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Comparison of phosphatidylcholines containing one or two docosahexaenoic acyl chains on properties of phospholipid monolayers and bilayers

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Abstract

Docosahexaenoic acid (DHA) is the longest and most unsaturated of the $n-3$ fatty acids found in membranes. Although a number of membrane properties have been demonstrated to be affected by the presence of this fatty acid, its mode of action has yet to be clearly elucidated. Prior reports on biological membranes have not distinguished the effect of mono-docosahexaenoyl phospholipids from those caused by phospholipids containing docosahexaenoic acid in both chains. This report compares properties of monolayers and bilayers composed of either 1-stearoyl-2-linolenoyl-*sn*-glycero-3-phosphocholine (as a control), 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine or 1,2-di-docosahexaenoyl-*sn*-glycero-3-phosphocholine. When compared to the mono-DHA phosphatidylcholine (PC), the di-DHA PC occupies a much larger area/molecule, supports a more fluid and permeable bilayer, and is less susceptible to peroxidation. Monolayers made from either phospholipid are not condensable by cholesterol. We suggest many of the membrane properties linked to the presence of DHA may be the result of phospholipids which have lost their normal positional selectivity and have incorporated DHA into both positions.

Keywords: Docosahexaenoic acid; Cholesterol; Di-docosahexaenoylphosphatidylcholine

1. Introduction

Docosahexaenoic acid (DHA, 22:6$\Delta^{4,7,10,13,16,19}$) is the longest and most unsaturated of the class of polyunsaturated fatty acids known as $n-3$. These fatty acids have recently received considerable attention because they have been linked with reduced heart disease, autoimmune diseases, multiple sclerosis, arthritis, peroxisomal disorders and cancer [1–6]. While the precise mode of action of $n-3$ fatty acids remains a mystery, their participation in seemingly unrelated disease states implies a fundamental role which would be common among many cell types. Three categories of activities have been suggested: $n-3$ fatty acids may be involved in the biochemical production of prostinoids; they may serve as the source of very potent peroxidation products; or they may serve unique structural roles as components of the hydrophobic interior of membranes. The experiments reported here explore the role of $n-3$ in membrane structure.

DHA is typically found in large amounts only in the rod outer segment, sperm and the gray matter of brain [7]. In these tissues DHA levels can occasionally exceed 50 mol% of the fatty acids found associated with a class of phospholipids. For example, Neil and Masters [8] found 78.8% of the phosphatidylcholine acyl chains in bovine spermatozoa were DHA, while Wiegand and Anderson [9] reported 62% of the acyl chains of frog outer segment phosphatidylethanolamine (PE) were DHA. The most common arrangement of acyl chains in phospholipids finds saturated chain fatty acids such as palmitic and stearic acid predominantly in the *sn*-1 position and unsaturated fatty acids such as oleic, linoleic or DHA in the *sn*-2 position [10]. The high levels of DHA reported for certain tissues implies the

Abbreviations: AS, (9-anthroyloxy)stearic acid; CF, carboxyfluorescein; DHA, docosahexaenoic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; MLV, multilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PROP-DPH, 3-*p*-(6-phenyl)-1,3,5-hexatrienylphenylpropionic acid; SUV, small unilamellar vesicles; 18:0,18:1 PC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; 18:0,18:3 PC, 1-stearoyl-2-linolenoyl-*sn*-glycero-3-phosphocholine; 18:0,22:6 PC, 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; 22:6,22:6 PC, 1,2-di-docosahexaenoyl-*sn*-glycero-3-phosphocholine.

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existence of significant amounts of phospholipids in which DHA must occupy both acyl chain positions. Dipolyenoic phospholipids have in fact been reported from several types of membranes, most notably from rod outer segments [11,12]. The frog ROS PE composition reported by Wiegand and Anderson [9] had 25% of the PEs containing two DHA chains. While it is assumed that $n-3$ fatty acids should provide a unique function, it has yet to be elucidated what that function might be.

