press

BBA 71217

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

III. DETAILED PHYSICAL AND BIOCHEMICAL ANALYSIS OF FATTY ACID-SUBSTITUTED EL4 PLASMA MEMBRANES *

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(Received November 11th, 1981)
(Revised manuscript received March 1st, 1982)

Key words: Lipid composition; Fatty acid substitution; ESR; (Plasma membrane)

Murine leukemia EL4 cells were modified by supplementation of culture media with fatty acids for 24 h. A plasma membrane-enriched fraction was prepared from substituted and normal cells. Analyses were performed to determine fatty acyl composition, phospholipid headgroup composition and cholesterol content. The two major membrane phospholipids, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were isolated by thin-layer chromatography and ESR measurements were done on liposomes prepared from these lipids as well as on the intact plasma membrane preparations. Slight perturbations in overall plasma membrane lipid composition were observed when EL4 cells were supplemented with a single exogenous fatty acid. This may be consistent with the idea that the incorporation of exogenous fatty acid induces compensatory changes in membrane lipid composition. On the other hand, we observed no significant difference in two ESR motional parameters between the unsubstituted control and various fatty acid-substituted plasma membranes. ESR measurements carried out on PE and PC liposomes derived from 17:0- and 18:2_c-substituted membranes also failed to detect major differences between these liposomes and those made from normal EL4 phospholipids. In the case of liposomes prepared from 18:2_t-substituted membranes, the order parameter was significantly changed from the normal. However, the change was in opposite directions in PE and PC, perhaps accounting for the fact that no change in order parameter is seen in intact 18:2_t-substituted plasma membrane. Measurements of order parameter (S) in mixed lipid vesicles showed that at up to 50 mol% mixture of a synthetic PC with plasma membrane PC, the value of S was only marginally different from that of the plasma membrane PC vesicles. We interpret these data as an indication that the two ESR parameters used are not sufficiently sensitive to detect changes due to modifications of the acyl chain composition of a complex biological membrane.

* For Part II see Ref. 4.

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Abbreviations: DSC, differential scanning colorimetry; ESR, electron spin resonance; LysoPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; S, order parameter; τ_0, approximate rotational correlation time; 17:0, heptadecanoate; 18:0, stearate; 18:1_c, oleate; 18:1_t, elaidate; 18:2_c, linoleate; 19:0, nonadecanoate.

0005-2736/82/0000–0000/$02.75

Introduction

We previously reported that alteration of the fatty acid composition of membrane phospholipids altered the rate of patching of cell surface antigens [1], and the cytotoxic function of alloimmune T lymphocytes [2,3]. Subsequently, we found a similar pattern of fatty acid effects on plasma membrane-restricted enzyme activities [4]. However, no corresponding change in plasma membrane physical properties, as measured either by fluorescence polarization [5] or electron spin resonance (ESR) spectroscopy [4] could be demonstrated. With both techniques it was found that in spite of the incorporation of exogenously supplied fatty acid to a substantial degree (up to 50% of total phospholipid fatty acid in some cases) motional parameters of probes embedded in altered plasma membranes stayed within a very narrow range of the values for probes embedded in normal control membranes. These observations suggested either that membrane physical properties are highly regulated, and able to remain within a fairly narrow range in spite of a modified supply of exogenous lipids, or that the physical techniques we used for measuring membrane 'fluidity' were not of sufficient sensitivity to detect induced changes, given the compositional heterogeneity and organizational complexity of the plasma membrane.

The studies in this paper were initiated in an attempt to better understand the difference effected by the incorporation of fatty acids on biological and biochemical properties of real cell membranes, and on physical properties as measured by ESR and fluorescence polarization. Quantitative analyses were done on headgroup composition of plasma membrane phospholipids; fatty acid composition of phosphatidylethanolamine (PE) and phosphatidylcholine (PC); and cholesterol content relative to total phospholipids. The goal of these studies was to determine whether the effect brought about by the incorporation of the exogenous fatty acid might be offset by changes in other elements in the lipid composition of the membranes. In addition, ESR motional parameters were measured in PE and PC liposomes derived from normal and substituted plasma membranes, to determine whether changes in the physical properties of these two major phospholipid groups could be detected in the absence of other membrane lipids.

Materials and Methods

Materials

14% boron trifluoride/methanol esterification kits were obtained from Applied Sciences. Fatty acids used as culture supplements and fatty acid methyl esters for gas chromatography reference standards were from NuChek Prep, Inc. Lipid mixtures for TLC standards were obtained from Supelco. All organic solvents were Mallinckrodt chromatographic grade. The two ESR probes, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (5-NS) and 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl (12-NS), were obtained from Syva Research Chemicals.

Cell culture and plasma membrane preparation

The murine T cell leukemia EL4 was maintained by serial passage in the peritoneal cavity of C57BL/6 mice.

