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The interaction of *N*-oleylethanolamine with phospholipid bilayers

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Long chain acylamides of ethanolamine were previously found to increase in the infarcted canine myocardium. Subsequent in vitro experiments established a number of interesting biological and physiological properties of these compounds including alteration of rabbit skeletal sarcoplasmic reticulum function and inhibition of permeability dependent calcium release from heart mitochondria. These results suggested an interaction between the *N*-acylethanolamines and biological membranes. In the present work we show that the most potent species in previous studies, *N*-oleylethanolamine, forms stable complexes with phospholipid vesicles, lowers diphenylhexatriene polarization ratios in dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine uni- and multilamellar bilayer vesicles, and also produces a concentration dependent decrease in the phase transitions of these lipid structures. In addition studies with parinaric acids also suggested that *N*-oleylethanolamine partitions preferentially into more fluid areas of the bilayer. The results are discussed in terms of possible effects on biological membranes.

Introduction

Lipid metabolites such as unesterified fatty acids, palmitylcarnitine, long chain acyl coenzyme A and lysophospholipids are increased during myocardial ischemia and infarction. These agents partition within myocardial membranes and other cellular processes as a result of their interaction with enzymes and lipids (for review, see Ref. 1). Long chain acylamides of ethanolamine [2] and their precursor *N*-acylphosphatidylethanolamine [3] reach high levels in infarcted areas of canine myocardium. Various fatty acyl derivatives of *N*-acylethanolamine prevent the permeability-dependent release of calcium from heart and liver mitochondria [4], alter rabbit skeletal muscle sarcoplasmic reticulum calcium transport activity [5] and inhibit the contractility decline of guinea pig atrium during hypoxic superfusion [6]. Of these compounds, the *N*-oleyl derivative is the most potent. Because the precise nature of the interaction of *N*-acylamides of ethanolamine with lipid membranes is unknown, we have investigated the effect of *N*-oleylethanolamine on lipid bilayer structure. In this study we show that the partitioning of *N*-oleylethanolamine within the phospholipid bilayer of unilamellar and multilamellar vesicles produces a decrease in membrane acyl chain order without significantly affecting membrane permeability.

Experimental procedures

Materials. [9,10-^{3}H]Dimyristoylphosphatidylcholine (10 Ci/mmol), [^{3}H]cholesterol (99 Ci/mmol) and [2-^{14}C]oleic acid (0.7 Ci/mmol) were

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obtained from New England Nuclear; 5,6-carboxyfluorescein from Eastman Kodak; 1,6-diphenyl-1,3,5-hexatriene, 1-[4-(trimethylammonium) phenyl]-6-phenyl-1,3,5-hexatriene, and *cis-* and *trans*-parinaric acids from Molecular Probes, Inc. (Junction City, OR). Dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine and egg phosphatidylcholine (Sigma Chemical Co., St. Louis, MO) were of above 95% purity as determined by thin-layer chromatography on silica gel in $ChCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v) and were used without further purification. *N*-[1-^{14}C]Oleylethanolamine (0.252 mCi/mmol) was synthesized from [1-^{14}C]oleic acid and ethanolamine free base and purified as previously described [2]. The synthetic compound gave a single radioactive spot on a silica gel H plate chromatographed in $CHCl_3/CH_3OH/$ concentrated NH_4OH (80 : 20 : 2, v/v) with non-radioactive standard. Unless stated otherwise, experiments were performed in a standard buffer of 10 mM Tris-HCl, 1 mM EDTA, 0.15 M NaCl, 0.01% NaN_3 (pH 7.4).

Preparation of liposomes and unilamellar vesicles. Dimyristoylphosphatidylcholine or dipalmitoylphosphatidylcholine (50 mg) were trace labeled with [^{3}H]dimyristoylphosphatidylcholine (final specific radioactivity 100–200 dpm/nmol). The lipids were co-solubilized by vortexing in a 20 × 120 mm glass conical centrifuge tube containing 3 ml of $CHCl_3$. The solvent was evaporated to dryness under nitrogen and residual solvent removed by lysophilization. Standard buffer was then added to give a final phospholipid concentration of 5 mg/ml. The mixture was vortexed vigorously producing a turbid multilamellar liposome suspension. These liposomes were either used after gel filtration on Sepharose CL-4B in some experiments or the suspension was then sonicated for 10 min under nitrogen above the phase-transition temperature of the phospholipid with a Heat Systems Ultrasonics sonifier (Model W-225R) at a setting of 3. Sonicated vesicles were then centrifuged at (100 000 × *g*) for one hour at ambient temperature to remove titanium metal and large multilamellar structures.