There have been a few reports of alterations in membrane properties caused by mixed acyl chain 18:0,22:6 PC [13–16]. With the exception of the recent differential scanning calorimetry study of Kariel et al. [17], however, there are no reports of bilayer properties affected by incorporation of phospholipids where both chains are DHA. Here we compare several properties of monolayers and bilayers made from phosphatidylcholines in which DHA occupies only the *sn*-2 position or where DHA occupies both positions.

2. Materials and methods

2.1. Materials

The phosphatidylcholines 18:0,18:1 PC, 18:0,18:3 PC and 18:0,22:6 PC were purchased from Avanti Polar Lipids, Alabaster, AL. CM cellulose, hydroxybenzotriazole, 4-(dimethylamino)pyridine, L-α-glycerophosphorylcholine, *N,N'*-dicyclohexylcarbodiimide and Sephadex G-50 were from Sigma (St. Louis, MO). *tert*-Butylhydroquinone was purchased from Aldrich (Milwaukee, WI), Rexyn 1300 from Fisher, carboxyfluorescein from Eastman Kodak (Rochester, NY) and the fluorescent membrane probes DPH, PROP-DPH, 2-AS, 6-AS, 9-AS, and 12-AS from Molecular Probes (Eugene, OR).

2.2. Synthesis of 1,2-di-docosahexaenoyl-sn-glycero-3-phosphocholine (22:6,22:6 PC)

A modification of the procedure of Rhodes et al. [18] was used to synthesize 22:6,22:6 PC. To minimize autooxidation, all procedures were performed in reduced light and under nitrogen. Water was scrupulously removed from all reagents and glassware. The reaction was carried out in two steps: synthesis of DHA-anhydride was subsequently followed by the esterification of L-α-glycerophosphorylcholine. DHA (100 mg, 0.305 mmol), *tert*-butylhydroquinone (12.45 mg, 0.075 mmol), *p*-hydroxybenzotriazole (10.12 mg, 0.075 mmol), *N,N'*-dicyclohexylcarbodiimide (79.30 mg, 0.380 mmol) were dissolved in 5 ml of dimethylformamide (doubly distilled) and the solution was stirred for 45 min at room temperature under an atmosphere of nitrogen. Then L-α-glycerophosphorylcholine (19.30 mg, 0.76 mmol) and 4-(dimethylamino)pyridine (18.33 mg, 0.152 mmol) were added. The mixture was stirred in the dark, under nitrogen for a period of 16 h. After acylation, the suspension was filtered, washed with chloroform and concentrated by rotary evaporation and then subjected to chromatography on a Rexyn 1300 column [19], eluted with 2 beds column of $CH_3OH/CHCl_3/H_2O$ (5:4:1). The column eluant was converted into the phase system of Folch et al. [20] and the lower phase collected. The resulting mixture, after concentration, was separated by CM-cellulose column chromatography using step-wise elution with solvents containing increasing percentages of methanol in chloroform [21]. Purity of the synthesized phospholipid was tested by silica gel TLC using $CHCl_3/CH_3OH/H_2O$ (65:25:4, v/v) and the total phospholipid content was determined by spectrophotometry assay for phosphorus according to Chen et al. [22]. The yield of 22:6,22:6 PC was between 40–45% of the starting glycerophosphorylcholine.

2.3. Preparation of small unilamellar vesicles (SUVs)

Phospholipids in chloroform were dried under nitrogen, then vacuum pumped overnight. Multilamellar vesicles (MLVs) were made by hydration in 10 mM Na_2HPO_4 buffer, pH 7.0. The turbid suspension was then sonicated on ice for 5 min at level 3 using a Heat System W-380 Cell Disrupter. The resultant clear suspension, comprised of small unilamellar vesicles (SUVs), was then centrifuged at low speed to remove titanium and non-liposomal particles and was immediately used for experimentation.

