Physical and Biological Properties of Cationic Triesters of Phosphatidylcholine

Robert C. MacDonald,* Gary W. Ashley, Miho M. Shida, Vera A. Rakhmanova, Yury S. Tarahovsky,# Dennis P. Pantazatos, Michael T. Kennedy, Edvin V. Pozharski, Kent A. Baker, Ramoun D. Jones, Howard S. Rosenzweig, Kenneth L. Choi, Ruozi Qiu, and Thomas J. McIntosh§

*Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60208 USA; #Institute for Theoretical and Experimental Biophysics, Pushchino 142292, Russia; and §Department of Cell Biology, Duke University, Durham, North Carolina 27710 USA

ABSTRACT The properties of a new class of phospholipids, alkyl phosphocholine triesters, are described. These compounds were prepared from phosphatidylcholines through substitution of the phosphate oxygen by reaction with alkyl trifluoromethylsulfonates. Their unusual behavior is ascribed to their net positive charge and absence of intermolecular hydrogen bonding. The O-ethyl, unsaturated derivatives hydrated to generate large, unilamellar liposomes. The phase transition temperature of the saturated derivatives is very similar to that of the precursor phosphatidylcholine and quite insensitive to ionic strength. The dissociation of single molecules from bilayers is unusually facile, as revealed by the surface activity of aqueous liposome dispersions. Vesicles of cationic phospholipids fused with vesicles of anionic lipids. Liquid crystalline cationic phospholipids such as 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine triflate formed normal lipid bilayers in aqueous phases that interacted with short, linear DNA and supercoiled plasmid DNA to form a sandwich-structured complex in which bilayers were separated by strands of DNA. DNA in a 1:1 (mol) complex with cationic lipid was shielded from the aqueous phase, but was released by neutralizing the cationic charge with anionic lipid. DNA-lipid complexes transfected DNA into cells very effectively. Transfection efficiency depended upon the form of the lipid dispersion used to generate DNA-lipid complexes; in the case of the O-ethyl derivative described here, large vesicle preparations in the liquid crystalline phase were most effective.

INTRODUCTION

Because the demonstration that membrane lipids and other amphipaths of similar structure form closed, membrane-bounded vesicles or liposomes (Bangham et al., 1965), these structures have met with wide application (Gregoriadis, 1995). Relative to most other uses, drug delivery has received the widest attention because of the number of potential medical applications. Recently, it has become apparent that lipid-like compounds can be used to deliver DNA to cells, and this application could become clinically important. The lipids that are most efficient in delivering DNA to cells are positively charged, and such cationic lipids are now being intensively studied (Farhood et al., 1994; Felgner, 1995).

The first applications of cationic lipids to deliver nucleic acids to cells involved the interferon inducer poly I/poly C, and it was demonstrated that animals injected with such preparations demonstrated reduced viral infectivity (Magee et al., 1976; Straub et al., 1974). At about the same time it was shown that cationic liposomes adhere to, fuse with, and deliver antigenic membrane components to cells as well as induce cell-cell fusion (Martin and MacDonald, 1974, 1976b,c). Cationic lipids were also shown to facilitate transfection of a bacteriophage into protoplasts in the presence of polyethylene glycol (Rodicio and Chater, 1982). A major advance was made with the demonstration that a cationic lipid called DOTMA was much faster, simpler, cheaper, and generally more effective than alternative methods in transfecting DNA into cultured cells (Felgner et al., 1987). Since that demonstration, there have been additional reports of different cationic lipids with transfection activity (e.g., Wheeler et al., 1996; Bichko et al., 1994; Akao et al., 1994; Solodin et al., 1995; Barthel et al., 1993; Leventis and Silvius, 1990; Gao and Huang, 1991; Felgner et al., 1994).

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Address reprint requests to Dr. Robert C. MacDonald, Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60201-3500. Tel.: 847-491-5062; Fax: 847-467-1380; E-mail: macd@nwu.edu.

Gary W. Ashley's present address is KOSAN Biosciences, Inc., Burlingame, CA 94010.

Howard S. Rosenzweig's present address is Wesley Jessen, Inc., Des Plaines, IL 60018.

Abbreviations used: DOTMA, dioleoyloxypropyl-trimethylammonium ion; DMEM, Dulbecco minimal Eagle's medium; DMPC, 1,2-dimyristoyl-*sn*-glycerol-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycerol-3-phosphocholine; DOTAP, 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane; DOPE, 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine; D-PBS, phosphate-buffered saline; DPPC, 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine; EDOPC, 1,2-dioleoyl-*sn*-glycerol-3-ethylphosphocholine, EDMPC, 1,2-dimyristoyl-*sn*-glycerol-3-ethylphosphocholine; EDPPC, 1,2-dipalmitoyl-*sn*-glycerol-3-ethylphosphocholine; GMEM, Glasgow minimal Eagle's medium; MLV, multilamellar vesicle; NBDPC, 1-oleyl-2-[12-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine; PBS, phosphate-buffered saline; PC^+, cationic derivative of phosphatidylcholine; RhPE, N-Lissamine rhodamine-B-sulfonyl-1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; SUV, small unilamellar vesicle; TLC, thin layer chromatography; TNS, toluidinonaphthalene sulfonate; triflate, trifluoromethane sulfonate.

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Aside from their uses as DNA delivery agents for gene therapy (Morgan and Anderson, 1993) cationic lipids may find important applications in delivery of antisense oligonucleotides (Bennett et al., 1992). Molecules other than DNA can be delivered to cells as cationic complexes (Zhou and Huang, 1994), for example, a transcription regulator protein (Debs et al., 1990), a viral transactivator protein (Huang et al., 1995), and a mushroom toxin (Barber et al., 1996).

The compounds described in the literature as "cationic lipids" are, in fact, rarely natural products but rather are almost always cationic amphiphiles or detergents, i.e., synthetic compounds that are physically similar but chemically different from natural polar lipids (an exception is a derivative of cholesterol (Gao and Huang, 1991)). To explore the possibility that cationic amphipaths consisting only of normal cellular metabolites linked with ester bonds would exhibit low toxicity and hence be well-suited for clinical applications involving gene and drug therapy, we have begun to synthesize a series of cationic analogs of natural phospholipids to assess their activity in DNA transfection and examine their physical properties and interactions with natural lipids in cell membranes. Compounds of this type are found to exhibit physical behavior that often differs considerably from the natural precursor, and some are effective transfection agents. One of these compounds has already been demonstrated to be an effective in vivo gene transfer agent (Gorman et al., 1997).

EXPERIMENTAL PROCEDURES

Materials

All negatively charged and zwitterionic lipids and some cationic lipids (chloride salts of the dipalmitoyl, dimyristoyl, dioleoyl, and palmitoyloleoyl derivatives) were obtained from Avanti Polar Lipids (Alabaster, AL). Other cationic phospholipids were synthesized according to the procedures described below. DNA for physical studies was either the plasmid used for transfection (pCMV-β-Gal, from Life Technologies, Rockville, MD, or probe-sonicated salmon or herring sperm from Sigma). COT-1 DNA was from Life Technologies. Most other biochemicals were purchased from Sigma. Organic synthesis intermediates were purchased from Aldrich. Tissue culture reagents were from Life Technologies. All solutions were prepared with deionized water from a Millipore Milli-Q water system.

Synthetic methods

1,2-dioleoyl-sn-glycerol-3-ethylphosphocholine

DOPC (0.20–1.0 g, 0.25–1.27 mmol), used as the $CHCl_3$ solution provided by Avanti (20 mg DOPC/ml), was treated at room temperature with 3 mol equivalents of ethyl triflate (freshly opened) and the mixture allowed to react for up to several hours under dry argon or nitrogen. When the reaction was essentially over according to thin layer chromatography on silica gel, the reaction mixture was placed directly on a silica gel column, typically 30–40 times the mass of the lipid product expected. The column was washed with several bed volumes each of $CHCl_3$ and $CHCl_3$/MeOH (9:1). The bulk of the product appeared in the 9:1 fraction. The yields ranged from 50 to 95%. The higher yields were obtained in the presence of 0.5–1 equivalent of the hindered base, 4-methyl-2,6-di-*tert*-butylpyridine, although the advantage of higher yield was partially offset by increased difficulty in purification (the base eluted just ahead of the product). Methyl and ethyl triflate are commercially available (Aldrich, Milwaukee, WI) and other triflate esters can be prepared from the corresponding alcohol (Stang et al., 1982) or halide (Burk et al., 1994).

The structure of a generic cationic phospholipid triester is shown in Fig. 1. The ^{1}H-NMR spectra and liquid secondary ion mass spectra obtained were consistent with the structure of the product for all cationic phospholipids described here. Mass spectra were obtained on a VG70 mass spectrometer (VG Analytical) with cesium ion as a primary source. The dominant peak in the mass spectrum corresponded to the mass of the expected product. Proton NMR spectra were obtained on a Varian Gemini 300 MHz spectrometer. The NMR spectral feature that is diagnostic for formation of the O-ethyl bond is a multiplet centered at 4.19 ppm resulting from an overlap of the double quartet arising from the diastereomeric methylene protons of the ethyl group attached to the phosphate oxygen (the site of alkylation with ethyl triflate) plus the triplet arising from the protons attached to the β-carbon with respect to the trimethylammonium group. Other features of the ^{1}H-NMR spectrum are a quintet centered at 5.35 ppm (4H, from integration) corresponding to the vinyl protons of the oleoyl ester group (not present in fully saturated compounds); a broad signal at 5.26 ppm (^{1}H), corresponding to the single proton of the central carbon in the glycerol backbone; broad signals at 4.51 ppm (2H) and 3.92 ppm (2H) corresponding to the protons of the terminal glycerol carbon (that connected to the phosphocholine headgroup) and to protons belong to the glycerol carbon (connected to the 1-acyl group), respectively; a multiplet centered at 4.32 ppm (2H) corresponding to the protons attached to the carbon adjacent to the trimethylammonium group; and a sharp singlet at 3.34 ppm (9H) corresponding to the protons of the three methyl groups attached to the quaternary nitrogen. ^{1}H-NMR samples were run in $CDCl_3$, with tetramethylsilane (TMS) as the internal standard. The most thoroughly studied cationic derivative was O-ethyldioleoylphosphatidylcholinium triflate (mol wt 964).

General procedures for preparing lipid dispersions

Lipids were stored at −20°C in chloroform. Aliquots were removed to vials where the bulk of the solvent was removed with a stream of argon. The vial was then placed under oil pump vacuum for at least 30 min/mg to remove residual chloroform. Next, the appropriate aqueous solution was added and the tube vortexed or bath-sonicated briefly. Lipid concentrations of stock solutions were determined using a phosphate assay (Bartlett, 1959).