Preparation of fluorescently-labeled vesicles. The fluorescent probes 1,6-diphenyl-1,3,5-hexatriene and 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene in tetrahydrofuran and dimethylformamide, respectively, were added to 13 × 100 mm glass tubes. The solvents were evaporated under nitrogen and 4 ml of dimyristoylphosphatidylcholine or dipalmitoylphosphatidylcholine sonicated (unilamellar) vesicles (200 μg phospholipid/ml) were added to give a probe: phospholipid molar ratio of 1 : 200. The probe was allowed to incorporate into the vesicles overnight at the phase-transition temperature of the phospholipids.

Unilamellar vesicles containing 5,6-carboxyfluorescein were prepared as previously described [7]. Briefly, egg phosphatidylcholine (5 mg) was dissolved in chloroform, dried under nitrogen and residual solvent removed by lyophilization. Standard buffer containing 0.25 M 5,6-carboxyfluorescein was degassed on a Virtis lyophilization apparatus and added to the dried lipid. The lipid and probe were cosonicated and then centrifuged as described above for the preparation of unilamellar vesicles. Free probe was removed by chromatography of the labeled vesicle preparation on a 1.5 × 21 cm column of sepharose CL-4B in standard buffer at ambient temperature. The isolated vesicles were kept on ice to minimize leakage of 5,6-carboxyfluorescein and used the same day of preparation.

Parinaric acid solutions were prepared in ethanol and the probes incorporated into lipid vesicles as described by Sklar et al. [8]. Various additions of *cis-* and *trans*-parinaric acid (from 1 μl to 20 μl) were made to solutions of multilamellar liposomes of dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylcholine (330 nmoles of phospholipid) containing *N*-oleylethanolamine at a 5 : 1 mole ratio. The incorporation of probe into the lipid was performed at 25 °C under argon.

Incorporation of N-oleylethanolamine into phospholipid vesicles and liposomes. *N*-[^{14}C]oleylethanolamine, isotopically diluted with *N*-oleylethanolamine to 100–200 dpm/nmol, was added to phospholipid vesicles at various mole ratios of fatty acid acrylamide and lipid. The vesicles were incubated for one hour at their phase-transition temperature and then chromatographed on a 1.5 cm × 21 cm water-jacketed column of Sepharose CL-4B in standard buffer; the column was maintained at the phase-transition temperature with a

Lauda K-2R temperature controlled recirculating bath.

In other experiments the effect of temperature on the incorporation of *N*-oleylethanolamine into sonicated phospholipid vesicles was investigated. *N*-[^{14}C]Oleylethanolamine was incubated with [^{3}H]dimyristoylphosphatidylcholine vesicles (1 : 5 mole ratio) for 10 min above and below the phase-transition temperature of the phospholipid. The samples were chromatographed on a water-jacketed column of Sepharose CL-4B maintained at the incubation temperature. In other experiments, various amounts of *N*-oleylethanolamine in ethanol were incubated for 10 min at 25 °C with egg phosphatidylcholine vesicles containing 5,6-carboxyfluorescein and dye leakage was monitored by fluorescence measurements [7]. An identical sample receiving ethanol and no *N*-oleylethanolamine served as control. During the experiment samples were maintained under an argon atmosphere in order to prevent lipid peroxidation.

Fluorescence measurements. Fluorescence measurements were made on a SLM 4800S spectrofluorometer. The instrument was equipped with a four-turret water-jacketed cuvette holder externally thermostatted to a Lauda 3 circulating bath. Phase-transitions of the various probe-labeled vesicles were monitored by fluorescence polarization measurements; sample temperatures were monitored with a digital thermocouple. Phase-transitions were measured as cooling curves in which the sample temperatures were decreased in 0.5–1.0 K gradations throughout the experiment. Fluorescence polarization studies were performed with the instrument configured in the T-format with Glan-Thompson polarizers. Triplicate readings for parallel and perpendicular polarized position were signal averaged × 10 and the triplicate readings averaged for the determination of the polarization ratio. 1,6-Diphenyl-1,3,5-hexatriene and 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene fluorescence polarizations were measured at excitation-emission wavelengths of 358–425 nm, respectively, with 5 nm slits. Fluorescence intensity measurements of 5,6-carboxyfluorescein and parinaric acids in lipid vesicles were made at excitation-emission wavelengths of 490 nm–520 nm and 300 nm–425 nm, respectively. Fluorescence measurements utilizing parinaric acids were made in the ratio mode with a 418 nm Schott cutoff filter in the emission to eliminate scattering of light by vesicles.