2.4. Lipid peroxidation

Lipid peroxidation of SUVs by Cu_2SO_4/H_2O_2 in 50 mM Tris, pH 7.0, was determined by measuring conjugated diene formation spectrophotometrically with tandem cuvettes [23,24]. The same amount of oxidizable polyunsaturated fatty acid was tested for each phospholipid. Since 22:6,22:6 PC has two oxidizable chains and the other two phospholipids, 18:0,18:3 PC and 18:0,22:6 PC, have only one, 22:6,22:6 PC was run at half the concentration of the other phospholipids. For this lipid the total lipid concentration was maintained by mixing 22:6,22:6 PC with non-oxidizable 18:0,18:1 PC (1:1, mol/mol) [24]. Prior to peroxidation, SUVs were diluted (1:5) in Tris buffer, then 1.5 ml of the diluted mixture was added to the first compartment of the tandem cuvettes in both the reference and the sample beam. The second compartment of both tandem cuvettes was filled with 1.5 ml of the buffer. Lipid peroxidation was initiated by addition of 20 μl of H_2O_2 and 20 μl of Cu_2SO_4 to the sample cuvette lipid compartment and to the reference cuvette buffer compartment. 40 μl of Milli-Q water was added to both the lipid-containing compartment of the reference cuvette and to the buffer-containing compartment of the sample cuvette. The final concentration of lipid in the cuvettes was 70 μM, and Cu_2SO_4 and H_2O_2 were 130 μM and 1.3

mM, respectively. Temperature of the cuvettes was 37° C. UV difference spectra (205–300 nm) were recorded continuously on an Aminco SLM 3000 diode array spectrophotometer.

2.5. Fluorescence anisotropy

Two types of fluorescent probe are used to monitor the effect of DHA on the property of membrane fluidity. Relative membrane fluidity was followed by steady-state fluorescence polarization of either the membrane interior (linear) probe 1,6-diphenyl-1,3,5-hexatriene (DPH) [25], the membrane surface (linear) probe 3-*p*-(6-phenyl)-1,3,5-hexatrienylphenylpropionic acid (PROP-DPH) [26] or a series of (9-anthroyloxy)stearic acids (non-linear probes) with the chromophore attached at the 2-, 6-, 9- or 12-position of stearic acid [27]. While the term membrane fluidity has proven to be very difficult to precisely define [28], the probes employed here report on some combination of degree of order and rate of motion. For the linear (DPH) probes, motion predominates while for the non-linear (AS) probes the dependency on degree of order becomes increasingly more significant. Although a precise definition of fluidity is not attainable, it is believed these probes in combination can be used qualitatively to report on the state of the bilayer at various positions down the acyl chains.

SUVs were made as described above from 18:0,18:3 PC, 18:0,22:6 PC or 22:6,22:6 PC mixed with the appropriate probe. Lipid concentration was between 1–1.5 mM with a probe to lipid ratio of 1:200. Anisotropy values (A) were measured at 25° C using a Perkin-Elmer MPF-66 Fluorescence Spectrophotometer interfaced to a Perkin-Elmer 7700 professional computer. The excitation and emission wavelengths were: DPH, 360 nm and 420 nm; PROP-DPH, 358 nm and 426 nm; and the AS probes, 361 and 446 nm, respectively. A values are the average of eight determinations for each sample.

2.6. Carboxyfluorescein permeability

Permeability of SUVs from 18:0,18:3 PC, 18:0,22:6 PC and 22:6,22:6 PC was followed by monitoring the increase in fluorescence intensity resulting from carboxyfluorescein (CF) leakage [29]. MLVs were made by hydrating the lipids in 60 mM CF, 30 mM KCl, 10 mM Na_2HPO_4, pH 7.0 then SUVs were made as described above. Non-sequestered CF was removed on a Sephadex G-50 column with 90 mM KCl and 10 mM Na_2HPO_4, pH 7.0. The resultant vesicles were kept on ice until used. CF permeability was initiated by rapidly mixing a small aliquot of SUVs with 2.5 ml of 90 mM KCl/10 mM Na_2HPO_4 pH 7 at 25° C. Fluorescence was followed on a Perkin-Elmer LS 50B Luminescence spectrometer at 25° C. Excitation was at 490 nm and emission at 520 nm. Total sequestered CF was obtained following SUV breakage by addition of 50 μl of 5% Triton X-100. Percent CF leakage was determined by:

$$\%CF = ((F - F_i)/F_{max}) \times 100$$

where F = fluorescence intensity at time t; F_{max} is the maximum fluorescence following Triton X-100 release; and F_i is the initial fluorescence at time $t = 0$.