Phase transition characterization

The phase transition temperature of gel-phase lipid was determined by differential scanning calorimetry or from changes in light scattering (Yi and MacDonald, 1973) or fluorescence (Sackmann and Trauble, 1972). In the latter case, 2 mg/ml EDPPC was sonicated for a few seconds in H_2O to reduce light scattering, made 10^{-5} M in toluene naphthylene sulfonate, and

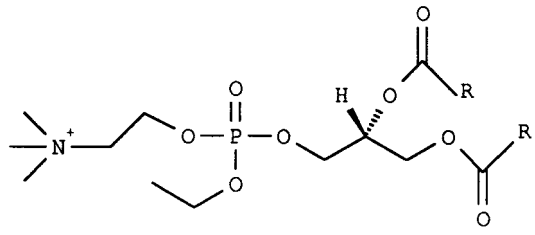

FIGURE 1 Structure of 1,2-diacyl-*sn*-glycerol-3-ethylphosphocholine. The alkyl portions of the fatty acyl substituents are represented by R.

subjected to heating and cooling cycles in a Farrand MK spectrofluorometer. The temperature was controlled with a Peltier-effect device and the rate of temperature change was 1°C/min or slower. Excitation was at 320 nm and emission was monitored at 445 nm.

Calorimetry on EDMPC suspensions in water or 0.1 M NaCl was done in a Microcal VP-DSC differential scanning calorimeter at a constant heating rate of 30°/h. The reference cell contained the same aqueous phase as the sample cell. Transition temperatures and enthalpies were determined with Microcal software.

Monolayer characterization

Surface tension of vesicle dispersions was measured using the detachment variation of the Wilhelmy method as given in MacDonald and Simon (1987). Measurements were initiated immediately after cleaning the surface by aspiration (Qiu and MacDonald, 1994). Surface potential was measured using an ionizing electrode as described (MacDonald and Simon, 1987).

Membrane fusion assays

Cell fusion

Erythrocyte fusion was done as described previously (MacDonald et al., 1978b) except that the cationic phospholipids were prepared as described above.

Vesicle-vesicle interaction assays: membrane mixing

Vesicles used for lipid mixing assays were DOPG-labeled vesicles containing 0.5% each of NBDPE and RhPE and EDOPC (chloride) unlabeled vesicles, both hydrated in deionized water or 100 mM NaCl 5 mM Tris pH 7.5 to a final concentration of 1 mg/ml. To 20 μg labeled vesicles in a 750 μl reaction volume, aliquots of unlabeled vesicles were added. Vesicle suspensions were stirred continuously. Fluorescence was monitored with an AlphaScan fluorimeter (PTI), using a bandpass of 4 nm, excitation at 460 nm, and emission at 534 nm, both before and after the addition of unlabeled vesicles.

To construct the transfer efficiency standard curve, DOPG vesicles with 0.5% NBDPE and an RhPE surface density ranging from 0 to 0.5% were prepared and their fluorescence measured. Calculation of the energy transfer was done according to a standard method (Struck et al., 1981) using the energy transfer expression $ET = 1 - F/Fo$, where Fo and F are the fluorescence with and without 1% Triton X-100. The percent lipid mixing was calculated with the equation % Lipid Mix $= (ET_i - ET_o)/(ET_i - ET_{st})$. The initial ($ET_i$) and observed ($ET_o$) transfer efficiencies represent energy transfer values before and after addition of unlabeled vesicles, and ET_{st} is the standard curve value. Percent fusion was then calculated from the observed lipid mixing and the theoretical maximum lipid mixing given the actual amounts of labeled and unlabeled vesicles that were used in a given experiment.

Vesicle-vesicle interaction assays: contents mixing

The procedure was based on that described by Kendall and MacDonald, 1982). One milligram of lipid was dried and placed under high vacuum for 1 h. The cationic lipid, EDOPC, was hydrated in 200 μl of 1 mM cobalt chloride/calcein, 10 mM Tris, 85 mM NaCl, pH 7.5. The anionic lipid, DOPG, was hydrated in 100 mM NaCl, 10 mM EDTA pH 7.5, respectively. After vortexing, the resultant liposomes were sonicated for a few seconds. Both cationic and anionic vesicles were eluted with 100 mM NaCl, 5 mM Tris, pH 7.5, the former from a Q-Sepharose anion exchange column and the latter from a Sepharose CL-4B column. Content mixing and vesicle leakage measurements were carried out in 100 mM NaCl, 300 μM $CoCl_2$, and 100 mM NaCl, 10 mM EDTA, respectively. Fluorescence increase was monitored at 520 nm with excitation at 490 nm. The maximum fluorescence (set as 100% fusion or leakage) was established by adding Triton X-100 to 1% in the absence of external cobalt ion.

Sequestration of DNA by cationic lipid and its release by anionic lipid

The procedure for examining the formation of the lipid-DNA complex was similar to that of Gershon et al. (1993) as modified by Eastman et al., 1997. Salmon sperm DNA (Sigma), 26 μM in 20 mM NaCl, was sonicated for several minutes with a probe sonicator to reduce the viscosity of the solution. By electrophoresis, the population of polynucleotides stained by ethidium bromide ranged from 400 to 700 nucleotides; 400 μl of the DNA solution were transferred to borosilicate glass culture tubes. EPOPC (1 mg/ml in 20 mM NaCl) was either dispersed by hand-shaking or by sonicating for 2 min in a bath sonicator. After the addition of increasing amounts of EPOPC to DNA solutions, the mixtures were incubated at room temperature for 30 min. Aliquots of a stock solution of ethidium bromide were then added to a final concentration of 30 μM and the fluorescence of the samples was determined at wavelengths of 530 nm for excitation and 580 nm for emission. Scans of the emission spectrum indicated that light scattering contributed negligibly to the signal.

Dissociation of the lipid-DNA complex was initiated by adding anionic lipid to DNA-lipid complexes prepared as described above except that, after ethidium bromide was added, aliquots of a stock solution of the sodium salt of oleic acid, phosphatidylserine, or phosphatidylcholine (the latter as control for a hydrophobic volume effect) were added and the fluorescence of the sample measured.

X-ray diffraction of cationic phospholipid and its complex with DNA

Two types of lipid systems were examined by x-ray diffraction, fully hydrated liposome suspensions, and partially hydrated multilayers. Liposome suspensions were made by hydrating the cationic lipid in excess water. To form the complex, lipid suspension was mixed 3:1 (wt/wt) cationic lipid/DNA (assuming an average nucleotide mol wt 330, this complex has 3% excess positive charge) and incubated with periodic vortexing for several hours at room temperature. DNA was from salmon sperm (Sigma), sonicated to reduce viscosity. These unoriented suspensions were concentrated by centrifugation, sealed in thin-walled x-ray capillary tubes, and mounted in a point-collimation x-ray camera. Partially hydrated, oriented multilayers were formed and examined by x-ray diffraction as described (McIntosh et al., 1987, 1989). After background subtraction, integrated intensities, $I(h)$, were obtained for each order h by measuring the area under each diffraction peak. For patterns from unoriented suspensions, the structure amplitude $F(h)$ was set equal to $[h2I(h)]^{1/2}$ (Herbette et al., 1977; Blaurock and Worthington, 1966). For the oriented line-focused patterns the intensities were corrected by a single factor of h due to the cylindrical curvature of the multilayers (Herbette et al., 1977; Blaurock and Worthington, 1966) so that $F(h) = [hI(h)]^{1/2}$.

Electron density profiles, $r(x)$, on a relative electron density scale, were calculated at a resolution of $d/2h_{max}$ Å from $r(x) = (2/d)S \exp[if(h)] \cdot F(h) \cdot \cos(2pxh/d)$, where x is the distance from the center of the bilayer, d is the lamellar repeat period, $f(h)$ is the phase angle for order h, and the sum is over h. Phase angles for Bragg reflections from the cationic lipid specimens were obtained by comparison with continuous transforms of liquid-crystalline phosphatidylcholine bilayers, and the phase angles for the patterns from liposomes containing DNA were chosen with the assumption that the hydrocarbon core of the bilayer was not markedly changed by the addition of DNA.

Electron microscopy of cationic phospholipid and its complex with DNA

Lipid-DNA complexes were prepared from EDOPC and ultrasound fragments (500 bp, avg. size) of calf thymus sperm DNA (Sigma) in propor-

tions of 3:1 and 10:1, by weight. Suspensions of samples were fixed with 1% osmium tetroxide in 100 mM Hepes buffer (pH 7.5) for 5–10 h. The fixed samples were centrifuged, the supernatants were discarded, and a drop of 1% agar solution applied to the pellets. The agar block was then washed five times in buffer and fixed with 1% tannic acid for 5–10 h, after which it was washed again in buffer, dehydrated in a series of water-ethanol solutions, and finally in propylene oxide. After embedding in Epon resin, sections were cut, stained in lead citrate and uranyl acetate, and examined in a JEOL 100B electron microscope. Freeze-fracture electron microscopy was done as described (Borovyagin et al., 1987).

Size characterization of cationic phospholipid vesicles and their complex with DNA by dynamic light scattering

Dynamic light scattering measurements were done with a Brookhaven Instruments BI-200SM goniometer and BI-9000 digital correlator (Brookhaven, NY). A Lexel 95, 3 Wt argon laser (Lexel Laser Inc., CA) was the source of 514 nm light.

EDOPC vesicle suspensions were prepared essentially as described below for transfection procedures. Lipid dispersions were vortexed to remove the lipid from the wall of the tube and, optionally, sonicated for 15 s in a bath sonicator. Generally, ~10 μl of a 1 mg/ml lipid suspension were mixed with the appropriate amount of plasmid DNA (dissolved in D-PBS), then D-PBS was added to produce a final sample volume of 200 μl. Measurements were made in 5 × 60 mm borosilicate glass culture tubes.

The NNLS (non-negatively least squares) algorithm provided with the instrument was used to obtain particle size distributions. In order to reduce artifacts, up to 20 correlation curves were obtained for each sample (5 m each) and the corresponding distribution functions were averaged. This procedure generates a mean and standard deviation of particles and a given peak. It should be emphasized that the distributions obtained by this procedure are *intensity* distributions. Because the size range represented by our samples is that in which Rayleigh and Mie scattering overlap and because we do not know the structure of all the contributing particles, we cannot determine what the distribution of particle number or particle mass is, even given the improbable assumption that the procedure itself is accurate for these heterogeneous samples.

Transfection of DNA into cultured cells

Transfection of BHK cells

BHK cells were transfected with plasmid coding for β-galactosidase (pCMV-βgal; Life Technologies) in 96-well microplates. Complete medium was GMEM containing 10% fetal bovine serum, 2 mM glutamine, 2% tryptose phosphate, and 50 μg/ml gentamycin. The cells were incubated at 37°C in 5% CO_2. For transfection, unless indicated otherwise, cells at ~70% confluence were provided with a medium lacking serum, tryptose, and antibiotics. Normally, the lipids were suspended in Dulbecco's PBS at 1 mg/ml and added to the plasmid at 0.1 mg/ml in PBS with gentle mixing and allowed to incubate at room temperature for 20–30 min. The DNA-lipid complex was added to the cells that were in medium lacking serum. After 4–6 h in the incubator, 1/10 volume of fetal bovine serum was added. Twenty hours after treatment with the DNA-lipid complex, the cells were assayed for β-galactosidase using a microplate fluorimetric assay (Rakhmanova and MacDonald, 1998). Three replicates were done for each condition. To date, optimum conditions yield 10–15 milliunits of enzyme activity per well of a 96-well plate. The X-gal procedure was used to determine the number of transfected cells histochemically (Sanes et al., 1986). Toxicity of cationic phospholipids and their DNA complexes was assessed by trypan blue staining. Using the same procedures, transfection of CHO cells was investigated, with results that were very similar to those obtained with BHK cells.