Other methods. Phospholipid was determined by the method of Bartlett [9]. Specific radioactivities of the lipids were determined by liquid scintillation with a Beckman LS 3800 scintillation counter. Samples were dissolved in 10 ml of Scintiverse (Fisher Scientific), 1 ml methanol and 1 ml 0.2% sodium dodecyl sulfate. Radioactivity was determined as dpm corrected for quenching and cross-channel spillover of ^{3}H and ^{14}C.

Results

As is shown in Fig. 1 the incorporation of *N*-[^{14}C]oleylethanolamine into ^{3}H-labeled unilamellar vesicles of dimyristoylphosphatidylcholine (panel A) and dipalmitoylphosphatidylcholine (panel C) resulted in stable complexes as evidenced by their coelution with vesicles alone (panels B and D). In these experiments an equivalent amount of *N*-[^{14}C]oleylethanolamine was incorporated into dimyristoyl- and dipalmitoylphosphatidylcholine vesicles.

The incorporation of *N*-oleylethanolamine into sonicated lipid vesicles of dipalmitoylphosphatidylcholine was temperature dependent as shown in Fig. 2. In this experiment *N*-oleylethanolamine was incubated with dipalmitoylphosphatidylcholine vesicles at various temperatures and then chromatographed on a column of Sepharose CL-4B at the incubation temperature. As is shown, incorporation of *N*-oleylethanolamine into the vesicles increased with increasing temperature and was maximal at the phase-transition temperature of the phospholipid.

We next investigated the effect of *N*-oleylethanolamine on phospholipid membrane permeability. Oleyl coenzyme A has been shown to cause the rapid leakage of 5,6-dicarboxyfluorescein from sonicated egg phosphatidylcholine vesicles [7]. In contrast to oleyl coenzyme A, no leakage of the dye was observed from sonicated egg phosphatidylcholine vesicles after three hours of incubation at a 5 : 1 mole ratio of egg phosphatidylcholine : *N*-oleylethanolamine (data not shown).