2.7. Lipid monolayer studies

Measurements of area/molecule were made on lipid monolayers using a KSV minitrough (KSV Instrument, Helsinki, Finland) interfaced to Epson 286 PC. Lipids, dissolved in triply distilled benzene at 1 mg/ml, were spread onto a subphase of 20 mM Na_2HPO_4, pH 7.0. After 10 min to allow evaporation of solvent, film compression (1 mN/m/min) at 22° C was initiated. Each compression was repeated a minimum of four times. Area/molecule determinations were made for single component 18:0,18:3 PC, 18:0,22:6 PC, 22:6,22:6 PC or cholesterol monolayers or for 80:20, 70:30, 60:40 or 50:50 mixtures of phospholipid/cholesterol (mol/mol). The extent of cholesterol condensation was estimated from the value of the sum of the ideal (noncondensable) areas for the lipid plus cholesterol and the experimentally derived value for the mixture [30,31]. The reported % condensation then reflects the deviation from ideal behavior.

2.8. Statistical analysis

For comparison of values, Student's t-test was employed in the cholesterol condensation and fluidity experiments. Statistical significance between all other groups was determined by analysis of variance (ANOVA) and the

Table 1
The calculated area/molecule (Å^2) for 18:0,18:3 PC, 18:0,22:6 PC, and 22:6,22:6 PC monolayers at lateral surface pressures of 10, 20, 30, and 40 mN/m

Phospholipid	Area/molecule (Å^2)			
	10 mN/m	20 mN/m	30 mN/m	40 mN/m
18:0,18:3 PC	80.75 ± 1.97 [a]	65.83 ± 1.74 [a]	54.50 ± 1.86 [a]	45.26 ± 0.70 [a]
18:0,22:6 PC	101.00 ± 3.10 [b]	80.67 ± 1.42 [b]	68.33 ± 2.78 [b]	54.50 ± 0.42 [b]
22:6,22:6 PC	125.75 ± 4.85 [c]	95.00 ± 1.42 [c]	82.20 ± 1.18 [c]	75.00 ± 1.26 [c]

The subphase was 20 mM Na_2HPO_4, pH 7.0. Values are means ± S.E. from four separate determinations. Values with different superscripts (a, b, c) are significantly different ($P < 0.05$, ANOVA).

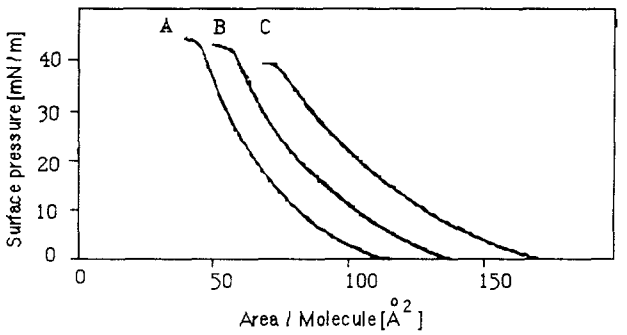

Fig. 1. Surface pressure–area curves for monolayers composed of: (A) 18:0,18:3 PC; (B) 18:0,22:6 PC and (C) 22:6,22:6 PC. Phospholipids were spread in benzene over a sub-phase of 20 mM Na_2HPO_4, pH 7.0 at 22° C. Compression rates were 1 mM/m/min.

Scheffe F-test. Differences were considered significant if $P < 0.05$.