Transfection of mouse 3T3 cells

3T3 cells were grown on 60-mM plates and split the day before transfection to generate cells at ~50% confluence. They were grown in DMEM supplemented with 10% fetal bovine serum and 1/100 PenStrep and incubated at 37°C in 5% CO_2. Just before transfection with pSV1-luc (coding for luciferase; provided by K. Lieuw and J. D. Engel), the complete medium was replaced with DMEM. Lipids were suspended in filter-sterilized 145 mM NaCl at 0.5 mg/ml, by manual shaking, and sometimes also by bath sonication. The appropriate volume of this stock solution was added to 1 ml DMEM and an appropriate volume of stock plasmid solution was added to a second 1-ml portion of DMEM, after which the two solutions were mixed and allowed to stand at room temperature for ~30 min. The complex was then added to cells from which medium had been removed and which had been washed with PBS. After 6 h, 2 ml of complete medium were added. Approximately 48 h later, the cells were assayed for luciferase activity using an automatic injection luminometer. Two to three replicate transfections were done.

RESULTS

Cationic phospholipid stability

To verify that phospholipid triesters are sufficiently stable for physical studies and treating cells, a variety of samples were stored under various conditions and examined by thin layer chromatography after various times. It was found that EDOPC could be stored without detectable degradation of the ester bonds (oxidation was not assayed) as an aqueous dispersion for several weeks at room temperature and as a chloroform solution at −20° for >5 years. With respect to chemical stability, this lipid is thus suitable for the kinds of physical studies commonly done on normal phospholipids and for laboratory transfections and, potentially, for applications as an in vivo delivery agent. In contrast to the O-ethyl compound, the O-methyl derivative undergoes detectable spontaneous demethylation within 2 days (to yield the corresponding PC) when incubated at 37°C as an aqueous dispersion. Longer homologs, which will be described in a subsequent publication (Rosenzweig et al., manuscript in preparation) appear to be as stable as the ethyl derivative. Only results on the ethyl derivative are presented in this communication.

Although stable to hydrolysis in the absence of a catalyst, O-ethyl phospholipids are degraded by some purified phospholipases and are metabolized by cells. Fluorescent derivatives (pyrene- and NBD-containing acyl chains) were degraded by phospholipase A at an easily measurable rate, by phospholipase D slowly, and by phospholipase C at an undetectable rate. Cationic phospholipids taken up by cells were initially hydrolyzed to release the fatty acid at the SN 2 position. More extensive results on biodegradability will be presented elsewhere (MacDonald et al., 1999).

Cationic phospholipids hydrate well and disperse readily

EDOPC readily hydrates at room temperature in water and forms very large, commonly unilamellar vesicles, as shown in Fig. 2. Dispersions of PC^+ derivatives become clear very

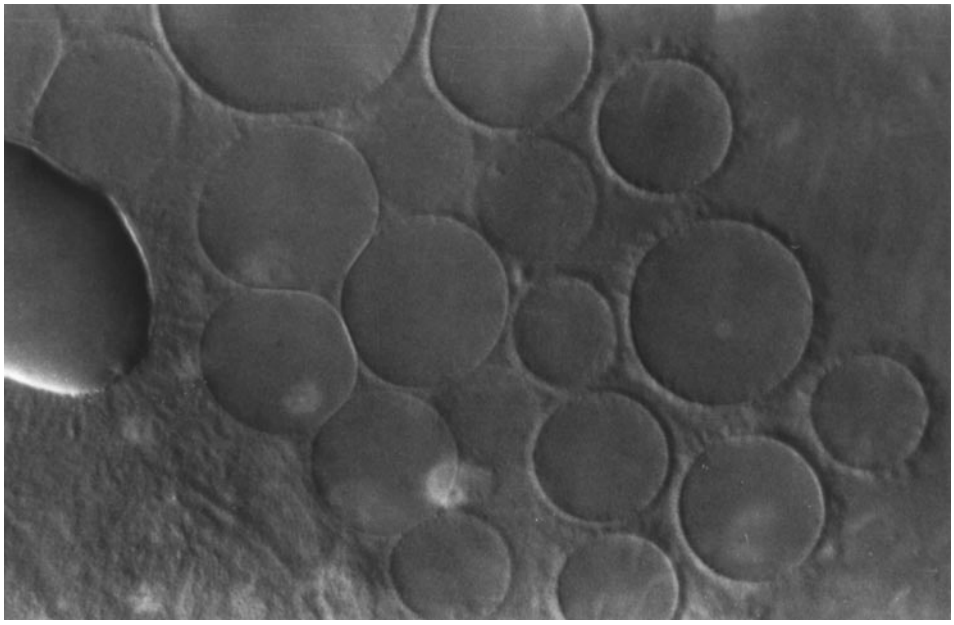

FIGURE 2 Cationic phospholipids hydrate to form bilayers with large interlamellar spacings. Several micrograms of EDOPC were sealed under water between a microscope slide and a coverslip and kept at room temperature overnight. The lipid imbibes water and generates large vesicles. The nonvesicular structure at the left is a portion of bulk lipid. The granular material is apparently small vesicles, many of which are not resolved. Some of these may be pinching off the larger vesicles. The large vesicles are ~25–50 μm in diameter. The image was obtained with differential interference optics.

rapidly—within tens of seconds at milligrams/milliliter—when sonicated in a bath sonicator (see also Fig. 9 and corresponding Results section). Hydration in 0.1 M salt solution was similar, although the proportion of unilamellar vesicles was considerably smaller than in water. EPOPC hydrated similarly, whereas EDPPC, which is in the gel phase at room temperature, did not form vesicles under these conditions.

Cationic phospholipids with saturated acyl chains exhibit gel→liquid crystal phase transition temperatures that are very similar to those of the corresponding phosphatidylcholine

Because the chain-melting phase transition of lamellar lipids involves an increase in area per molecule, one expects that the larger the net charge on the headgroup, the lower the phase transition temperature, other structural relationships being equal. As measured by fluorescent dye partitioning, the phase transition of EDPPC was centered at 37°C. This is only a few degrees below that of DPPC, which occurs at 41°C. Because the presence of the dye may have a small effect on the phase transition temperature as measured by fluorescence measurements (Blume and Eibl, 1979), we also determined cationic phospholipid transition characteristics by differential scanning calorimetry, in this case, of EDMPC. As seen in Fig. 3, the phase change was quite sharp, indicating that the two diasteromers pack in bilayers in very similar and, presumably, mutually compatible ways. The transition temperature and enthalpy of EDMPC in water were 25.4°C and 43.5 kJ/mol, both larger than the corresponding values of 23.5°C and 26 kJ/mol of DMPC (Cevc, 1993). In 0.1 M NaCl, the transition temperature and enthalpy were both lower than for water dispersions, at 23.4°C and 37.2 kJ/mol, respectively.

Surface activity of cationic phospholipids: EDPPC is released from bilayers much more rapidly than is DPPC

The rate transfer of molecules from a bilayer to the air-water interface was established by monitoring the surface tension of a suspension of vesicles (MacDonald, 1996). The surface

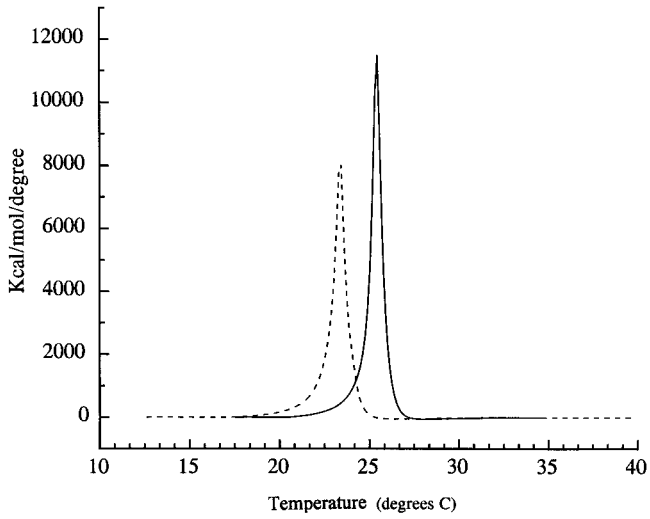

FIGURE 3 The main phase transition temperature of EDMPC is similar to that of DMPC. Multilamellar preparations of EDMPC, hydrated in either H_2O (*solid line*) or 0.1 M NaCl (*dashed line*) were subjected to differential scanning calorimetry in a MicroCal VP-DSC calorimeter at a constant heating rate of 30°C/h. Excess heat capacity is plotted against temperature. Charged lipids are expected to have a lower phase transition temperature because the electrostatic repulsion is reduced as the membrane expands in area upon melting. In fact, the positively charged EDMPC melts at only a few degrees below the transition temperature of the neutral DMPC (23.5°C). Also unexpected is that the sample in water exhibited a higher transition temperature than that in salt solution.

tensions of suspensions of DPPC and EDPPC dispersed in water and 0.1 M NaCl are shown as a function of time in Fig. 4. The experiment was done at room temperature, hence both DPPC and the cationic derivative were below their chain-melting phase transition temperature. According to the difference in initial rates of change of surface tension, and given the difference in concentrations of the dispersions, the rate of transfer of EDPPC molecules from a bilayer to a monolayer at the air interface of the vesicle suspension in NaCl solution is ~200 times greater than the corresponding rate of transfer of DPPC. The ratio of monolayer formation rates is even larger in water, in which DPPC forms a monolayer at a rate so slow that it is not readily measured. Preliminary experiments indicate that, as would be expected, a liquid crystalline form (EDOPC) is even more surface-active than a gel phase cationic phospholipid. The surface potential of EDPPC was ~150 mV greater than that of DPPC, as would be expected from the additional presence of a double layer of anions adjacent to a cationic surface.