In the next experiment, fluorescence polariza-

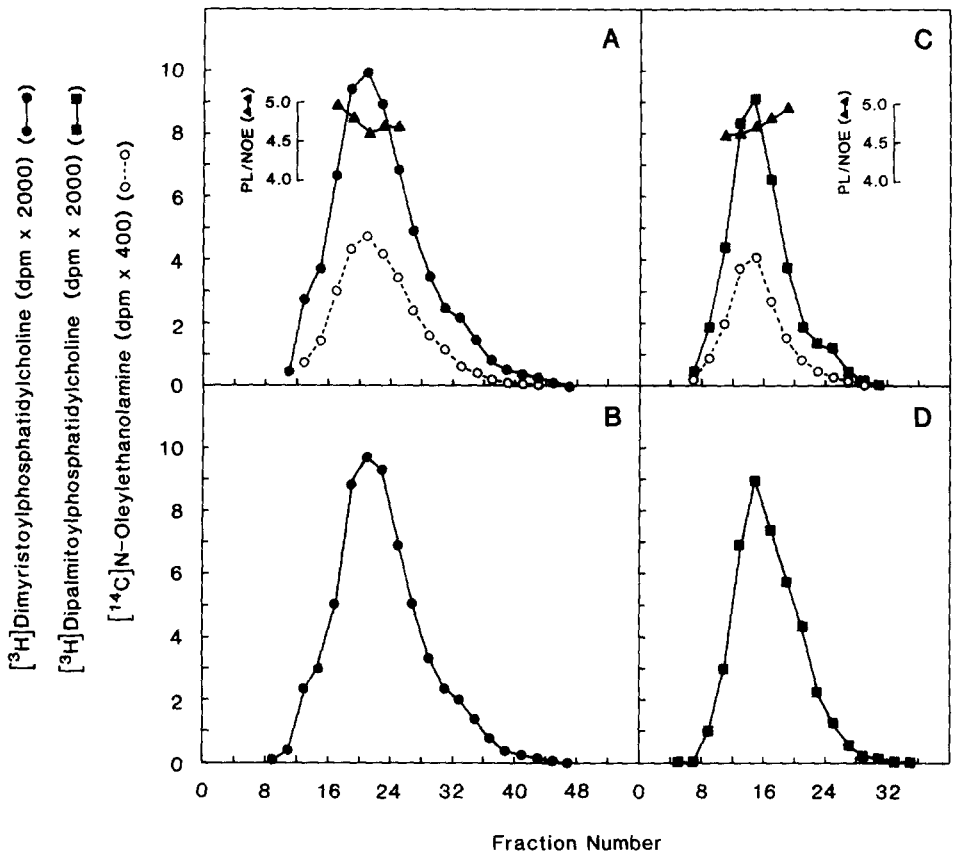

Fig. 1. Column chromatography of dimyristoyl- and dipalmitoylphosphatidylcholine unilamellar vesicles. [^{3}H]Dimyristoyl- and dipalmitoylphosphatidylcholine vesicles were incubated with *N*-[^{14}C]oleylethanolamine at a phospholipid : *N*-oleylethanolamine mole ratio of 5. The column was eluted with standard buffer and 1.5 ml fractions were collected. Panel A: dimyristoylphosphatiodylcholine + *N*-oleylethanolamine (5.1); Panel B: dimyristoylphosphatidylcholine; Panel C: dipalmitoylphosphatidylcholine + *N*-oleylethanolamine (5 : 1); Panel D: dipalmitoylphosphatidylcholine.

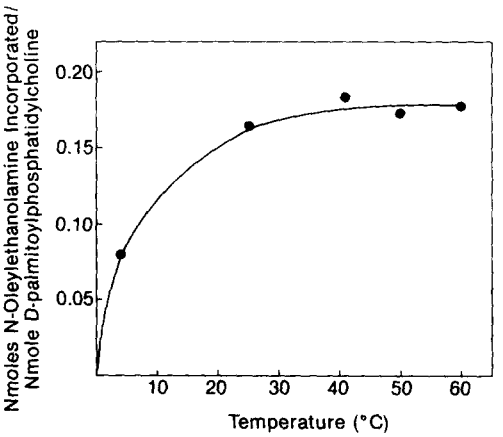

Fig. 2. Temperature dependence of the incorporation of *N*-oleylethanolamine into sonicated unilamellar dipalmitoylphosphatidylcholine vesicles. See Methods for details.

tion measurements of 1,6-diphenyl-1,3,5-hexatriene was used to monitor the effect of *N*-oleylethanolamine on the phase-transition of dimyristoyl- (Fig. 3A) and dipalmitoylphosphatidylcholine (Fig. 3B) unilamellar vesicles. Over the temperature range investigated, *N*-oleylethanolamine decreased the fluorescence polarization and lowered the phase-transition temperature of the vesicles approx. 2 K. Moreover, *N*-oleylethanolamine broadened the temperature range over which the phase transition of these lipids occurred.

The effect of *N*-oleylethanolamine on the fluorescence polarization of 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene incorporated into unilamellar vesicles of dimyristoyl- and dipalmitoylphosphatidylcholine was investigated.