3. Results

Lipid monolayers and phospholipid bilayer vesicles (SUVs) were made from 18:0,18:3 PC, 18:0,22:6 PC or 22:6,22:6 PC. Area/molecule and cholesterol-induced condensation determinations were made using the lipid monolayers while lipid peroxidation, fluidity, and bilayer permeability were monitored with lipid vesicles.

Monolayers were made from 18:0,18:3 PC, 18:0,22:6 PC or 22:6,22:6 PC over an aqueous subphase of 20 mM Na_2HPO_4, pH 7.0. Compressions were made at 1 mN/m/min on a KSV minitrough (Fig. 1) and the area per molecule calculated for each lipid at 10, 20, 30 and 40 mN/m (Table 1). At all pressures di-22:6 PC occupied about 1.3-times the area as did 18:0,22:6 PC and about 1.75-times the area as did 18:0,18:3 PC. Upon addition of 30, 40 and 50 mol% cholesterol, monolayers made from 18:0,18:3 PC were shown to exhibit statistically significant condensation at 15, 20 and 25 mN/m lateral pressure (Fig. 3). An increase in % condensation was measured with increasing % cholesterol at all lateral pressures tested. In contrast, at these same pressures, neither the 18:0,22:6 PC nor the 22:6,22:6 PC could be condensed by cholesterol (Figs. 2 and 4).

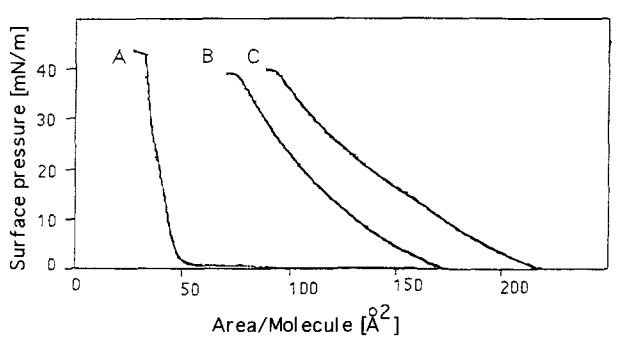

Fig. 2. Surface pressure–area curves for monolayers composed of: (A) cholesterol; (B) 22:6,22:6 PC and (C) cholesterol/22:6,22:6 PC 1:1 (mol/mol). Phospholipids were spread in benzene over a sub-phase of 20 mM Na_2HPO_4, pH 7.0 at 22° C. Compression rates were 1 mM/m/min.

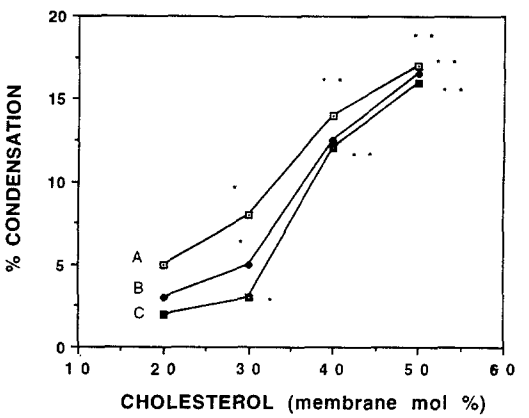

Fig. 3. Cholesterol-induced condensation of 18:0,18:3 PC monolayers as a function of membrane cholesterol content. Cholesterol/18:0,18:3 PC (mol/mol) were 20:80, 30:70, 40:60, 50:50. Lateral pressures are: (A) 15 mN/m; (B) 20 mN/m; and (C) 25 mN/m. Phospholipids were spread in benzene over a sub-phase of 20 mM Na_2HPO_4, pH 7.0 at 22° C. Compression rates were 1 mM/m/min. (Average ± S.E., $n = 6$ individual determinations. * $P < 0.01$, ** $P < 0.001$ compared to the sum of the area/molecule of individual lipid.)