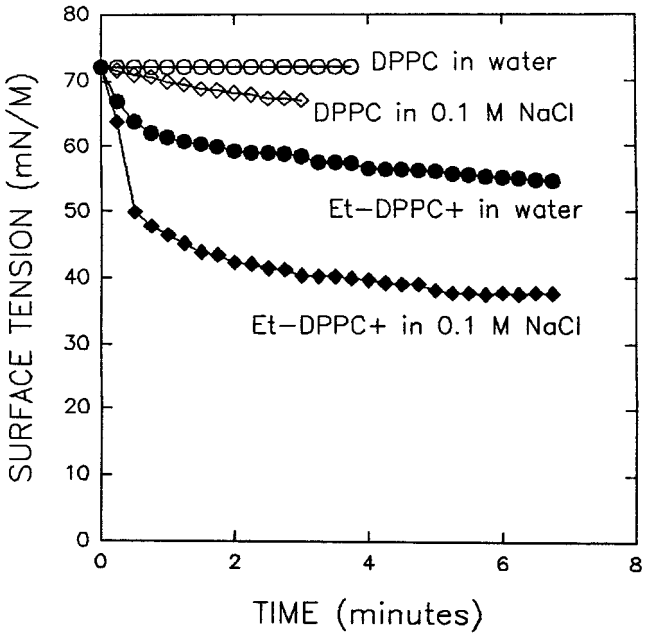

FIGURE 4 The surface activity of EDPPC vesicles is much higher than that of DPPC vesicles. The rate of formation of a monolayer at the air-water interface of a dispersion of vesicle is a measure of the ease with which molecules can be transferred from a bilayer to the surface of the dispersion. It is determined by measuring the surface tension of a liposome suspension as a function of time. As shown, dispersions of DPPC vesicles (10 mg/ml) generated a monolayer at the air-water interface quite slowly. EDPPC vesicles generated a monolayer faster even at a much lower concentration (1 mg/ml). Per unit vesicle concentration, the initial rate of transfer (following cleaning the surface) of EDPPC molecules from a bilayer to a monolayer at the air interface of the vesicle suspension was ~200 times greater that the corresponding rate of transfer of DPPC when both lipids were dispersed in 0.1 M NaCl. In water, this ratio is even larger because of the extremely low rate of transfer of DPPC under such conditions. The temperature of the experiment (RT) was below the main chain melting transition of both lipids.

Membrane fusion activity

Induction of erythrocyte fusion

Like other cationic liposome preparations we have examined (MacDonald et al., 1978a), dispersions of EDOPC induce fusion of erythrocytes. When a few microliters of a 1 mg/ml hand-shaken dispersion of EDOPC in 150 mM saline buffered with 5 mM phosphate was incubated with a drop of washed human red blood cells on a microscope slide under a Vaseline-sealed coverslip, aggregation of the cells followed by conversion to spherocytes was observed. Many of the contacting cells then fused with each other. Other amphipathic transfection agents have also been observed to possess this activity (Li and Hui, 1997). Although large multilamellar vesicles sometimes adhered to cells, vesicle-cell fusion has not been observed. If the vesicles were labeled with Rh-PE, the cells slowly became fluorescent. Thus, it is clear that molecules can transfer from the cationic phospholipid bilayer to the cell surface membrane, although it is not yet clear whether fusion is involved.

Fusion of cationic phospholipid vesicles with anionic phospholipid vesicles

A common method of investigating vesicle-vesicle fusion is to determine whether fluorophores, initially in different vesicles, mix; if the membranes do not merge, fusion is ruled out, although the converse is not proven. As shown in Fig. 5 *A*, there is very extensive mixing of membranes of vesicles of DOPG and EDOPC. In a few seconds after addition of an aliquot of positive vesicles, the mixing process is complete. Mixing is less extensive in 100 mM NaCl (*bottom curve*) than in water (*top curve*), as would be expected from reduced electrostatic interactions in the former solution.

Because membrane mixing assays do not rule out processes in which fluorophores move among membranes without fusing, and in particular do not distinguish between full fusion and hemifusion (fusion of outer monolayers only), we also applied a bulk population contents mixing assay to assess the extent to which the contents of initially separate sets of vesicles come together. We mixed cationic vesicles (of EDOPC and containing cobalt calcein) with anionic vesicles (of DOPG and containing EDTA). Upon fusion, EDTA removes the fluorescence quencher Co^{2+} from the calcein, which then acquires full fluorescence. A small amount of Co^{2+} in the external phase assures that no fluorescence increase occurs as a consequence of leakage, because any released calcein remains as the quenched cobalt chelate. As shown in Fig. 5 *B*, addition of anionic vesicles to cationic vesicles induces a definitive increase in fluorescence, much of which occurs within the first few tens of seconds. Based on the total calcein present in the cationic vesicles, (see Fig. 5 *C*; the intensity value after addition of detergent represents the fluorescence that would have been measured had all cationic vesicles fused with anionic vesicles), the amount of stable fusion, in contrast to the amount

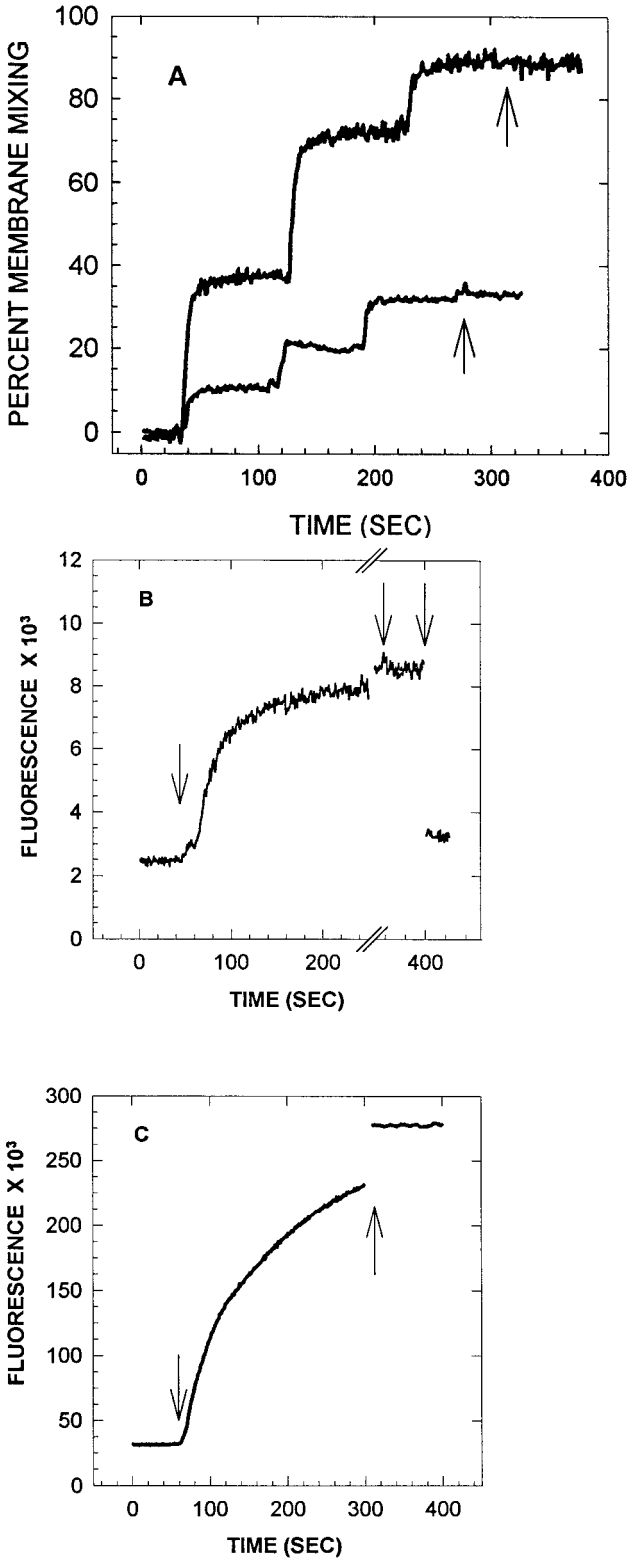

FIGURE 5 Interaction of positively and negatively charged phospholipid vesicles. (*A*) Membrane mixing. DOPG vesicles containing NBD and rhodamine probes were treated with increasing amounts of EDOPC vesicles. Measurements of fluorescence resonance energy transfer allowed estimation of the extent of membrane mixing, as plotted in the figure. *Top curve:* vesicles in water. *Bottom:* vesicles in 0.1 M NaCl. Four additions of cationic vesicles were made, corresponding to the three steps in curves and a fourth, marked by an arrow where no effect was seen. The molar ratio of cationic to anionic lipid at each addition was 0.5, 1.0, 2.0, 3.0. (*B*) Contents mixing. EDOPC vesicles containing cobalt calcein and suspended in 0.1 M NaCl and 100 μM $CoCl_2$ were treated (*first arrow*) with DOPG vesicles containing EDTA. The fluorescence rose, indicating that EDTA extracted the cobalt ion, relieving its quenching of the calcein. After a short delay, enough $CoCl_2$ (*second arrow*), was added to double the initial external concentration. There was essentially no change in fluorescence, verifying that the fluorescence was not due to external calcein. The suspension was then made 1% in Triton X-100 (*third arrow*) to lyse the vesicles. The fluorescence fell, showing that the amount of cobalt ion present was enough to quench essentially all available calcein. Fluorescence intensity is given in detector counts/s. This experiment was done under exactly the same conditions as that in (*C*), so the upper limit for the contents mixing scale may be taken to be the fluorescence intensity obtained after addition of detergent in (*C*). (*C*) Lysis. An aliquot of EDOPC vesicles containing cobalt calcein used in the experiment of (*B*) was added to 0.1 M NaCl containing EDTA and DOPG vesicles were injected (*first arrow*). Under these conditions the fluorescence rose somewhat slowly, indicating release of the vesicle contents into the external, EDTA-containing phase. This leakage was nearly complete, as revealed by the modest increase when the vesicles were completely lysed by adding Triton X-100 (*second arrow*). Fluorescence intensity is given in detector counts/s.

of membrane mixing, represents a small proportion of the vesicles, only a few percent. When the vesicles were lysed by adding detergent, the fluorescence intensity fell below the initial (premix) baseline, showing that sufficient Co^{2+} was present externally to maintain any released calcein in the quenched state. Thus, the rise in fluorescence upon vesicle mixing does not contain any contribution from contents release and must be attributed to contents mixing. Parallel measurements with the same vesicles in which EDTA instead of Co^{2+} was present in the external phase revealed that most of the vesicles did, in fact, eventually lyse.

DNA is sequestered in a complex by cationic phospholipids and is released from that complex when the lipid positive charge is neutralized with anionic lipid

The accessibility of DNA to ethidium bromide is reduced when the DNA is treated with DOTMA (Gershon et al., 1993) or other long-chain cations (Eastman et al., 1997). The DNA seems to become fully sequestered when most of the charge on the DNA has been neutralized by lipid positive charge. As shown in Fig. 6, *top*, a similar phenomenon occurs when DNA is neutralized by the cationic phospholipid, EPOPC. Formation of a complex with a larger size than either the vesicles or the DNA is also revealed by light scattering (data not shown). Fluorescence and light-scattering experiments have been done on both sheared and plasmid DNA and no significant differences were observed.

Given the obvious electrostatic nature of the cationic phospholipid-DNA complex, it would be expected that, if some of the positive charge of the lipid were neutralized by exogenous negative charge, the complex would dissociate.