Fatty acid supplementation and culture conditions have been described previously [5]. Fatty acid concentrations during the 24 h culturing period were 20 μg/ml 17:0, 20 μg/ml 19:0, 20 μg/ml $18:1_t$, 35 μg/ml $18:2_c$ and 50 μg/ml $18:2_t$. These dosages were chosen to give maximum incorporation of the fatty acid while maintaining a minimum viability of 95%. The fatty acids were added to the spinner cultures as ethanol solutions. The final concentration of ethanol was less than 1%. Starting cell concentration was $(1.0–1.5) \cdot 10^6$ cells/ml. Following culture, EL4 cells were disrupted either by nitrogen cavitation at a pressure of 125 lb/inch^2 for 15 min or by two passages through a Stansted Cell Disruptor using a forward pressure of 40 lb/inch^2 and a back pressure of 32 lb/inch^2 [4]. Plasma membrane was isolated using essentially the fractionation scheme of Crumpton and Snary [6] and was stored in 10 mM Tris-HCl, pH 7.4, under nitrogen at −20°C until use.

Lipid separation and analysis

The extraction method described by Bligh and Dyer [7] was followed. The organic phase was

removed, dried under nitrogen and redissolved in a small amount of chloroform/methanol (2:1, v/v). Phospholipids were separated by thin-layer chromatography using a developing solvent of chloroform/methanol/ammonia (65:35:10, v/v) as described by Wisnieski et al. [8]. The bands corresponding to PE and PC were identified against phospholipid standards included on the same plate with the experimental sample. The standards (only) were visualized by exposure to iodine vapors. PE and PC were eluted from the silica gel, using chloroform/methanol (2:1, v/v), and used either for spin label studies or transmethylated with boron trifluoride for analysis of fatty acid composition. The latter was done by gas-liquid chromatography as described previously [5].

Separation of all major phospholipid classes was by one-dimensional thin-layer chromatography using the solvent described by Skipski and Barclay [9] (chloroform/methanol/acetic acid/water (25:15:4:2, v/v)). Identification was made by comparison with commercial TLC standards aided by the use of a rat liver lipid extract and the R_F values reported by those authors [9]. The separated phospholipids were eluted from the silica gel. Quantitation of each phospholipid class was done by determination of phospholipid phosphate using an ashing method [10]. Cholesterol content was measured directly on the Bligh-Dyer extract according to the method of Bowman and Wolf [11].

ESR measurements and sample preparation

The ESR probe molecules dissolved in ethanol at 10^{-2} M were added to plasma membrane samples directly. Sample handling and ESR instrumentation were the same as described previously [4].

The chromatographically separated PE or PC was dried down under nitrogen in 6×50 mm Kimble tubes. 100 μl of 10 mM Tris-HCl buffer was added to each dried sample. The tubes were flushed with nitrogen, corked and sonicated in a sonicator bath for two periods of 7 min each. The temperature of the bath did not rise above 40°C during sonication. The aqueous dispersion of PE and PC were used for ESR measurements as described above for plasma membrane samples, unless otherwise indicated.

For mixed lipid vesicles, the plasma membrane- or microsome-derived lipids and synthetic lecithin (Supelco) were mixed together in organic solvent in the appropriate proportions. The mixture was dried down under nitrogen and sonicated to clarity in 0.5–1.0 ml 10 mM Tris-HCl buffer, pH 7.4, with a standard microtip. When necessary, the vesicles were concentrated by pelleting in a Beckman airfuge and the buffer volume reduced before resuspension. Samples were prepared from these vesicles for ESR studies as described above. Vesicles of pure synthetic lecithin, EL4 plasma membrane PC or microsomal lipids were also sonicated using a microtip for comparative studies in the same experiment.

Two motional parameters were calculated according to the following equations:

$$\text{order parameter } (S) = \frac{T'_{\parallel} - T'_{\perp}}{T_{zz} - T_{xx}}$$

where 2 $T'_{\parallel}$ and 2 $T'_{\perp}$ are the separations between the outer and inner hyperfine extrema on the 5-NS spectra [12], and where $T_{zz} = 32$ gauss and $T_{xx} = 6$ gauss. Approximate rotational correlation time

$$(\tau_0) = \left[(h_0/h_{-1})^{1/2} - 1\right] W_0 \kappa$$

where h_0 and h_{-1} are the heights of the mid- and highfield lines, respectively, and W_0 is the width of the midfield line in gauss of the 12-NS spectra [13]. κ is the constant ($6.5 \cdot 10^{-10}$) calculated from crystal parameters.

Results

Phospholipid composition of fatty acid substituted plasma membranes

The ratios of different phospholipid classes present in normal and fatty acid-substituted plasma membrane are presented in Table I. In all cases PE and PC together made up the bulk of the membrane phospholipids, about 80% for normal, 19:0- and 18:1_t-substituted plasma membranes and slightly lower, approx. 75%, for 17:0-, 18:2_c- and 18:2_t-substituted plasma membranes. In the latter group the increase in the minor phospholipid classes was mainly in phosphatidylinositol (PI). The PE to PC ratio was not significantly changed

TABLE I

PHOSPHOLIPID COMPOSITION OF NORMAL AND FATTY ACID-SUBSTITUTED PLASMA MEMBRANES

Normal and fatty acid substituted EL4 plasma membranes were extracted by the method of Bligh and Dyer [7]. The different classes of phospholipids were separated by thin layer chromatography using the solvent described by Skipski and Barclay [9]. The separated phospholipids were quantitated as μmol of phospholipid phosphate. The data is expressed as % of total phospholipids. Numbers in parentheses indicate the number of different samples tested. Sph, sphingomyelin.