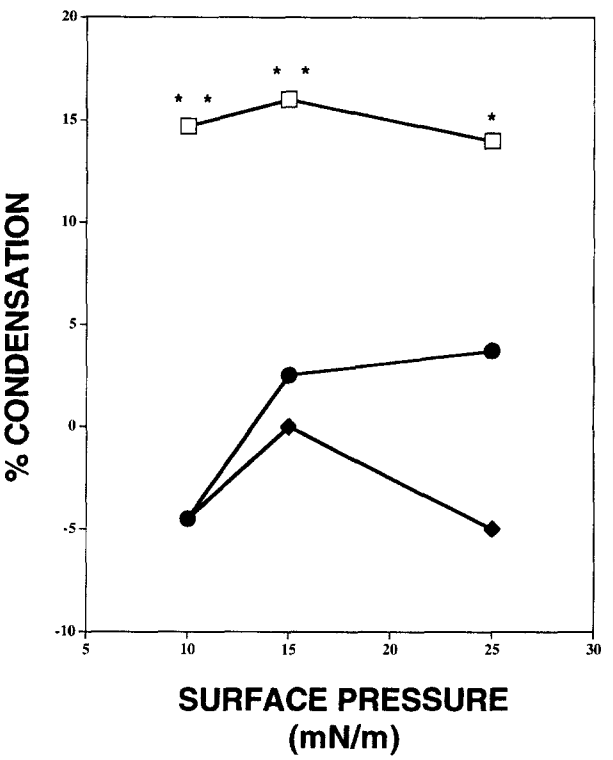

Fig. 4. Cholesterol-induced condensation of: 18:0,18:3 PC (□); 18:0,22:6 PC (◆); and 22:6,22:6 PC (●) at lateral pressures of 10, 15 and 25 mN/m. Lipids (cholesterol/phospholipid, 1:1) were spread in benzene over a sub-phase of 20 mM Na_2HPO_4, pH 7.0 at 22° C. Compression rates were 1 mM/m/min. (Average of four separate determinations; all S.E. < 0.5%. Significantly different than the sum of the area of lipid + cholesterol, * $P < 0.01$, ** $P < 0.001$, one tailed Student's t-test.)

Table 2
Rates of lipid peroxidation and carboxyfluorescein (CF) leakage from SUVs made from 18:0,18:3 PC, 18:0,22:6 PC and 22:6,22:6 PC

	Phospholipids		
	18:0,18:3 PC	18:0,22:6 PC	22:6,22:6 PC
Peroxidation ΔABS (233 nm/min $\times 10^{-4}$)	15 $\pm$3 [a]	60 $\pm$4 [b]	30 $\pm$6 [c]
CF leakage (%/min)	0.3 $\pm$ 0.01 [a]	0.6 $\pm$ 0.03 [b]	1.2 $\pm$ 0.04 [c]

Rates of peroxidation by $CuSO_4/H_2O_2$ (37° C) are reported as absorbance changes (232 nm) due to conjugated diene formation. Carboxyfluorescein leakage is reported as an increase in fluorescence (excitation at 490 nm, emission at 520 nm). (Average $\pm$ S.E., $n = 4$ individual determinations, values with different superscripts a, b, c are significantly different from each other, $P < 0.05$ ANOVA.)

Polyunsaturated fatty acids are known to be particularly susceptible to peroxidation. Table 2 compares the rates of Cu_2SO_4/H_2O_2 induced peroxidation for phospholipid vesicles made from either polyunsaturated 18:0,18:3 PC, 18:0,22:6 PC or 22:6,22:6 PC. As discussed above, 18:0,18:1 PC vesicles were added as a non-peroxidizable lipid [24] to the 22:6,22:6 PC vesicles in order to maintain the same amount of lipid in each experiment. With lipid vesicles containing equivalent amounts of polyunsaturated acyl chains, the rate of 18:0,22:6 PC peroxidation is about 2-times greater than 22:6,22:6 PC and about 4-times greater than 18:0,18:3 PC.