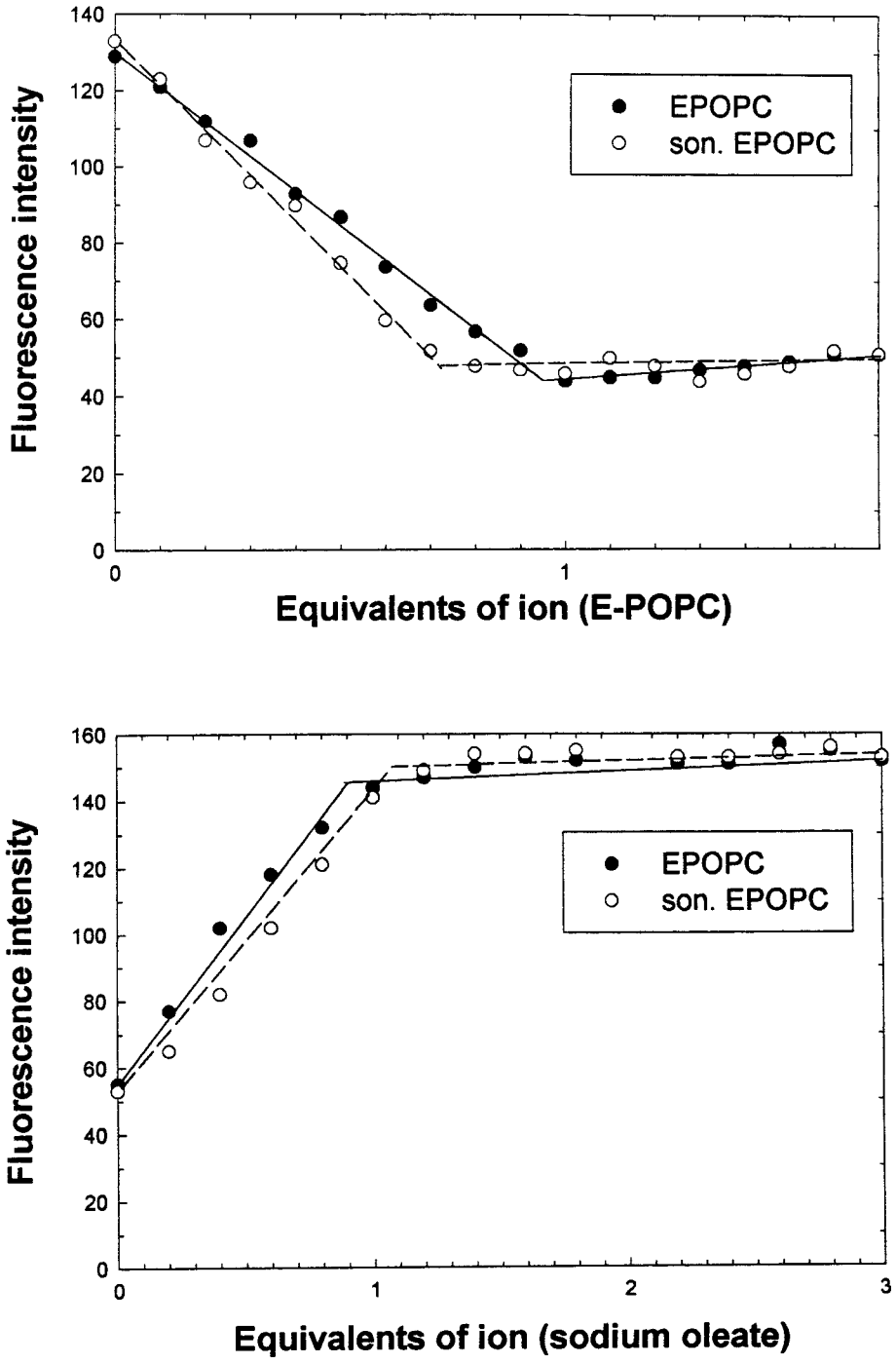

FIGURE 6 DNA becomes sequestered upon interaction with cationic phospholipid. The DNA is released again when the positive charge is neutralized with anionic lipid. *Top:* accessibility of DNA is tested by measuring the fluorescence of ethidium bromide added to a dispersion of cationic lipid-DNA complex. A series of solutions containing the same concentration of DNA were treated with various amounts of EPOPC dispersion (plotted as equivalents of positive charge relative to negative charge). Then ethidium bromide was added and the fluorescence measured. The fluorescence progressively fell to near the neutralization point where a plateau value (due to free ethidium) was reached, at which point the DNA became sequestered and inaccessible to the dye in the aqueous phase. *Bottom:* if, after a 1:1 DNA-lipid complex had been formed, oleate was added (*open circles*) and the accessibility of the DNA is again probed with ethidium bromide, it was found that the complex opened up in direct proportion to the amount of cationic lipid that had been neutralized by oleate. As indicated by the open symbols, sonication had little effect other than to cause the lipid to be somewhat more efficient in interacting with the DNA, perhaps by eliminating multilamellar vesicles.

Such dissociation is shown in the bottom panel of Fig. 6, which depicts the result of adding sodium oleate to the suspension of the complex. DNA is essentially completely released when enough oleate was added to neutralize all of the cationic lipid. If the original complex was prepared with a larger ratio of cationic lipid to DNA, then a correspondingly larger amount of anionic lipid was required. Release of DNA from the complex was also induced by phosphatidylserine, although in that case, detection of complete release of DNA was frustrated by a large increase in turbidity of the sample. Using a fluorescence resonance assay, others demonstrated that several anionic phospholipids release DNA from complexes containing the long-chain cations DOTAP and DOGS (Xu and Szoka, Jr., 1996). Phosphatidylcholine, with no net charge, does not release the DNA from the complex (not shown).

In view of the effect that sonication of EPOPC had in reducing its activity as a transfection agent (see below), we also examined the formation and dissociation of DNA from complexes of sonicated lipid. Those data are represented in

Fig. 6 by open circles. Sonication reduced somewhat (15–20%) the amount of lipid needed to sequester DNA, but there was no obvious change in the shape of the titration curves that would suggest an explanation for the effect on transfection of sonicating the lipid. All of the experiments in Fig. 6 have also been done with EDOPC as the cationic lipid; its behavior was very similar to that of EPOPC.

DNA and lipid form a lamellar complex in which strands of DNA separate bilayers of lipid

All cationic lipid and cationic lipid/DNA specimens prepared from O-ethyl derivatives gave a very broad wide-angle diffraction band centered at [1/4.5] Å, characteristic of liquid-crystalline phase lipids (Tardieu et al., 1973). For the cationic lipids alone, the low-angle diffraction patterns depended strongly on the water content; fully hydrated suspensions gave a single broad band centered at ¹⁄₃₄ Å, whereas partially hydrated multilayers gave several sharp orders of a lamellar repeat period. The lamellar repeat period was 46.4 Å at 98% relative humidity and 43.3 Å at 79% relative humidity. In the case of 3:1 cationic lipid/DNA specimens, several orders of lamellar repeat periods were recorded from both fully hydrated liposomes and partially hydrated multilayers. The observed lamellar repeat periods were 62.5 Å in excess water, 61.2 Å in 98% relative humidity, and 58.7 Å at 79% relative humidity. Thus, for each of these relative humidities, the repeat period for the lipid/DNA multilayers was ~15 Å greater than the repeat period from the cationic lipid multilayers. Although the most extensive x-ray diffraction was done on the complex formed of lipid with short, linear DNA in excess water, essentially the same lamellar pattern, with a repeat period of 62.6 Å, was seen for the complex formed in excess D-PBS with the mostly supercoiled β-gal plasmid that we use for transfection.

Fig. 7 shows electron density profiles of EDOPC alone and as 3:1 lipid/DNA multilayers at 98% relative humidity. In each profile the center of the bilayer is located at the origin. The low-density region around the bilayer center corresponds to the low-density terminal hydrocarbon core of the bilayer, and the two electron density peaks near ±17 Å correspond to the lipid headgroups. The low-density region at the outer edges of the profile correspond to the fluid spaces between apposing bilayers in the multilamellar system. In the case of 3:1 lipid/DNA, the region of the profiles from ~−17 Å to +17 Å was quite similar to the profile of the cationic phospholipid alone (Fig. 7, *top*). However, the presence of DNA gave rise to very large peaks at the edges of the profiles in the fluid spaces between adjacent bilayers (Fig. 7, *bottom*).

Thin-section electron micrographs of cationic lipid at a very low ratio of lipid to DNA (Fig. 7, *bottom*) and at a 3% excess of positive charge (3:1 lipid to DNA by weight, *top*) are shown in Fig. 8. The lipid alone was largely in the form

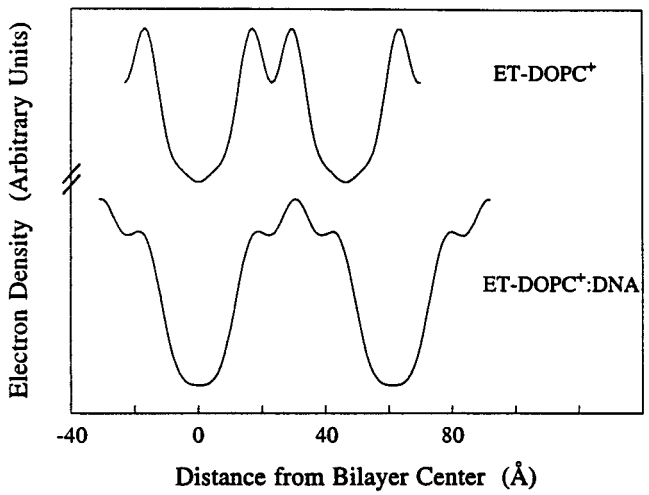

FIGURE 7 Calculated electron density profile normal to the cationic lipid bilayer in the presence and absence of DNA. The figure shows the electron density profile of cationic lipid (*top curve*) and cationic lipid/DNA multilayers (*bottom curve*), both at 98% relative humidity. The proportions of cationic lipid and DNA were such that positive charge was in slight excess. The origin corresponds to the center of the bilayer where the electron density is low due to the presence of the hydrocarbon core. The two electron density peaks, near ±17 Å, correspond to the lipid headgroups. The bilayer region is similar in the presence and absence of DNA; however, the region between the bilayers changes greatly when DNA is included. In the purely lipid system (*top*), there are low-density regions at the outer edges of the profile which correspond to the water between opposing bilayers. In contrast, in the presence of DNA (*bottom*), there are very large peaks at the edges of the profiles in the fluid spaces between adjacent bilayers, indicating that the DNA lies between relatively unperturbed bilayers.

of unilamellar vesicles which, in the presence of a small amount of DNA, sometimes adhered to one another (*bottom image*). When lipid and DNA nucleotides were present in approximately equimolar amounts, the liposomes were entirely replaced with a multilamellar structure that had a repeat distance of 62 ± 3 Å (*top image*), which is the same as that observed by x-ray diffraction for the corresponding situation, namely, complex in excess water.

The structure of the complex of EDOPC with DNA appears to be similar to that of other cationic lipids where compact lamellar structures have been observed by x-ray diffraction (Boukhnikachvili et al., 1997; Lasic et al., 1997; Rädler et al., 1997), but is clearly not hexagonal, as was observed for the cationic transfection agent DOTAP when it was combined with the co-lipid, DOPE (Koltover et al., 1998). Cationic lipid-DNA complexes have also been examined by electron microscopy and a variety of different structures have been observed (Sternberg, 1996; Gershon et al., 1993; Sternberg et al., 1994; Gustafsson et al., 1995; Eastman et al., 1997), and in one case (Gustafsson et al., 1995), structures similar to those we observed were also seen. Considerably more comparative research will be needed to determine what, if any, relationship exists between complex structure and transfection efficiency.