Lipid analyzed	Fatty acid used in culture supplement					
	None (5)	17:0 (4)	19:0 (3)	18:1_t (3)	18:2_c (3)	18:2_t (3)
PE	34.8 ±8.1	44.7 ±1.3	31.5 ±3.0	38.3 ± 1.8	38.7 ±5.6	35.9 ±6.4
PS	1.4 ±0.6	1.8 ±0.9	2.0 ±1.9	1.7 ± 0.6	1.0 ±0.1	1.1 ±0.9
PI	5.7 ±2.7	7.0 ±2.5	5.6 ±2.7	6.6 ± 1.8	9.7 ±1.4	8.5 ±5.3
PC	45.6 ±5.5	37.6 ±4.7	51.7 ±5.9	42.2 ±22.6	37.9 ±5.5	40.6 ±5.1
Sph+LysoPC	11.1 ±3.3	11.8 ±1.4	9.3 ±2.6	11.1 ± 0.9	12.7 ±2.7	13.8 ±4.7
PE:PC	0.76±0.13	1.20±0.16	0.62±0.13	0.91± 0.09	1.05±0.31	0.89±0.18

for 19:0-, 18:1_t-, 18:2_t- and 18:2_c-substituted membranes. The increase in PE:PC was real for 17:0-substituted plasma membrane at a 0.05 level of confidence in the *t* test.

Cholesterol content of fatty acid substituted plasma membranes

The total cholesterol contents of normal and fatty acid substituted membranes were normalized to total membrane phospholipid (Table II). Statistical analysis of the data, using the T test, showed no significant difference, at a 0.05 level of confidence, between the unsubstituted plasma membrane and any experimental group.

TABLE II

PLASMA MEMBRANE CHOLESTEROL CONTENT

A Bligh Dyer extract [7] was made on normal and fatty acid substituted EL4 plasma membrane. The cholesterol content of the extracts was determined by the method of Bowman and Wolf [11]. C/PL, cholesterol to total phospholipid ratio.

Fatty acid	No. of expts.	C/PL (mean ± S.D.)
None	9	0.69±0.13
17:0	7	0.61±0.13
19:0	10	0.64±0.10
18:1_t	6	0.69±0.07
18:2_c	9	0.68±0.11
18:2_t	9	0.81±0.18

Fatty acyl composition of plasma membrane phospholipids, PE and PC

The fatty acyl compositions are presented separately for PE and PC in Tables III and IV. The degree of incorporation is substantial for all five fatty acids studied, ranging from 11% of total phospholipid fatty acid in 19:0-substituted, to 50% in the PE of 18:2_t-substituted membranes. Except for 19:0, the degree of incorporation into PE and PC was generally unequal, being higher in PE for 18:1_t and 18:2_t, and lower in PE for 17:0 and 18:2_c.

Incorporation of any fatty acid into PE appeared principally to have an effect on the level of the C_{18} acyl chains (18:0 and 18:1_c) lowering either one (18:2_c-substituted) or both (17:0-, 19:0-, 18:1_t- and 18:2_t-substitution). 18:1_t and 18:2_t fatty acids, in addition, slightly decreased the level of 18:2_c acyl chains in PE while 17:0 increased it somewhat. Some minor changes were also seen in the C_{20} unsaturated acyl chains in PE for most fatty acid supplements used.

The incorporation of an exogenous fatty acid into PC, as for PE, was largely acccomodated by reduced levels of 18:0 and 18:1_c acyl chains. 18:1_t and 18:2_t fatty acids also lowered the percent of 18:2_c acyl chains in PC while 17:0 slightly increased it as was seen in PE. 17:0-, 19:0- and 18:1_t-substitution also moderately decreased the level of 16:0 acyl chains in PC. 17:0-, 19:0-, and 18:1_t-substitution induced detectable levels of

TABLE III

FATTY ACYL COMPOSITION OF PLASMA MEMBRANE PHOSPHATIDYLETHANOLAMINE

Phosphatidylethanolamine was separated from other plasma membrane phospholipids by thin-layer chromatography as described by Wisnieski et al. [8]. After elution from the silica gel, the phosphatidylethanolamine was transmethylated with a mixture of 14% boron trifluoride in methanol (Applied Sciences, Inc.) The methylated fatty acid esters were separated by gas liquid chromatography [5] and identified by comparison with methylated standards. Peak areas and retention times were measured on a Hewlett-Packard Model No. 5830A gas chromatograph and integrator. Data is presented as percent of total phospholipid fatty acid. Degree of unsaturation was calculated as Σ (% unsaturated acyl chain × number of double bonds). Numbers in parentheses indicate the numbers of samples run.