Membrane fluidities of phospholipid vesicles composed of 18:0,18:3 PC, 18:0,22:6 PC or 22:6,22:6 PC were followed using the fluorescent probes 2-AS, 6-AS, 9-AS and 12-AS as well as the membrane interior probe DPH and the membrane surface probe PROP-DPH (Fig. 5). The shape of the fluidity gradient profiles, measured with the AS probes, looked similar for both 18:0,22:6 PC and 22:6,22:6 PC. The di-22:6 PC, however, had lower anisotropy values (higher relative fluidity) than did the 18:0,22:6 PC at all four membrane depth positions tested. The DPH and PROP-DPH probes supported these observations, as 22:6,22:6 PC had lower A values than did 18:0,22:6 PC at the membrane surface (PROP-DPH) and the membrane interior (DPH). In agreement with our previous report [32], fluidity of 18:0,18:3 PC bilayers was similar to that of membranes composed of 18:0,22:6 PC (results not shown).

Finally, we compared the permeability rates for phospholipid vesicles composed of 18:0,18:3 PC, 18:0,22:6 PC or 22:6,22:6 PC (Table 2). 22:6,22:6 PC was 2-times more permeable to carboxyfluorescein than was 18:0,22:6 PC and was 4-times more permeable than 18:0,18:3 PC.

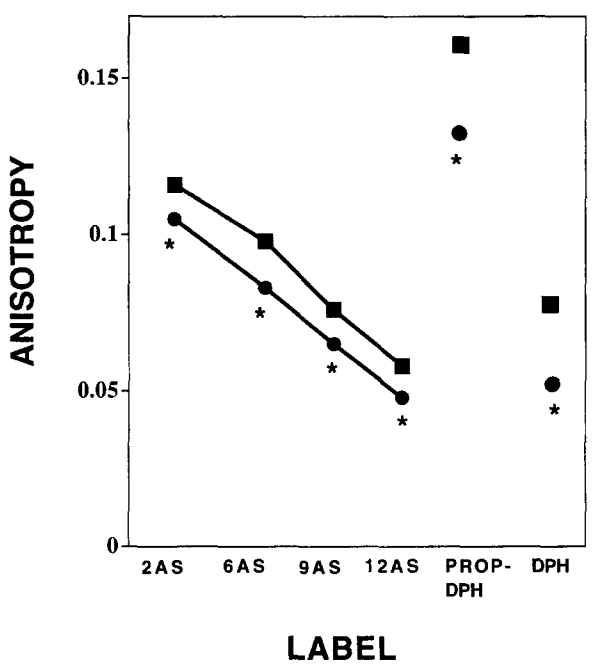

Fig. 5. Relative membrane fluidities of SUVs made from 18:0,22:6 PC (■) or 22:6,22:6 PC (●) as monitored by fluorescence anisotropy (A) of the membrane probes 2-AS, 6-AS, 9-AS, 12-AS, DPH or PROP-DPH (25° C). The excitation and emission wavelength were: DPH, 360 nm and 420 nm; PROP-DPH, 358 nm and 426 nm; and the AS probes, 341 and 446 nm, respectively. (Average of six separate determinations; all S.E. < 0.5%. * Significantly different than SUVs made from 18:0,22:6 PC, $P < 0.001$, Student's t-test.)

4. Discussion

To date most studies involving DHA and membranes have reported DHA-related changes in a membrane function without identifying the chemical species in which DHA was found. Even in those experiments in which DHA was shown to be associated with a specific phospholipid, there usually has been no distinction made between the mono and di-DHA species. DHA levels are consistently high in three tissues, the rod outer segment, sperm and gray matter of the brain [7], where some of the phospholipids most certainly contain DHA in both acyl chains [17]. DHA levels in other membranes can vary over a wide range depending on the diet. In membranes altered by dietary DHA, it is possible that di-DHA phospholipids also exist in significant amounts and account for the reported DHA-induced alterations in membrane function. Whatever effect DHA-incorporation into phospholipids has on membrane structure–function, it may be amplified when the fatty acid is found in both chains. The only report on the effect of 22:6,22:6 PC on a membrane property is that of Kariel et al. [17] who measured phase behavior by differential scanning calorimetry. The work presented here

extends the differential scanning calorimetry report of Kariel et al. [17] on the phase behavior of bilayers composed of 22:6,22:6 PC to a variety of membrane properties.