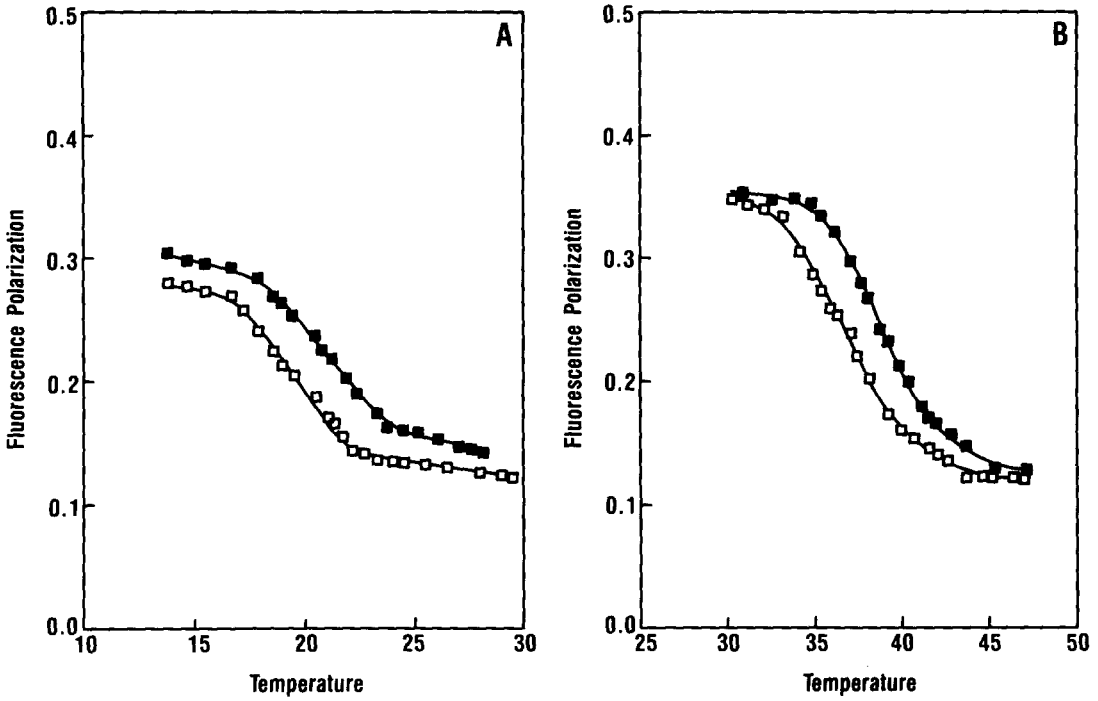

Fig. 3. Effect of *N*-oleylethanolamine on the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene incorporated into sonicated unilamellar phospholipid vesicles. Panel A: dimyristoylphosphatidylcholine (■) and dimyristoylphosphatidylcholine/*N*-oleylethanolamine (5 : 1) vesicles (□); Panel B: dipalmitoylphosphatidylcholine (■) and dipalmitoylphosphatidylcholine/*N*-oleylethanolamine (5 : 1) vesicles (□). See Methods for details.

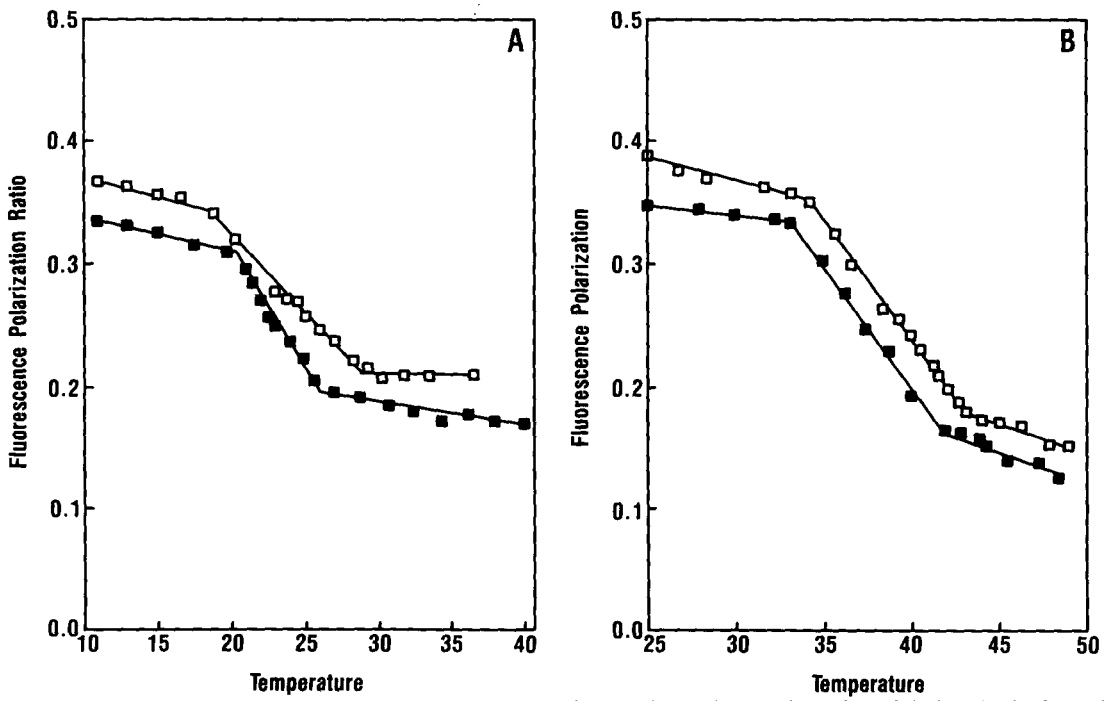

Fig. 4. Effect of *N*-oleylethanolamine on fluorescence polarization of 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene in sonicated vesicles. Panel A: dimyristoylphosphatidylcholine (■) and dimyristoylphosphatidylcholine/*N*-oleylethanolamine (5 : 1) vesicles (□); Panel B: dipalmitoylphosphatidylcholine and (■) dipalmitoylphosphatidylcholine/*N*-oleylethanolamine (5 : 1) vesicles (□).

The charged trimethylammonium group localizes the probe near the phospholipid polar head groups whereas 1,6-diphenyl-1,3,5-hexatriene partitions within the fatty acyl chain region of the phospholipid bilayer [10]. As is shown in Fig. 4, *N*-oleylethanolamine causes a significant increase in the fluorescence polarization of the probe in these vesicle preparations. The increase in fluorescence polarization of the probe was accompanied by a broadening of the phase-transition range and an increase in the phase-transition temperature of both dimyristoyl- and dipalmitoylphosphatidylcholine sonicated vesicles; however, the effect of *N*-oleylethanolamine was greater on the fluorescence polarization of the probe in dimyristoyl than in dipalmitoylphosphatidylcholine.

