O-Ethylphosphatidylcholine: A Metabolizable Cationic Phospholipid Which Is a Serum-Compatible DNA Transfection Agent

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Abstract □ 1,2-Dioleoyl-*sn*-glycero-3-ethylphosphocholine was prepared in a one-step reaction from phosphatidylcholine by reaction with ethyl trifluoromethanesulfonate. This and related *O*-alkyl phosphatidylcholines constitute the first chemically stable triesters of biological lipid structures and the first cationic derivatives of phospholipids consisting entirely of biological metabolites linked with ester bonds. The complex of cationic phospholipid and plasmid DNA transfected cells with high efficiency. Maximum efficiency of transfection was obtained with complexes in which the positive charge was a few percent in excess over the negative charge. Modest stimulation of transfection of common cell lines was obtained by continuous culture in the presence of 10% serum. Incubation of the phospholipid complex for at least 2 h at 37 °C in nearly pure serum had no deleterious effects on transfection efficiency. The lipid has low toxicity; BHK cells tolerated amounts of 2 mg/2 × 10^6 cells at concentrations of 1 mg/mL. The lipid is biodegradable; it was hydrolyzed by phospholipase A_2 in vitro and was metabolized with a half-life of a few days in cells in culture. The synthetic route to cationic phospholipids is well suited to the preparation of derivatives that are tailor-made to have a wide variety of different properties.

Introduction

Since the demonstration that membrane lipids and other amphipaths of similar structure form closed, membrane-bounded vesicles or liposomes,[1] these structures have been used in many applications.[2] Most recently, dispersions of cationic amphipaths have been used to deliver DNA to cells,[3,4] an application that could become important clinically. Although cationic liposomes were reported to be potential cellular delivery vehicles many years ago,[5−8] it was not until their efficiency in DNA delivery was recognized[9,10] that they have been widely used for that purpose. Cationic lipids are particularly effective in packaging DNA because interactions with the positive surfaces lead to formation of a rather compact particle with a high content of DNA, now frequently termed a "lipoplex".[11] In addition to their uses as DNA delivery agents for gene therapy,[12] cationic lipids may find important applications in the delivery of antisense oligonucleotides.[13] Proteins and small molecules can also be delivered to cells as cationic complexes.[14−17]

The mode of delivery of DNA to the nucleus by cationic lipids remains to be elucidated, although endocytosis is generally accepted as the major route of cellular uptake. For in vitro applications, the cationic lipids are often combined with 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), without which activity is frequently low; however, in vivo[18] or in vitro in the presence of serum,[19] cholesterol is a much more effective helper lipid. The need for DOPE has been rationalized on the basis of the tendency of this lipid to destabilize lamellar phase lipids, and it could be such an activity that facilitates escape from endosomal compartments.[20] However, some form of neutralization, perhaps by cellular lipids[21] or other cellular molecules of the cationic lipid must occur for the DNA to be released from the complex.

The correlation between in vivo and in vitro activity is generally very poor, in part because many barriers are presented to the delivery vehicle by the whole organism that do not exist in the in vitro situation. One set of such factors is due to serum, which contains, among others, proteins that can bind to the surface of the complex, lipids that can mix with or extract the lipid, and nucleases that can degrade the DNA. One test of whether the lipoplex can survive exposure to serum is to measure transfection of cultured cells in the presence of 10% serum. Even at this dilution, serum inactivates many lipoplexes, however, methods have been devised to modify the formulation of some lipoplexes so that their resistance to interference by serum is greatly enhanced.[19,22−25] Recent evidence is beginning to suggest that the complex may remain intact in serum and that the main effect of serum is to reduce cellular uptake.[19,26]

With few exceptions,[27] most cationic "lipids" are synthetic compounds that are physically similar but chemically different from natural polar lipids. The compound described here is an ethyl ester of the common cellular lipid phosphatidycholine, and hence consists of only normal cellular metabolites linked with ester bonds. It exhibits low toxicity and, given its resistance to serum, may be well suited for clinical applications involving gene and drug therapy. A closely related cationic lipid has, in fact, been found to be an efficient gene delivery agent in vivo.[28]