Fatty acid analyzed	Fatty acid used in culture supplement					
	None (11)	17:0 (5)	19:0 (4)	18:1_t (3)	18:2_{cis} (8)	18:2_t (6)
14:0	0.77±0.5	0.7±0.6	1.0±0.7	3.0±2.9	0.5±0.2	0.4±0.1
15:0						
16:0	11.0±4.3	5.6±0.9	12.8±2.6	8.4±4.7	10.9±1.8	8.7±1.5
16:1_c			0.7±1.4			
17:0		19.9±4.4				
18:0	29.6±4.3	19.4±0.8	18.5±1.8	11.5±4.0	30.2±5.7	16.4±5.6
18:1_c	29.4±4.3	21.0±0.1	19.7±3.4	16.0±4.4	14.8±3.0	14.3±2.9
18:1_t				45.7±7.2		
18:2_c	11.0±2.4	16.1±4.0	13.7±0.8	6.5±5.0	29.2±4.8	7.0±1.8
18:2_t						51.1±4.8
19:0			10.9±1.8			
20:0		0.3±0.2				
20:1	1.6±0.6		1.0±1.4	1.1	0.6±0.2	
20:2	1.1±0.9	1.4±0.9	0.5±0.2	1.1	7.6±2.0	
20:3	2.6±1.8	1.2±1.0	2.9±0.3	1.0	1.3±0.5	2.3±2.2
20:4	12.3±5.2	14.0±0.9	12.3±3.7	7.3	6.0±4.0	4.0±1.8
% Saturated fatty acids	41.4	45.9	43.2	22.9	41.6	25.5
% Unsaturated fatty acids						
cis	58	53.7	50.8	33	59.5	27.6
trans				45.7		51.1
Degree of unsaturation	110	116	108	65	117	51

16:1_c acyl chains. Again, changes in the level of C_{20} unsaturated acyl chains were seen but not necessarily for the same fatty acid supplements as in PE nor in the same pattern.

ESR measurements on plasma membranes

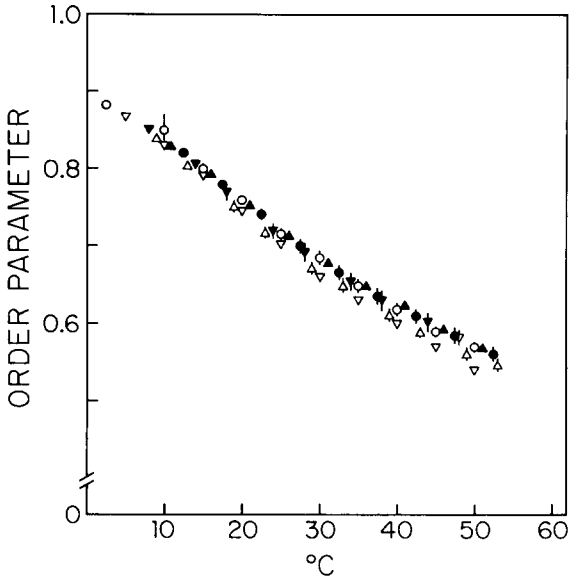
We have previously reported on the temperature dependence of two ESR motional parameters in fatty acid-substituted and normal EL4 intact plasma membrane [4]. Further data is presented in Figs. 1 and 2. The order parameter (S), which reflects the packing order or flexibility of the phospholipid acyl chains, was not significantly different between fatty acid-substituted and normal unsubstituted controls. The numerical agreement between substituted and control membranes around the physiological temperature (37°C) was especially tight, considering the number of fatty acids we have examined, including three different chain lengths and different degrees and configurations of unsaturation. Similarly, the approximate rotational correlation time for all six membrane preparations fell within a strikingly narrow range over most of the temperature range recorded (Fig. 2). The differences below about 15°C are probably more apparent than real, since the values of this parameter approached the lower limit of sensitivity of the ESR technique used. Thus the

TABLE IV

FATTY ACYL COMPOSITION OF PLASMA MEMBRANE PHOSPHATIDYLCHOLINE

All conditions were the same as in Table III.