Previously we reported that DHA did not affect membrane fluidity to a degree significantly different than did other polyunsaturated fatty acids [32]. 18:0,18:3 PC vesicles and 18:0,22:6 PC vesicles had almost identical fluidities as monitored by the membrane probes DPH and 2-, 6-, 9-, and 12-anthracene stearic acids [32]. In contrast the 18:0,22:6 PC vesicles were far more fusogenic and permeable than were vesicles made from 18:0,18:3 PC [32]. We [13] and others [30,31] have also reported that 18:0,22:6 PC monolayers are not condensable by cholesterol while many other PC monolayers are. In fact most phosphatidylcholines commonly found in biological membranes are cholesterol condensable. Using differential scanning calorimetry, Kariel et al. [17] have recently reported that cholesterol at up to 50 mol% has little or no effect on the position, width or ΔH of the 22:6,22:6 PC transition in bilayers. They conclude that cholesterol does not affect the packing of di-22:6 PC in bilayers and that perhaps it is excluded from domains that might be rich in this phospholipid. Our lipid monolayer data presented here concur with the conclusions of Kariel et al. [17] and support our previous proposal that DHA may function in membranes by inducing lateral phase separation into DHA-rich, cholesterol-poor and DHA-poor, cholesterol-rich domains [13]. If such lipid domains do indeed exist, their unique compositions would induce individual protein segregation resulting in local variations in biochemical activity. The existence of cholesterol domains has been suggested from several studies [33,34].

Here we report that incorporation of DHA into both chains makes phosphatidylcholine bilayers more fluid than those in which DHA is incorporated into just one chain (Fig. 5). The increased bilayer fluidity is felt from the aqueous interface (as monitored by PROP-DPH) through to the bilayer interior (as followed by 12-AS and DPH). These fluidity measurements are consistent with reported phase transition temperatures obtained from differential scanning calorimetry (18:0,22:6 PC, -7° C and 22:6,22:6 PC, -68.4° C [17]). The increase in bilayer fluidity parallels an increase in permeability as we have shown that di-DHA PC bilayers are 2-times as permeable to carboxyfluorescein as are bilayers made from mono-DHA PC (Table 2). We anticipate the large enhancement in membrane permeability we previously reported for lipid bilayers [16,32], tumor cell plasma membranes [15,16,35] and mouse and rat liver mitochondria (Stillwell et al., unpublished results) caused by 18:0,22:6 PC supplementation would be even further enhanced by incorporation of 22:6,22:6 PC. Experiments confirming this hypothesis are currently in progress.

From lipid monolayer studies (Fig. 1 and Table 1) we report that replacing the *sn*-1 stearic acid chain with a second DHA chain increases the area/molecule for phosphatidylcholine containing DHA in the *sn*-2 position by about 1.3-fold. Like 18:0,22:6 PC monolayers, di-DHA PC monolayers are also not condensable with cholesterol. If our prediction that mono-DHA phospholipid can induce lateral phase separation into DHA-rich, cholesterol-poor domains is correct [13], then we would expect that the di-DHA lipid would also induce lateral phase separation.

A major problem with proposing a biologically significant membrane structural role for DHA-containing phospholipids is the extreme susceptibility of DHA to peroxidation. In Table 2 we report that incorporation of a second DHA into phosphatidylcholine significantly decreases DHA's oxidation potential.

We predict that if significant levels of di-DHA PC do exist in biological membranes, they might be found in cholesterol-poor domains which would be very fluid, very permeable and would be partially resistant to peroxidation.

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