The effect of *N*-oleylethanolamine on the incorporation of *cis*- and *trans*-parinaric acids into liposomes of dipalmitoylphosphatidylcholine is shown in Fig. 5. The incorporation of *cis* (panel A) and *trans* (panel B) parinaric acids is linear up to a probe : phospholipid mole ratio of 0.015 and saturation occurred at mole ratios of approx. 0.05 (closed squares). The incorporation of the *cis* isomer into mixed vesicles of dipalmitoylphosphatidylcholine and *N*-oleylethanolamine was enhanced relative to the *trans* isomer (open squares, Panels A and B). In fact, the presence of *N*-oleylethanolamine (dipalmitoylphosphatidylcholine : *N*-oleylethanolamine, 5 : 1) had little effect on the incorporation of *trans*-parinaric acid in the lipid vesicles. However, as is shown in Fig. 6, *N*-oleylethanolamine produced a concentration-dependent effect on the phase-transition temperature of dipalmitoylphosphatidylcholine multilamellar structures as observed by fluorescence polarization of *cis*-parinaric acid (Fig. 6). Increased mole fractions of *N*-oleylethanolamine broadened the phase-transition temperature of dipalmitoylphosphatidylcholine and decreased the polarization values. Similar results (not shown) were noted for *N*-oleylethanolamine incorporated into dimyristoylphosphatidylcholine vesicles. As shown in Fig. 7, *N*-oleylethanolamine reduced the phase-transition temperature from 41° C in liposomes of dipalmitoylphosphatidylcholine vesicles

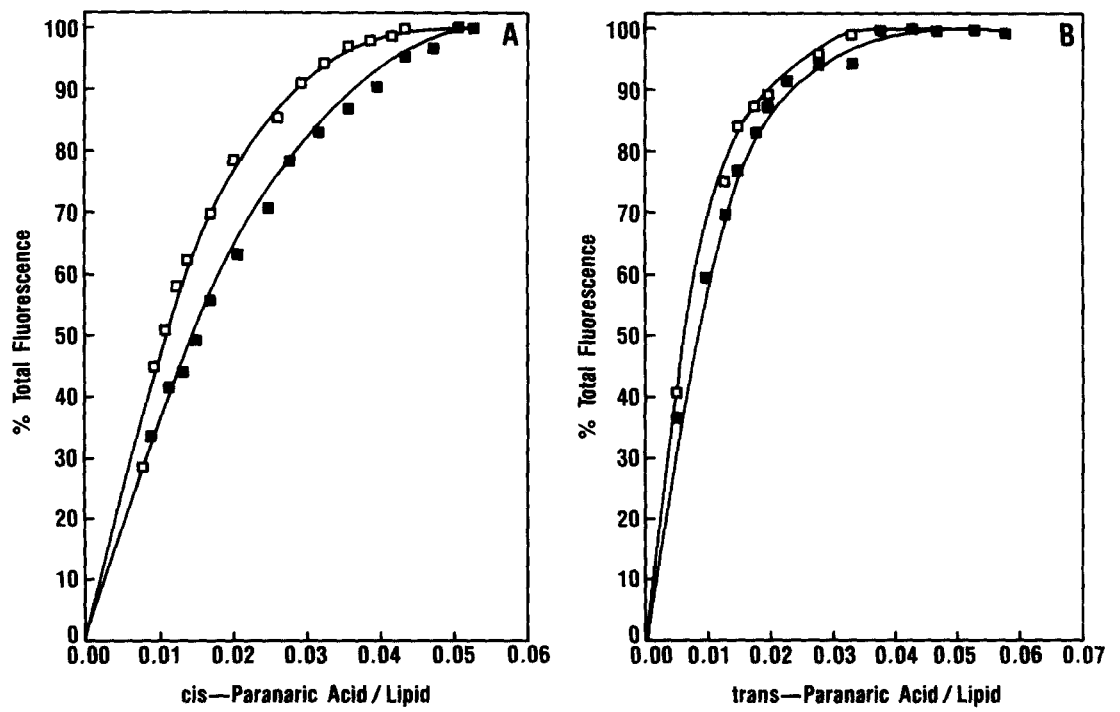

Fig. 5. The effect of *N*-oleylethanolamine on the incorporation of *cis*- and *trans*-parinaric acids into non-sonicated multilamellar vesicles of dipalmitoylphosphatidylcholine. The figure shows the incorporation of *cis* (panel A) and *trans* (panel B) parinaric acids into multilamellar vesicles of dipalmitoylphosphatidylcholine (■) and dipalmitoylphosphatidylcholine : *N*-oleylethanolamine (mole ratio 5 : 1) (□).