Fatty acid analyzed	Fatty acid used in culture supplement					
	None (11)	17:0 (5)	19:0 (4)	18:1_t (3)	18:2_{cis} (8)	18:2_t (6)
14:0	1.8±0.9	1.2±0.8	1.7±0.4	1.8±0.9	0.8±0.6	1.4±1.3
15:0						
16:0	30.4±5.5	12.9±1.1	22.4±3.2	14.1±5.7	30.7±2.6	25.0±9.7
16:1_c		0.9±0.8	1.0±2	7.4±1.4		
17:0		26.4±5.7				
18:0	23.3±3.2	14.0±2.7	14.4±1.4	16.1±3.4	15.7±2.4	16.6±2.4
18:1_c	27.6±3.2	26.9±0.1	24.1±1.6	15.1±7.5	11.3±1.3	13.1±4.0
18:1_t				31.9±2.0		
18:2_c	10.1±3.4	14.6±1.6	15.6±2.9	5.2±3.3	36.1±5.1	4.3±2.4
18:2_t						35.8±9.2
19:0			11.0±1.7			
20:0		1.3±1.1				
20:1	2.2±1.3		1.3±0.6		0.4±0.3	
20:2	1.8±1.5	1.4±1.1	1.3±0.2		3.9±1.2	
20:3	1.3±0.9	0.3±0.3	1.8±0.3			
20:4	2.0±2.2	1.1±0.9	2.4±0.2			2.9±0.5
% Saturated fatty acids	55.6	55.8	49.5	32	47.2	43
% Unsaturated fatty acids						
cis	45.0	45.2	47.5	27.7	51.7	20.3
trans				31.9		35.8
Degree of unsaturation	66	67	75	33	92	33



chemical modification of the plasma membrane phospholipids through the incorporation of an exogenous fatty acid was not accompanied by detectable changes in the physical properties of the intact membranes as measured by these two motional parameters.

Fig. 1. Temperature dependence of the order parameter, *S*, in normal and fatty acid substituted EL4 plasma membranes. Plasma membrane was isolated and labeled with the stearic acid spin probe, 5-NS, as described in Methods. Each spectrum was recorded as a 4 min 100 G scan at 20 mW power. *S* was calculated using the equation given in Materials and Methods. Normal EL4 plasma membrane (○). ●, Substituted plasma membranes: 18:2_c-; ▼, 18:2_t-; ▲, 18:1_t-; △, 17:0-; and ▽, 19:0-substituted. The number of experiments performed for each group was: normal EL4 plasma membrane, 5. Substituted plasma membranes: 18:2_c-, 3; 18:2_t-, 3; 18:1_t-, 1; 17:0-, 3; and 19:0-substituted, 1.

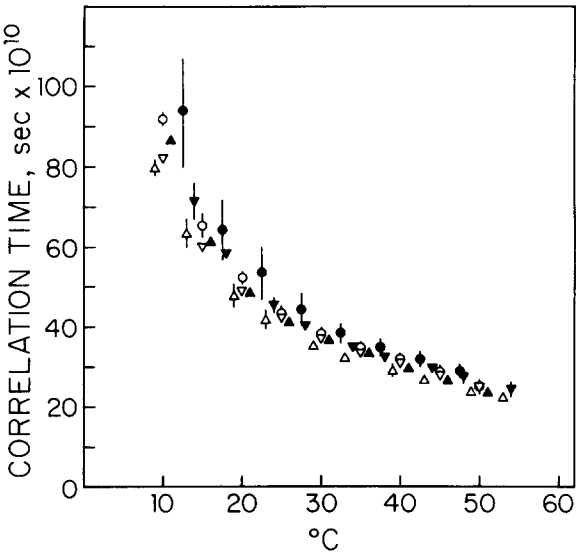


Fig. 2. Temperature dependence of the approximate rotational correlation time, τ_0, in normal and substituted EL4 plasma membranes. Samples were prepared as in Fig. 1, except that the stearic acid spin probe used was 12-NS. Each spectrum was recorded as a 4 min 40 G scan at 20 mW power. τ_0 was calculated using Kivelson's equation (see Materials and Methods). Symbols are the same as in Fig. 1. The data are the means and standard deviations of three separate experiments except for 18:1_t and 19:0 which were performed only once.

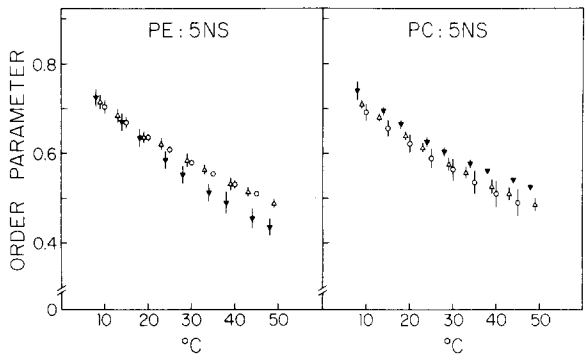


Fig. 3. Temperature dependence of the order parameter, S, of phosphatidylethanolamine (left panel) and phosphatidylcholine (right panel) liposomes derived from normal (○), 18:2_t- (▼) and 17:0- (△) substituted EL4 plasma membranes. See Materials and Methods for sample preparation. Spectrum recording was the same for Fig. 1. Each data point is the mean and standard deviation of four experiments except for normal PC liposomes which were done three times.