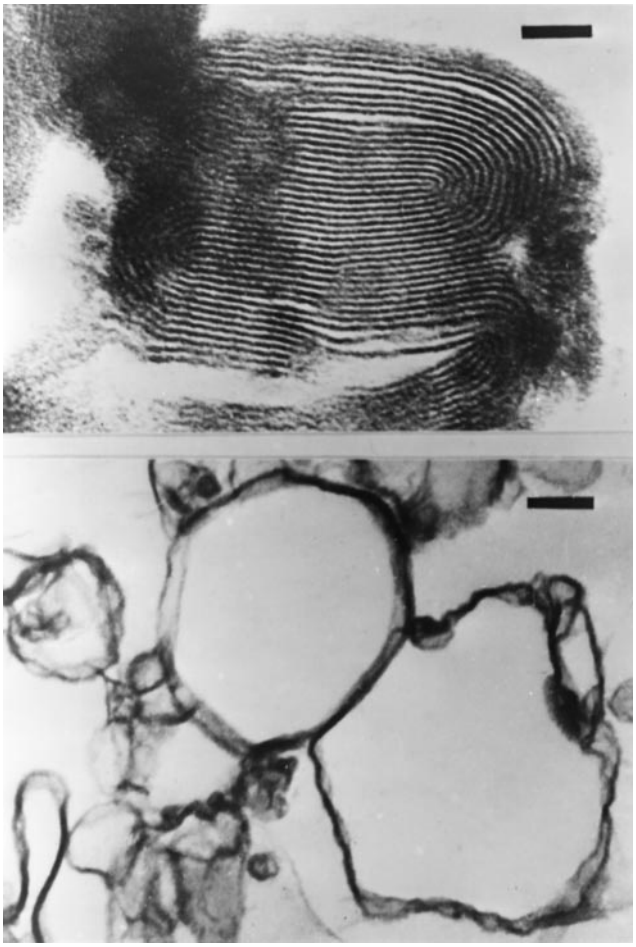

FIGURE 8 Electron microscopy of lipid-DNA complex. The panel on the bottom shows EDOPC vesicles that had been treated with sufficient DNA to neutralize ~10% of the lipid charge. Aggregates formed, but the vesicles remained largely intact. The bar corresponds to 200 nm. The panel on the top came from a similar experiment in which the net charge on the complex was near neutrality (3% excess positive charge). The vesicles were completely disrupted and replaced by a lamellar complex in which the spacing between the bilayers increased by an amount that is approximately the diameter of double-stranded DNA. The bar represents 50 nm.

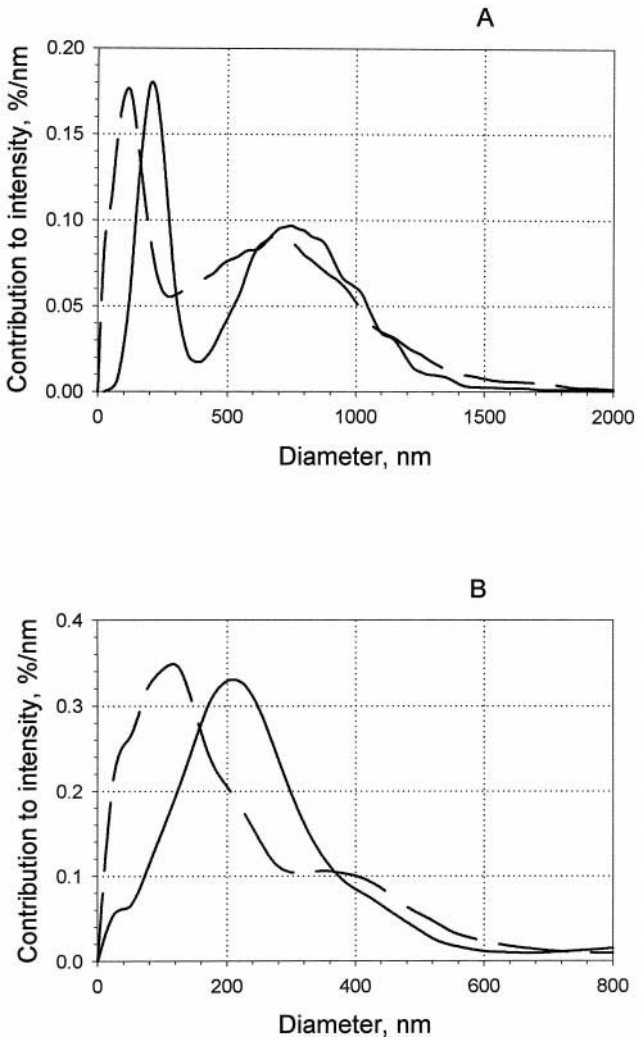

FIGURE 9 Distribution of particle sizes of lipid vesicles and their complex with DNA as determined by dynamic light scattering. The distribution of particle sizes of lipid vesicles as well as of the complex formed between those vesicles and plasmid DNA is shown in (*A*) for lipid suspended by vortexing and in (*B*) for lipid that was vortexed and subsequently sonicated for 15 s. The dashed line represents the lipid alone and the solid line represents the complex. The composition of the complexes and their method of formation were the same as those used for transfection complexes. Sonication of EDOPC for 15 s had little effect on transfection activity.

Particle size distribution of EDOPC vesicle suspensions and of their complexes with DNA

EDOPC vesicles

When lipid films were simply resuspended in D-PBS solution, a broad bimodal distribution of particle sizes was obtained (Fig. 9 *A, dashed line*). The mean size and relative contributions to scattering intensity of these two particle populations was highly reproducible, although given the uncertainties of dynamic light scattering in the case of heterogeneous populations, the size measurements must be regarded as approximate. Mean diameters of particles in populations were 150 ± 20 nm and 790 ± 70 nm, while contributions to scattering intensity were 36 ± 3% and 64 ± 3%, respectively (four independent samples). Sonication eliminated the population of larger particles and caused the distribution of particle sizes to become unimodal, being more narrow and shifted to smaller sizes (Fig. 9 *B, dashed line*). Light microscopy suggested that the larger particle population represented both large multilamellar vesicles and vesicle clusters. Such structures were also seen by negative stain (uranyl acetate) electron microscopy.

EDOPC complexes with DNA

The near-neutral EDOPC/DNA complex (3:1 by weight, 3% excess positive charge) was investigated in detail because this composition was used for most transfection experiments. As shown in Fig. 9 *A* (*solid line*), the particle size distribution remained bimodal for the DNA complex with

mean diameters of 260 ± 40 nm and 920 ± 270 nm. The contributions of the small and large populations to the scattering intensity were 31 ± 3% and 69 ± 3%, respectively (samples were processed from the same four described above). The unimodal distribution obtained for the briefly sonicated EDOPC sample remained unimodal after DNA addition, but the mean size shifted from 230 to 280 nm (Fig. 9 *B, solid line*).

Transfection activity of cationic phospholipids: efficiency and dependency upon the physical properties of their dispersions

Because a variety of cationic amphipaths are effective DNA transfection agents, it was not surprising that cationic phospholipids were also found to exhibit this activity. Because of stability and ease of preparation, we concentrated our attention on cationic phospholipids of the ethyl phosphotriester type, particularly EDOPC and EPOPC.

Dependence of transfection efficiency on dose

Cationic phospholipids effectively transfected a variety of standard culture cell types, but most of experiments were performed on BHK cells. The cells were grown in 96-well plates and assayed for β-galactosidase reporter gene by microplate fluorimetry. Transfection by cationic phospholipids was found to be quite weakly dependent upon dose and complex composition. The dependence of activity on dose of complex exhibited a broad peak. For a 3:1 or 4:1 ratio of EDOPC to DNA (wt/wt), the activity was maximal at 3 and 1.5 μg lipid per well. Weight ratios of lipid to DNA higher than 5:1 led to significantly reduced activity.

Comparison of transfection mediated by cationic phospholipid and by Lipofectamine

Lipofectamine (Life Technologies, Inc.) is perhaps the most commonly used transfection agent in molecular biology laboratories. It was therefore of interest to compare the PC^+ compounds with Lipofectamine. As shown in Fig. 10, optimal activity of EDOPC is slightly greater than that of Lipofectamine, however twice as much DNA is needed for comparable activity. EDOPC was also much less toxic than Lipofectamine (see below). In addition to low toxicity, an important advantage of the PC^+ compounds for many cells is that their activity increases in the presence of 10% serum, whereas that of Lipofectamine is virtually eliminated, at least under our conditions. Not only are PC^+ compounds active in 10% serum, but when the complex is under slightly modified conditions, the complex survives 37° incubation in 95% serum for at least 2 h (MacDonald et al., 1999). Activities of the dimyristoyl and palmitoyloleoyl derivatives of PC^+ were similar to those of the dioleoyl compound when prepared under standard procedures, although there were differences when sonicated lipid was used (see below).

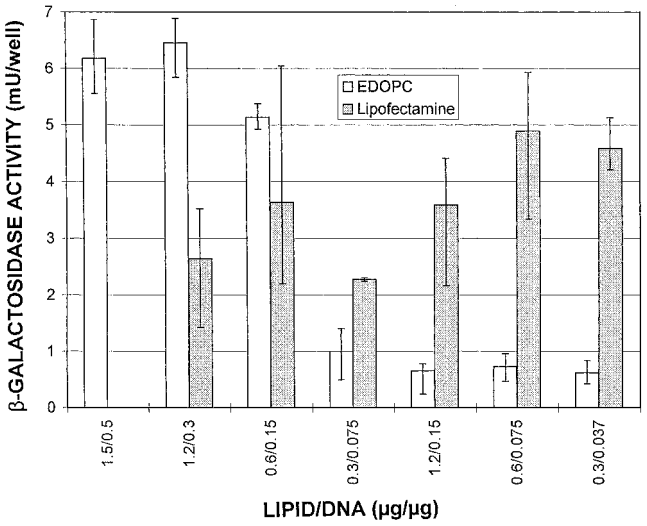

FIGURE 10 EDOPC is an effective transfection agent. Expression of β-galactosidase by BHK cells treated with plasmid complexed with either EDOPC or Lipofectamine in the amounts (μg per well) shown. Enzyme activity was determined using a microplate fluorimeter assay. Optimal activity of EDOPC was found with complexes that have a small excess of positive charge; more cationic phospholipid reduces transfection efficiency. Optimal activity with Lipofectamine, in contrast, was found at higher ratios of cationic charge to DNA charge.