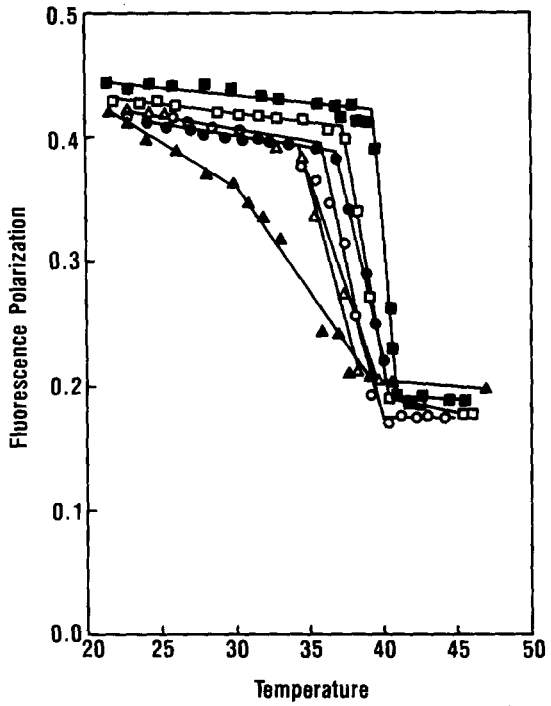

Fig. 6. The effect of *N*-oleylethanolamine concentration on the phase transition of dipalmitoylphosphatidylcholine multilamellar vesicles. Phase-transitions were measured with *cis*-parinaric acid as probe at the following mole ratios of dipalmitoylphosphatidylcholine: *N*-oleylethanolamine: dipalmitoylphosphatidylcholine alone (■); 50:1 (□); 25:1 (●); 15:1 (○); 25:1 (△); 5:1 (▲).

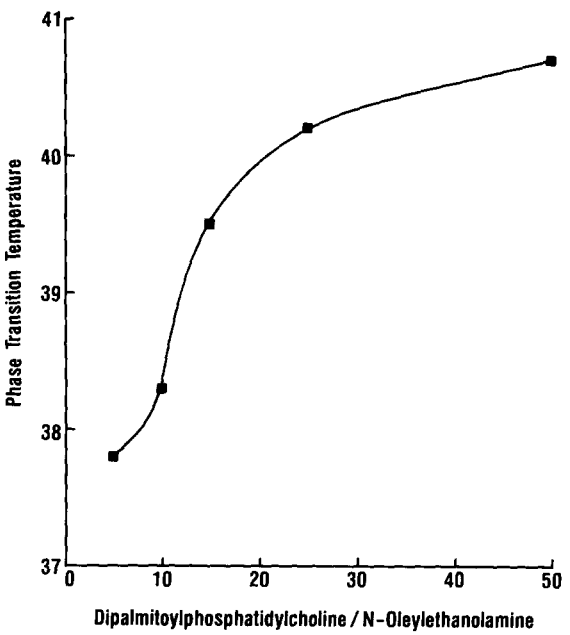

Fig. 7. The effect of *N*-oleylethanolamine concentration on the phase-transition temperature of dipalmitoylphosphatidylcholine.

to 37.8°C in liposomes having a dipalmitoylphosphatidylcholine: *N*-oleylethanolamine ratio of 5:1.

Discussion

Amphiphiles such as local anesthetics [11], fatty alcohols [12,13] lysophospholipids [14] and oleyl coenzyme A [7] have been demonstrated to alter lipid head group orientation, phase transitions, fluidity, and packing density of phospholipid bilayer membranes. Because *N*-acetylethanolamines accumulate in the infarcted myocardium [2] and in other tissues [15,16] we investigated the interaction of this compound with lipid bilayers. The present results show that *N*-oleylethanolamine is highly soluble in phospholipid bilayers and forms stable complexes. Incorporation does not compromise bilayer integrity as evidenced by the dye leakage study using 5,6-carboxyfluorescein. In contrast oleyl coenzyme A, a cardiac amphiphile, was shown to cause time dependent leakage of the dye from egg phosphatidylcholine vesicles [7]. Although *N*-oleylethanolamine is not disruptive to lipid bilayers at mole fractions of 0.2 or less, we have observed an increase in vesicle size from ≈ 290 Å to ≈ 350 Å as determined by electron microscopy (data not shown). This effect is similar to that observed with local anesthetics [17].