Order parameter of plasma membrane derived liposomes

We next carried out ESR studies on PE and PC liposomes derived from lipid-altered plasma membranes. These measurements were made to determine whether any alteration in physical properties induced by the incorporation of the fatty acids might become more easily detectable as the complexity of the system is reduced. In Fig. 3 order parameter is compared among fatty acid-substituted and normal EL4 liposomes. No difference between liposomes from normal and 17:0-substituted plasma membrane could be detected. However, the 18:2_t-substituted PE liposomes had lower S values than normal PE liposomes above 25°C, while the 18:2_t-substituted PC liposomes displayed slight but consistently higher S values over the entire temperature range tested. A large difference is apparent between the S values of 18:2_t-substituted PE and 18:2_t-substituted PC liposomes, which was not observed for the other two fatty acids or for the normal control. Possible interpretations of these results will be presented in Discussion.

Approximate rotational correlation times for ESR probes in plasma membrane-derived liposomes

The temperature dependence of the approximate rotational correlation time for PE and PC liposomes are shown in Fig. 4. As with the intact plasma membranes, the values of τ_0 fell within a strikingly narrow range over most of the temperature scale including the physiological temperature (37°C). For the reason cited above for the data on intact plasma membranes, the apparent differences in τ_0 with large standard deviation at the lower end of the temperature scale are considered to be of dubious significance. The overall observation is that the incorporation of 17:0, 18:2_c or 18:2_t into these membrane phospholipids did not result in detectable changes in the approximate rotational correlation time for probes embedded in these liposomes of relatively simple composition.

ESR measurements in mixed vesicles of synthetic and EL4 membrane-derived lipids

Given the extensive incorporation of some of the substituted fatty acids into membrane phospholipids (see below and Tables III and IV), the relative lack of impact of substitution on the be-

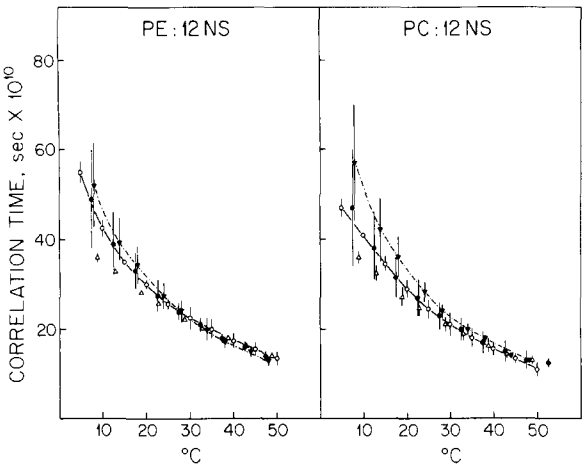


Fig. 4. Temperature dependence of the approximate rotational correlation time, τ_0, of phosphatidylethanolamine (left panel) and phosphatidylcholine (right panel) liposomes derived from normal and fatty acid substituted EL4 plasma membranes. See Materials and Methods for sample preparation. Symbols and conditions for spectrum recording were the same as in Fig. 2. Each data point is the mean and standard deviation of three separate experiments.

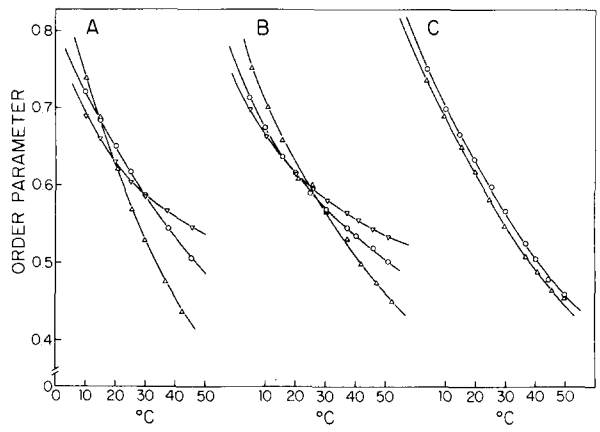


Fig. 5. Temperature dependence of the order parameter, S, in synthetic, cellular and mixed lipid vesicles. In panel A the lipids used were EL4 microsomal lipids (△), di-18 : 1_c-PC (▽) and 50 mol% mixture of the two (○). Panel B, EL4 plasma membrane phosphatidylcholine (△), di-18 : 2_c-PC (▽) and 50 mol% mixture of the two (○). Panel C, EL4 plasma membrane phosphatidylcholine (△) and 20 mol% mixture with di-18 : 0-PC (○).