Sonication reduced transfection efficiency of EPOPC

The transfection activity of complexes formed from EPOPC that had been sonicated was significantly reduced for both 3T3 cells (Fig. 11) and BHK cells. Transfection activity fell rather rapidly with increasing sonication time, reaching a plateau at 10% of the unsonicated value within 20 s. Sonicated EPOPC, although much less effective than EPOPC, nevertheless had about the same activity as the commercial product, Lipofectin (Life Technologies, Inc.). Although 20 s is not long for sonication treatments, EPOPC vesicles are reduced in size very quickly by sonication; the turbidity at 400 nm fell from an initial value of ~0.3 to a lower plateau of ~0.03 absorbance units within 20 s (lipid dispersion at 0.5 mg/ml in 145 mM NaCl). The change in activity of the lipid relative to formation of transfection complexes is almost certainly structural and not chemical, for extraction of the lipid from sonicated suspensions and analysis by thin layer chromatography revealed no degradation of the lipid, even at much longer sonication times (many minutes) than those needed to reduce transfection activity. Additional evidence for the structural hypothesis is that sometimes activity of sonicated preparations could be recovered by freezing and thawing. Finally, it is noted that the DNA-lipid complex could be sonicated for 15 s without loss of transfection activity (although prolonged sonication did eventually eliminate activity).

The magnitude of the effect of sonication depended both upon the lipid and its ionic form. The effect of sonication was larger for EPOPC than for EDMPC and EDOPC. The triflate form of all cationic phospholipids was more active than the chloride form, and sonication of EPOPC had the

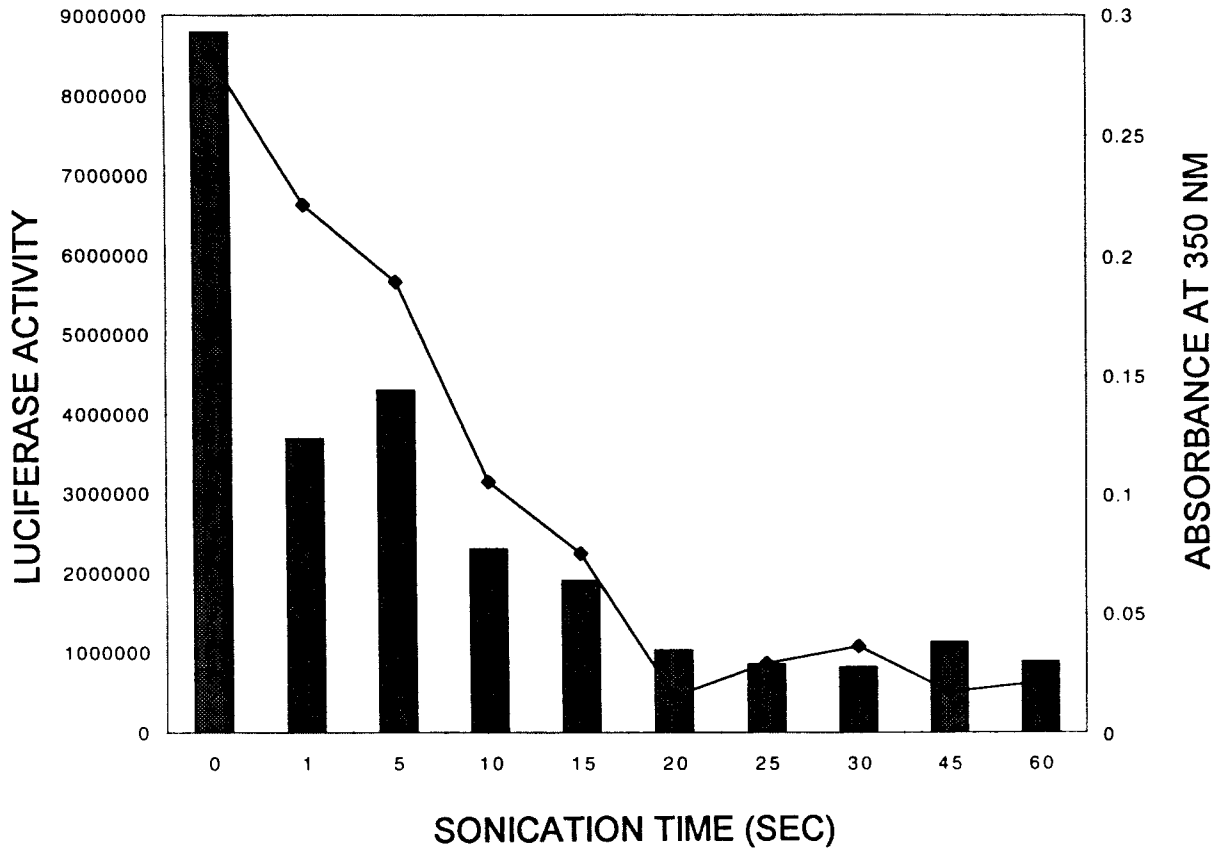

FIGURE 11 Effect of sonication on transfection efficiency of EPOPC. In the dilute solutions used for transfection, EPOPC was readily reduced to small vesicles by mild sonication, as shown by absorbance decrease with increasing sonication time. Sonicated suspensions of EPOPC generate complexes with DNA that had substantially reduced transfection efficiency.

effect of depressing the activity of the triflate, thus reducing the difference between the two ionic forms. (These experiments were done on BHK cells only.) Greater activity of the triflate than the chloride has been observed for other transfection agents (Aberle et al., 1996). A final observation with respect to sonication is that although they are not considered in this communication, some cationic phospholipids (those with long-chain alkyl groups) *require* sonication for generation of effective DNA transfection complexes. It is thus clear that the physical state of the lipid presented to DNA to form transfection complexes is likely to be one of the most important variables in determining the efficiency of transfection by those complexes.

Lipid phase transition temperature effects

EDPPC, which at room temperature is well below its liquid crystalline transition temperature, was ineffective in transfecting DNA into either 3T3 or BHK cells. EDMPC, with a transition temperature near room temperature, was as effective as EDOPC and EPOPC. It thus appears that a cationic phospholipid residing definitively in the gel state is incapable of transfecting DNA. It is likely that such lipids cannot be dispersed sufficiently well to interact with DNA. Consistent with the well-known effect of cholesterol in promoting formation of a liquid crystalline phase with DPPC, addition of equimolar cholesterol to EDPPC did generate a lipid mixture with modest transfection activity.

Colipid effects

The cationic amphipaths described in the literature as transfection agents tend to be more effective in the presence of DOPE (Farhood et al., 1995; Felgner et al., 1994; Leventis and Silvius, 1990), although there are exceptions (Barthel et al., 1993; Wheeler et al., 1996). For cationic phospholipids, addition of DOPE or cholesterol was not helpful and, in fact, reduced transfection efficiency.

Viability of cells treated with EDOPC-DNA complex under transfection conditions

Cationic phospholipids have very low toxicity; the dose at which cell recovery was reduced by half was 30–40 $\mu g/cm^2$

of area for BHK cells. This is, for example, 10–30× less toxic than Lipofectamine, based on Life Technologies' literature. Whether or not the low toxicity of the cationic phospholipids is related to their natural lipid-like structure remains to be determined. Cationic phospholipids are metabolized by cultured cells, and this may also be relevant to their low toxicity (MacDonald et al., 1999).

DISCUSSION

Alkylation of zwitterionic phospholipids provides a facile route to a new class of positively charged, bilayer-forming molecules

Amphipathic molecules have many scientific, medical, technical, and cosmetic applications. Such molecules of natural origin may be of advantage, either because of diminished toxicity or because of the characteristic required for the natural function. Aside from modification of the fatty acid substituents and derivatizing the headgroup to incorporate markers such as fluorophores, there has been relatively little research involving basic changes in the structure of natural phospholipids. We found that it is straightforward to change zwitterionic phospholipids to cationic phospholipids; in the case of alkyl derivatives, the reaction is simple and the yields are good. Although the investigation of these compounds has only begun, some unusual properties have already been revealed. The primary effect of conversion of a phosphatidylcholine to an alkyl phosphatidylcholinium ion is elimination of the negative charge, but an important secondary effect is a drastic reduction of the hydrogen bond accepting potential of the molecule. The synthetic route allows, in addition, the option to alter the size and polarity of the headgroup by varying the size and character of O-alkyl substituent. For example, the O-stearyl derivative of DOPC has been synthesized; because the stearyl moiety must lie along with the acyl groups, this compound effectively has three chains and, not surprisingly, it forms a hexagonal phase (Rosenzweig et al., manuscript in preparation). Furthermore, because cationic phospholipids are very rare in nature, they are anticipated to have unusual physical and biochemical interactions with cells. Some of their characteristics make them attractive candidates for agents to deliver nucleic acids and other pharmacological and physiological agents to cells (Barber et al., 1996).

Ready hydration of EDOPC

The O-ethyl phosphatidylcholinium compounds appear to be highly hydrophilic in the liquid-crystalline phase. EDOPC and EPOPC both hydrate very rapidly in aqueous solutions, and in water generate giant vesicles or lamellar phases with large separations. The ability of cationic phospholipids to generate giant vesicles provides an important property, because it enables investigations of positively charged bilayers under the light microscope (Panazatos and MacDonald, 1999). We are unaware of any other cationic amphiphile that exhibits this behavior without the requirement of adding a considerable proportion of neutral phospholipid. (As is typical for other charged phospholipids, cationic phospholipids do not generate giant vesicles as abundantly in solutions of high ionic strength as in low ionic strength.) The formation of individual bilayers is evident from light microscopy as well as from x-ray diffraction, which reveals a diffuse diffraction ring at a spacing corresponding to scattering from a single bilayer but no repeat, as would be expected from stacked lamellae. Furthermore, hand-shaken liposomes made from these lipids can be reduced greatly in size by brief sonication. Evidently this characteristic is a result of the presence of a net charge on the molecule (giving rise to in-plane repulsion as well as interlamellar repulsion) and perhaps also to diminished hydrogen bonding capacity. These influences may also lower the viscosity of the bilayer and lead to reduced resistance to flow.

High surface activity of O-ethylphosphatidylcholinium.

The surface activity of cationic phospholipid is revealed by the fact that hand-shaken dispersions form suds, an indication of rapid transport of molecules to a newly created surface. We chose to study the dipalmitoyl derivative because gel phase lipids generally do not transfer rapidly from bilayer vesicle to monolayer at the air-water interface (MacDonald, 1996). The rate of transfer of the cationic version was found to be more than two orders of magnitude greater than that of the precursor phosphatidylcholine (Fig. 4). Assuming that the transfer is by single molecules through the aqueous phase (Martin and MacDonald, 1976a), then qualitatively, at least, this can be explained by the change in electrostatic interactions. Removal of a PC^+ from a bilayer composed of the same lipids is encouraged by electrostatic repulsion and hence will be favored over dissociation of a zwitterionic lipid from a dipolar array. Insertion of a positive molecule into a positively charged monolayer will also be disfavored by that same charge repulsion; however, this is less important in the overall transfer process because the removal step, not the insertion step, has the higher activation energy (Nichols, 1985). The reduction of the activation energy for removing a molecule can easily be estimated, because it should equal the electrostatic energy per molecule in a charged monolayer, and the latter can be approximately calculated (Cevc, 1993). For typical areas per molecule, we calculate an energy of ~3 kcal/mol. Because the measured rate enhancement factor of 200 would correspond to a reduction in activation energy of 3.2 kcal/mol, it appears the difference in surface activity between a bilayer of PC^+ and one of PC is accounted for by the electrostatic *repulsive* forces in the former. The apparent ready removal of molecules from cationic phospholipid bilayers, at least relative to removal of molecules from zwitterionic bilayers, may be relevant to their function as transfection agents, as discussed below.