Materials and Methods

Synthetic Methods—1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) in $CHCl_3$ was treated at room temperature with 3 (typically) mole equivalents of ethyl triflate (ethyl trifluoromethanesulfonate, Aldrich), and the mixture was allowed to react for several hours under dry argon. In some cases, 0.5−1 mol equivalent of the hindered base, 4-methyl-2,6-di-*tert*-butylpyridine, was included to ensure more complete reaction. The reaction mixture was applied to a silica gel column that was washed with $CHCl_3$, and the product was eluted with $CHCl_3$:MeOH (9:1). Purity and identity of the product were verified by thin-layer chromatography (TLC) and mass spectral (MS) analysis, respectively.

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This reaction was also used to convert 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (Avanti), 1,2-dipyrenbutanoyl-*sn*-glycero-3-phosphocholine (DPYPC), and 1-alkyl-2-pyrenebutanoyl-*sn*-glycero-3-phosphocholine to the corresponding fluorescent cationic phospholipids, 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-ethylphosphocholine (ENBDPC), 1,2-dipyrenbutanoyl-*sn*-glycero-3-ethylphosphocholine (EDPYPC), and 1-alkyl-2-pyrenebutanoyl-*sn*-glycero-3-ethylphosphocholine, respectively. The latter two compounds were prepared by standard methods. The dipyrene compound was prepared by diacylation of glycerophosphorylcholine[29] and the monopyrene compound was prepared by monoacylation of alkyl lysophosphatidylcholine,[30] both with pyrenebutanoic anhydride that was synthesized from pyrene butanoic acid (Aldrich) according to Selinger and Lapidot.[31]

Lipid Dispersions—Lipids were stored at −20 °C in chloroform. Solvent was removed with a stream of argon followed by high vacuum for at least 30 min. The appropriate aqueous solution was then added, and the tube was vortexed. When used for transfection, normally the aqueous phase was Dulbecco's phosphate-buffered saline (D-PBS; containing calcium and magnesium) and the lipid concentration was 1 mg/mL. The molecular weight of EDOPC triflate is 964 g/mol, so that a 1 mg/mL dispersion is very close to 1 mM.

Enzyme-Catalyzed Hydrolysis—*Activity of Known Phospholipases*—To examine the enzymatic hydrolysis of a cationic phospholipid, ENBDPC, dispersed as liposomes, was incubated with partially purified enzymes,[32] and the reaction was followed by TLC. The enzymes examined were: phospholipase A_2 from *Naja naja* venom, phospholipase C from *Clostridium perfringens*, and phospholipase D from *Streptomyces chromofuscus*, Savoy cabbage, peanuts, and Brussels sprouts. All enzymes were from Sigma except the last, which was prepared according to Christie.[32]

Phospholipase A_2 Activity In Vitro—The procedure used is standard.[33] In brief, 5 mg of lipid in 100 μL of ethyl ether was combined with 10 μL of borate buffer containing 250 μg of calcium acetate and 6 units of phospholipase A_2 from either snake venom (Naja naja) or bee venom (*Apis mellifera*). The mixture was incubated at 37 °C, and samples were removed at desired intervals and analyzed by TLC on silica gel plates developed in chloroform: methanol:water (65:25:4). Reaction products were visualized by iodine staining, fluorescence quenching of fluor incorporated in the plates, or by intrinsic fluorescence of the compound itself.