havior of ESR probes seemed somewhat surprising. In order to gain an impression of the expected impact, we carried out experiments in which pure synthetic phospholipids containing some of the substituent fatty acids were mixed with the phospholipids isolated from normal EL4 cells prior to ESR analysis. Representative results of these experiments are shown in Fig. 5. The synthetic lipids used in Panels A and B were di-18 : 1_c-PC and di-18 : 2_c-PC, respectively, and the cellular lipids were whole microsomal lipids and plasma membrane phosphatidylcholine, respectively. Both of the synthetic lecithin vesicles have much shallower curves for order parameter vs. temperature than either the microsomal lipid or the plasma membrane PC vesicles. This is probably because both unsaturated synthetic PC have transition temperatures well below the temperature ranged recorded. The cellular lipids would be expected to have a broad transition within the recorded temperature range. The microsomal lipids appear significantly more fluid than the plasma membrane PC over most of the temperature range. This may be attributable to the greater heterogeneity (particularly with respect to cholesterol) in the composition of the microsomal lipids. At 50 mol%, di-18 : 1_c-PC has significantly altered the S values compared to 100% microsomal lipids, while at the same concentration di-18 : 2-PC altered the S values only marginally above the standard deviation seen for different plasma membrane PC samples. It was also noted that the curves for both mixtures crossed the corresponding curves for the cellular lipids, apparently having opposite effects at different parts of the temperature scale (ordering at high and disordering at low). In panel C the order parameter curves for plasma membrane PC vesicles and vesicles of plasma membrane PC phospholipids and 20 mol% synthetic di-18 : 0-PC are shown. The two curves are parallel and the slight difference between them is probably within the standard deviation from one plasma membrane PC sample to another (Fig. 3).

Discussion

We have observed that culture supplementation with a single fatty acid alters the patching rate of cell surface antigens [1], cytotoxic function of lymphocytes [2,3] and the plasma membrane enzyme activities of EL4 cells [4]. Previous studies using fluorescence polarization [5] and ESR tech-

niques [4], on the other hand, showed no correlation between the functional effects observed and the apparent physical properties of lipid-altered plasma membranes. The studies in this report were directed toward understanding these seemingly contradictory findings.

We report here for the first time details of the alterations in overall lipid composition caused by the introduction of inordinate amounts of normally occurring fatty acids, or the introduction of 'unnatural' fatty acids, into EL4 plasma membranes. Several potentially important points were noted concerning fatty acid-induced alterations in overall plasma membrane lipid composition. It is clear that $18:1_t$ and $18:2_t$, which are configurationally similar to saturated fatty acids, were incorporated preferentially into PE. While the mean incorporation of 17:0 and $18:2_c$ into PE and PC is not dramatically different, we have observed the same uneven pattern of incorporation suggested for these two fatty acids (Tables III and IV) in every plasma membrane preparation we have analyzed. Both are incorporated less into PE than PC. Since PE has fewer saturated fatty acids than PC in the normal EL4 plasma membrane, the preferential incorporation of $18:1_t$ and $18:2_t$ into PE may be an attempt to regulate the overall effect of incorporation of these fatty acids. Incorporation of these two fatty acids also led to a marked decrease in the total percent saturated fatty acids in both PE and PC, although the degree of *cis* unsaturation was also reduced. The fatty acid supplements, 17:0, 19:0 and $18:2_c$, appeared to have very little influence on the percent saturated fatty acid or the degree of unsaturation. The only exception is an increase in the degree of unsaturation in $18:2_c$ substituted PC.

The incorporation of a single exogenous fatty acid induced changes in phospholipid headgroup composition in some cases. There was a significant increase in the level of PI in $18:2_c$-substituted plasma membrane, and in PE:PC ratio for 17:0-substituted plasma membrane. These alterations, together with the fatty acid profile changes noted above, could be consistent with a directed metabolic response to the changes in physical properties that may accompany the incorporation of an exogenous fatty acid (see further discussion below). On the other hand, none of the fatty acids, after incorporation into plasma membrane phospholipids, significantly alters the phospholipid:cholesterol ratio.

As previously reported, we again found no change in two ESR motional parameters in fatty acid-altered EL4 plasma membranes. The incorporation of a single exogenous fatty acid up to 40–50% of total membrane phospholipid fatty acid could not be detected by changes in the thermotropic behavior of the ESR probes used. King and Spector [14], using the same probes in a similar study on fatty acid enriched Ehrlich ascites cell plasma membrane, arrived at the conclusion that fatty acid substitution did alter plasma membrane physical properties as defined by the same two ESR parameters. We have addressed this discrepancy thoroughly in a previous publication [4]. Briefly, the magnitude of change they reported is within the standard deviation in our own experiments, and the fact that they used an $18:2_c$-substituted plasma membrane as a control, instead of normal unsubstituted plasma membrane, makes a direct comparison with our data difficult. In our studies, ESR measurements were also done on PE and PC liposomes derived from fatty acid-substituted and normal EL4 cell plasma membranes, to see whether in a less complex system changes resulting from the incorporation of a fatty acid supplement might be more readily detectable. Using the fatty acids 17:0 and $18:2_c$, we could detect no significant change in either S or τ_0 in either PE or PC liposomes. Substitution with $18:2_t$ was somewhat different. Incorporation of $18:2_t$ into PE (51%) decreased the order parameter, while incorporation into PC (36%) increased the order parameter with respect to the control. At the same time no change was found in the order parameter of intact $18:2_t$-substituted plasma membrane. This suggests the possibility that the same fatty acid incorporated into the two different phospholipid classes induces off-setting physical changes resulting in no net effect at the level of the intact plasma membrane.