The incorporation of *N*-oleylethanolamine into bilayers of dipalmitoylphosphatidylcholine decreased below the phase-transition temperature of the saturated lecithin. At these temperatures the phospholipid acyl chains are primarily in the rigid *trans* configuration. *N*-Oleylethanolamine contains a *cis* 9,10-double bond that prevents its hydrocarbon chain from assuming the *trans* configuration and this may decrease the ability of *N*-oleylethanolamine to interact with the rigidly packed dipalmitoylphosphatidylcholine chains at the lower temperatures [18]. The change in phase-state that occurs with heating reflects a cooperative acyl chain melt of the phospholipid with the mole fraction of fluidized lipid increasing with temperature. The solubility of *N*-oleylethanola-

mine in dipalmitoylphosphatidylcholine increased with temperature and was optimal above the phase-transition temperature of the phospholipid suggesting the preferential incorporation of *N*-oleylethanolamine into more fluid domains. Since bilayer fluidity influences the lipid solubility of *N*-oleylethanolamine we next investigated the effect of *N*-oleylethanolamine on the physical properties of the bilayer.

N-Oleylethanolamine decreased the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene in both dimyristoyl- and dipalmitoylphosphatidylcholine unilamellar vesicles. These data indicate an increased rotationial freedom of the probe consistent with a decreased rigidity of the bilayer. The fluidizing effect of *N*-oleylethanolamine may possibly relate, in part, to the ability of the *cis* double bond to decrease the van der Waals interactions between individual phospholipid acyl chains [19]. In a study of various long-chain fatty alcohols it was found that long-chain saturated alcohols increased the phase-transition temperature of phospholipid bilayers whereas *cis* unsaturated fatty acid alcohols lowered the phase-transition temperature; *trans* unsaturated fatty alcohols behaved similarly to saturated fatty alcohols [12]. *N*-Oleylethanolamine has a free terminal hydroxyl group and thus resembles an unsaturated long-chain alcohol. By analogy, it may be expected to lower the phase transition temperature. The decrease in the dipalmitoylphosphatidylcholine phase-transition temperature was dependent on the concentration of *N*-oleylethanolamine and is similar to the concentration dependent effect of long-chain *n*-alkanols on the phase-transition temperature of the phospholipid [20,21]. Moreover, the nonlinear effect of *N*-oleylethanolamine concentration on the phase-transition temperature (Fig. 7) is similar to that described for *n*-alkanols [19].

The fluidizing effect of *N*-oleylethanolamine is consistent with the increased incorporation of *cis*-parinaric acid into dipalmitoylphosphatidylcholine multilamellar vesicles containing the fatty acyl amide. *N*-Oleylethanolamine had little if any effect on the incorporation of *trans*-parinaric acid into the bilayer. It is inferred from these studies that *N*-oleylethanolamine creates more fluid or liquid-like regions for the partitioning of *cis*-parinaric acid. The lack of enhanced *trans*-parinaric acid partitioning is consistent with fluidizing rather than solidifying effects of *N*-oleylethanolamine on phospholipid bilayers.

The charged quarternary amine moiety of 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene orients the probe near the phospholipid head group region [10]. The increase in fluorescence polarization of this probe may be due to the ordering of the phospholipid head groups in the bilayer and possibly, from the relief of repulsions between head groups induced by *N*-oleylethanolamine [20]. Hydrogen bonding of the *N*-oleylethanolamine head group hydroxyl to the phospholipid head groups would hinder motional freedom in the phosphoglycerol backbone region and may possibly account for the observed increased rigidity.

With respect to biological properties, *N*-oleylethanolamine was found to effect sarcoplasmic reticulum function [5], prevent the permeability dependent release of calcium from mitochondria [4] without inhibiting phospholipase A_2, and to alter the response of guinea pig heart muscle to hypoxic superfusion [6]. These effects possibly reflect alterations in cellular or subcellular membrane organization. In further work (unpublished results) we found the effect of *N*-oleylethanolamine on rabbit skeletal sarcoplasmic reticulum function to be associated with the inhibition or stimulation of passive calcium permeability. In fact all of the reported in vitro and in situ activities of *N*-oleylethanolamine may result from modulation of the permeability of biological membranes to ions. Fluidization of the membrane should favor increased permeability to small molecules (ions). In the present study, addition of *N*-oleylethanolamine to preformed egg phosphatidylcholine vesicles did not result in the leakage of 5,6-carboxyfluorescein. However, 5,6-carboxyfluorescein is a large molecule relative to ions of biological importance and subtle changes in the packing density induced by *N*-oleylethanolamine could increase or decrease diffusion rates of monovalent and divalent cations. Whether changes in the fluidity and order of membrane lipid constitute the predominant mechanism for alteration of enzyme and ion transport processes by *N*-oleylethanolamine is under investigation.

Acknowledgements

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