Lack of effect of positive charge on the temperature of the chain-melting phase transition

Because chain melting in a gel phase lipid is associated with an increase in area per molecule, increasing the charge on a bilayer lipid is expected decrease the phase transition temperature, which for a single ionic charge per molecule could amount to up to 20°C at low ionic strength (Jähnig, 1983; Träuble et al., 1976), a prediction that is accurately realized in the case of phosphatidylserine (MacDonald et al., 1976). We observed very little difference between the transition temperature of PC and PC^+, which means that the difference between the free energy of PC^+ and PC in the gel phase is not as large as expected or the difference between the lipids in the liquid crystalline phase is larger than expected. Thus, the presence of the ethyl group must either stabilize the gel phase or destabilize the liquid crystal phase (see Fig. 8.8 of Cevc and Marsh, 1987). Because the ΔH of PC^+ melting is significantly greater (11–17 kJ/mol) than that of PC melting, because the T_M values are similar, the ΔS of PC^+ melting must be correspondingly larger than that of PC. This is a reasonable expectation, because the charge density of the PC^+ surface is reduced by area dilation at the phase transition, which reduces the surface potential and thus releases counterions (Trauble et al., 1976). This effect does not appear to be very large, however, and an additional entropy increase would be expected from a release of electrostricted water that must accompany the area expansion at the phase transition.

Cationic phospholipids should represent a good system for examining electrostatic effects in bilayers, for in contrast to anionic lipids, their degree of ionization is fixed for all useful ranges of pH. They are also distinguished from the anionic phospholipids in that the latter engage in hydrogen bonding which, furthermore, is a function of the extent of protonation, i.e., charge on the molecule.

Membrane-membrane interactions

Like some other cationic amphipaths (Stamatatos et al., 1988; Bailey and Cullis, 1997; Düzgüneş, et al., 1989), cationic phospholipid vesicles undergo membrane merging upon contact with anionic vesicles. This mixing is extensive in water, but still significant in high-ionic-strength solutions, in contrast to that observed with another cationic amphipath, which appears to behave anomalously with respect to the influence of ionic strength (Stamatatos et al., 1988). Some of the vesicle-vesicle interactions involve fusion, as is suggested by the fact that a small proportion of the vesicles undergo contents mixing. Lysis was also common, and after a few minutes most of the vesicles had released most of their contents. Whether such lysis was because most events involve leakage or were due to processes following the initial event is not elucidated by these experiments; however, examination of individual fusion events by video fluorescence microscopy indicates that, per event, fusion is more likely than rupture (Pantazatos and MacDonald, 1999). The microscopy experiments also indicate that the increased fluorescence recorded in Fig. 5 *B* is not likely to be due to contents mixing from broken vesicles in the interior of an aggregate, as may be possible with other fusion systems in which an aggregating agent is added to a homogeneous population of vesicles (Kendall and MacDonald, 1982). The cationic-anionic fusion system has the advantage that after a few rounds of fusion, the electrostatic charges become neutralized and the vesicles become resistant to further fusion.

It remains to be elucidated how much membrane fusion contributes to the transfection activity of the cationic phospholipid complexes with DNA. Membrane fusion involving the complex could obviously be advantageous in transporting the DNA to the nucleus. Fusion of lipid vesicles was not observed with red cells, but transfer of fluorescent dye from cationic vesicles to red cell membranes was observed, which may suggest that both probe and cationic phospholipid may exchange across the contacting membrane. If this occurred at a limited area membrane such an endosome, the content of cationic lipid could rise to the point where the endosome ruptured, releasing the DNA.

DNA induces multilamellar organization of the bilayers of cationic lipid vesicles

No lamellar repeat period is observed in the x-ray diffraction pattern of hydrated EDOPC. The one feature of the pattern is a broad low-angle band at ⅓4 Å; it corresponds to the first peak in the continuous transform of a bilayer (Wilkens et al., 1971; McIntosh and Simon, 1986; Lewis and Engelman, 1983), which implies that this sample consists of bilayers with large fluid spaces between them. This is consistent with images obtained under the light and electron microscopes. The large separation is evidently due to electrostatic repulsion between the positive lamellae. When water in the preparation is partially removed, a lamellar repeating unit is observed. The electron density profile thus obtained is similar to that previously observed from quite disordered liquid-crystalline bilayers such as diarachidonoyl-PC in two aspects: 1) the high-density peak-to-peak separation across the bilayer (34 Å); 2) the absence of a prominent terminal methyl trough in the geometric center of bilayer (McIntosh et al., 1995). Such reduced order is consistent with the finding that EDOPC is ~10% more expanded in a monolayer at the air-water interface than is DOPC (R. C. MacDonald and H. L. Brockman, manuscript in preparation), as well as with the ease of dispersion of these lipids by sonication. Although there have been several electron microscope studies of DNA-cationic amphipath complexes (Gershon et al., 1993; Sternberg et al., 1994; Gustafsson et al., 1995), the molecular arrangement of the complex has not been elucidated, with the exception of the structure in which a lipid bilayer covers a strand of DNA (Sternberg et al., 1994).

The addition of DNA to cationic phospholipid causes the formation of a multilamellar system even in excess water.

DNA must therefore neutralize the lipid charge to bring apposing bilayers close together. As water is removed in an atmosphere of reduced humidity, the repeat period decreases only slightly. Repeat periods at both 98 and 79% relative humidity are ~15 Å larger for cationic lipid/DNA complexes than for cationic lipid, implying that DNA takes up ~15 Å of repeat period. This corresponds reasonably well with the 20 Å maximum diameter of hydrated DNA (Zimmerman, 1982). Furthermore, electron density profiles show extremely high density peaks between bilayers, which very strongly indicates the presence of DNA between bilayers. Observations that DNA 1) causes condensation of fully hydrated cationic phospholipid and 2) adds ~15 Å to the repeat period of cationic lipid at both 98 and 79% relative humidity implies that DNA lies parallel to the bilayer surface and cross-links apposing bilayers (15 Å is probably large enough for one layer of DNA molecules, but NOT for multiple layers between bilayers).

Although the electron density distribution was not determined for the complex prepared with plasmid, it is clear from the diffraction pattern that the EDOPC-plasmid complex is also a sandwich structure in which the bilayers are separated by strands of DNA. We presume, therefore, that it is a sandwich array that is taken into cells and is active in transfection, although because only a fraction of the DNA taken up by cells is transcribed (Zabner et al., 1995), it is possible that a minor component in the transfection complex suspension is actually the active agent.

Transfection activity of cationic phospholipids

It is evident from fluorescence microscopy observation of cells in the presence of fluorescent cationic phospholipids that uptake of both lipids and DNA is rapid and extensive; this step in the transfection process is therefore very unlikely to be rate-limiting. Transport of the DNA from the lipid complex to the nucleus, however, is probably quite slow and almost certainly incomplete as well (Zabner et al., 1995). It is possible that incorporation of DNA into the nucleus only occurs upon reformation of the nuclear membrane after cell division, but if not, then the DNA must not only escape from the endosome, but also cross the nuclear membrane. Escape from the endosome would appear to be facilitated by the propensity of cationic lipids to fusion with anionic membranes, although protein at the surface of anionic membranes clearly hinders fusion, as the absence of fusion of cationic vesicles with erythrocytes illustrates. Possibly the inner surface of the endosome is depleted of proteins relative to the cell surface whence it arises, but the anionic charge density may also be too low to support fusion. The high surface activity of cationic phospholipids could play a role in the delivery process for, if fusion is slow or infrequent, diffusion of the lipid from the external surface of the complex might be extensive enough to destabilize the endosomal membrane.

As suggested in a preliminary communication, dissociation of cationic phospholipid-DNA complexes and release of DNA might be assisted by neutralization of the cationic charge by cellular anions (Ashley et al., 1996). It has recently been shown that a number of anionic bilayer-forming lipids cause dissociation of DNA from DOTAP in vitro (Xu and Szoka, Jr. 1996) and it was suggested that direct contact between the complex and the endosomal membrane initiates destabilization of the membrane such that anionic membrane lipids undergo flip-flop from the cytoplasmic surface of the endosomal membrane to its inner surface and subsequently into the complex, leading to its dissociation. Other hydrophobic anions from the cell could equilibrate across the endosomal membrane and enter the lipid-DNA complex, facilitating DNA release. Or, as suggested above, the cationic lipid may fuse or partition into the endosomal membrane and thereby weaken it.

The finding that a only a slight excess of positive over negative charge on the complex gave optimal transfection efficiency is not unexpected. Presumably only the external surface of the complex need be positively charged—to provide for binding to the cell—and any excess lipid is a hindrance, because it must be displaced by cellular molecules before the DNA can be released.

Osmotic effects may also be important in the release of DNA from endosomes. The DNA-cationic lipid complex is composed of very large polyelectrolytes of opposite charge, hence it is essentially osmotically inactive. If the complex begins to dissociate, say, by import of cellular anions, then the released DNA, as a free polyelectrolyte, will give rise to an enormous increase in osmolarity. Thus, the escape of DNA from endosomal vesicles could be driven in significant part by water uptake.

Small vesicle preparations are less effective transfection agents than dispersions of larger vesicles, although the extent of the effect depended markedly on the lipid and its counterion. Hand-shaken preparations of other cationic amphipaths have also been reported to be most effective (Felgner et al., 1994; Holmen et al., 1995). A possible explanation for lower activity of small vesicles is that the high curvature reduces DNA-bilayer interaction such that small vesicles are less likely to be disrupted than larger ones. Aggregation without disintegration would lead to incomplete neutralization of charge and such aggregated complexes, being less osmotically inactive than fully neutralized complex, would be less able to undergo osmotic expansion upon capture of hydrophobic anions. Although data are limited, it currently appears that the smaller the lipid vesicle, the smaller the complex, and it could also be that smaller complexes are simply less likely to settle out and hence come into contact with cells during normal incubation procedures.

It has been proposed that an important function of the amino group of the cationic transfection agents is to neutralize the protons that are pumped into endocytic compartments (Boussif et al., 1995). This is not possible in the case of quaternary ammonium-containing PC^+, but a limited

capacity for accepting incoming protons would be available in the DNA (as a conjugate base) if the proton concentration were high enough to displace the cationic lipid.

Cationic phospholipids offer opportunities for a variety of new surface chemical investigations

Cationic phospholipids exhibit physical properties that, because they differ considerably from those of their zwitterionic precursors, offer opportunities for new kinds of membrane biophysical investigations. Because their synthesis allows conversion of phosphatidylcholines with any fatty acid substituents to cationic versions, they can be constructed to have minimal steric influences on membranes. Their charge should make them useful in the study of electrostatic effects on membrane stability and protein or enzyme function. Combinations of cationic phospholipids with natural anionic lipids will generate electrostatically linked arrays that will have unusual mechanical and phase transition properties (Stamatatos et al., 1988). Finally, the properties of the molecules that allow them to deliver DNA and other molecules to cells may be tailored for optimal effects in specific cells by taking advantage of the large number of available natural precursors and modifying them with any of the many compounds that may be converted into triflates.

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