The ESR experiments on mixed lipid vesicles show that 20 mol% di-18:0-PC did not significantly alter the S values of EL4 plasma membrane PC over the entire temperature range analyzed. Even at as high as 50 mol%, di-$18:2_t$-PC produced effects only marginally significant when standard

deviations observed for repeated measurements of pure plasma membrane PC vesicles is considered (Fig. 3). These results suggest that altering the fatty acid composition may not affect the order parameter as much as might be expected.

It seems to us that there are two major questions that must be addressed by our study, and the many others in the literature, on lipid alteration of biological membranes. (1) Knowing even in considerable detail the compositional changes brought about by lipid alteration, can one truly predict changes to be expected in either the physical or biochemical properties of the altered membrane? (2) Do physical techniques such as fluorescence polarization and ESR, which give elegant and useful information about membrane structure in relatively simple synthetic membrane systems, give interpretable and useful information about the physical state of complex biological membranes?

Much of our current impression of the relation of membrane lipid composition to physical properties comes from studies on temperature acclimation in various cell types. Rapid shifts in growth temperatures have been shown to alter the lipid composition of different organisms including *Fusarium oxysporum* [15], *Acholeplasma* [16], *Tetrahymena* [17], goldfish [18], and the plasma membranes of mouse LM cells [19] and rat epididymal adipocytes [20]. These changes in lipid composition could be interpreted as compensatory responses to an environmental pressure that would modify or shift the membrane physical properties away from the physiological optimum. These findings lend credibility to the concept of self regulation of membrane physical properties, which has been termed 'homeoviscous adaptation' by Sinensky [21]. Martin et al. [22] suggested fatty acid desaturase as the molecular mechanism for this self regulation of membrane properties because the activity of this enzyme monitored as the incorporation of [^{14}C]acetate into unsaturated fatty acids increased upon lowering of growth temperature.

It is tempting to extrapolate this concept of self-regulation of membrane 'fluidity' to our own system where the experimental variable of temperature shift is replaced by the incorporation to varying degrees of exogenous fatty acids of differing physical properties. Ferguson et al. [19] and Esko et al. [23] interpreted their data on lipid substitution of LM cells as suggestive of such a compensatory response. We feel it is not possible with our present state of knowledge of biological membranes to predict with any certainty the effect a given fatty acid, when incorporated into membrane phospholipids, will have on membrane physical properties. Constituent phospholipid molecules, and not individual fatty acid chains, are the units that, together with cholesterol, will determine membrane physical properties. Even our knowledge of the physical properties of synthetic lipid vesicles made from phospholipids of defined fatty acyl composition is extremely limited. It has been shown that in a synthetic mixed-function phospholipid, two neighboring acyl chains on the glycerol backbone together determine the transition temperature of the molecule, and this is not necessarily the arithmetic average of the transition temperatures of the corresponding single-function phospholipids [29,31,33–35]. Moreover, although in naturally occurring phospholipids the second position preferentially incorporates unsaturated fatty acids, reversing the positions of two given acyl chains results in molecules having significantly different transition temperatures [35]. In a biological membrane, the number of possible different phospholipid species, differing in head group and in content or position of acyl chains, is very large. As we report here, introduction of an exogenous fatty acid can perturb the overall lipid composition of the membrane in ways that are difficult to predict on a priori grounds. Whether or not the perturbations we observed in overall lipid composition in some cases was an attempt by the cell to reestablish homeostasis is unclear. The results of our mixing experiments (Fig. 5) suggest that the physical properties of the membrane may not be greatly perturbed by most fatty acid substitutions.

The answer to our second question is of course related to the answer to the first. Given the relatively modest perturbations in lipid composition observed after fatty acid substitution, it may be the case that only relatively minor alterations in physical properties are in fact induced. That the methods used in this study are responsive to changes in the physical properties of membrane lipids is seen by the changes in motional parameters as a function of temperature in all systems tested. We also observed marked differences in the

order parameter of PE and PC vesicles containing $18:2_t$ (Fig. 3). But the fact remains that in the vast majority of measurements we have made of fatty acid-substituted membranes we have been unable to detect measurable alterations in the behavior of our ESR probes. We reported essentially identical results from analysis by fluorescence polarization [5], as did Stubbs et al. [24]. The results of our mixing experiments (Fig. 5 and unpublished observations) suggest that introduction of a fatty acyl function in the form of phospholipids into plasma membranes, and even into purified plasma membrane phospholipids, may have only a modest effect on the motional parameters ordinarily measured by ESR. Intact biological membranes are obviously highly complex lipid-protein mosaics, with potentially many different lipid domains ordered to varying degrees by interactions with surrounding molecules, and the whole of it modified by the presence of cholesterol. Any spectroscopic measurement will at best report an average of the various environments among which the probe used partitions. It is not possible to predict how the substituent fatty acids used will distribute among the various domains to which the probe distributes. We thus feel that particularly for studies involving lipid alteration of biological membranes, and perhaps for complex biological membranes generally, physical techniques such as ESR and fluorescence polarization are of limited value, and should be used with extreme prudence.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (USPHS AI 14747). WRC was supported by an NIH Career Development Award (AI 00009).

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