The rate of hydrolysis of EDPYPC by phospholipase A_2 was quantified with a fluorescence assay,[34] modified to include detergent.[35] The hydrolysis of 0.6 μM EDPYPC in 0.1 M NaCl, 5 mM $CaCl_2$, 5 mM Tris (pH 7) buffer containing 10 mM cholate by phospholipase A_2 from *Apis mellifera* bee venom (Sigma) was measured with a Farrand spectrofluorometer with 5-nm slit widths. Various amounts of enzyme, up to 160 units, were used. At an excitation wavelength of 350 nm, emission spectra were recorded to encompass the monomer (400 nm) and excimer (480 nm) emission peaks, as well as sufficient additional lower wavelength portions of the emission spectrum to allow subtraction of the background from the peak values for the monomer. Enzyme activity was determined from changes in fluorescence intensity using 4-(1-pyrenyl) butyric acid as a standard. Porcine pancreas lipase gave measurable, but lower activity than bee venom. Triton X-100 was also tested as a detergent in this assay and found to be inferior to cholate.

Phospholipase Activity in Cells—To test for degradation of the cationic phospholipid in cultured cells, 50 μg of fluorescent ENBDPC combined with 50 μg of 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (EDOPC) was incubated with BHK cells in 25-cm^2 culture dishes. A second set of experiments was done using lipid complexed 3:1 by weight with DNA (COT-1 DNA from Life Technologies). The lipid or lipid−DNA complexes were incubated with cells for 6 h in the presence of serum-free medium, following which bovine serum was added to 10%. After 1, 3, and 7 days, the cells and the medium (separately) were extracted to isolate total lipids. After removing the medium, the cells were washed with 2.5 mL of D-PBS and then removed from the dish by scraping with a rubber policeman. Then 2.5 mL of chloroform−methanol (2:1) were added to both the medium and cell suspensions. The lower chloroform phase was removed, and the aqueous phase was re-extracted with 3.0 mL of chloroform−methanol−water (2:1:0.8). The chloroform phases were combined, the solvent removed under an argon steam, and the residue dissolved in a known volume of chloroform.[33] The lipids were analyzed by TLC and visualized using long-wave ultraviolet (uv) light. Cells treated and grown under the same conditions were also examined by fluorescence microscopy to determine the distribution of the fluorescent cationic lipid. Similar experiments were done with L-cells.

To verify that the degradation observed was not limited to the NBD derivative, the procedure just described was also carried out with two other fluorescent derivatives of a generic cationic phospholipid (PC+), EDPYPC, and 1-alkyl-2-pyrenebutanoyl-*sn*-glycero-3-ethylphosphocholine.

Transfection Mediated by Cationic Phospholipids—*Standard Procedure*—BHK cells were transfected with plasmid coding for green fluorescent protein (pGreen Lantern-1, Life Technologies) or with plasmid carrying the β-galactosidase gene (pCMV-βgal; Life Technologies). Complete medium was Glasgow minimal Eagle's medium (GMEM) containing 10% fetal bovine serum, 2 mM glutamine, 2% tryptose phosphate, and 50 mg/L of Gentamycin. The cells were seeded at densities to give ∼70% confluence at the time of transfection and incubated at 37 °C under 5% CO_2. Normally, the lipids were suspended in D-PBS at 1 mg/mL and added to the plasmid DNA at 0.1 mg/mL in D-PBS, except as stated otherwise, and incubated at room temperature for 20−30 min. The DNA−lipid complex was added to the cells that were either in medium with 10% fetal bovine serum or in medium lacking serum (total volume bathing cells was 100 μL). In the latter case, 1/10 volume of bovine serum was added to each well after 4−6 h in the incubator. Then, 20−24 h after treatment with the DNA−lipid complex, the cells were assayed for expression of the reporter gene. Expression of green fluorescent protein was monitored by fluorescence microscopy. Measurement of β-galactosidase level in transfected cells was performed by a microplate fluorimetric assay.[36] To date, optimum conditions yield 10−15 milliunits of enzyme activity per well of a 96-well plate. The X-gal (5-bromo-4-chloro-3-indolyl β-galactopyranoside) procedure was used to determine the number of tranfected cells histochemically.[37] Viability of cells after treatment by cationic lipids and lipid−DNA complexes was assessed by staining cells with trypan blue and counting them in a hemacytometer.

For comparison of toxicity and transfection efficiency in the same experiment, replicates of four were used. Twenty hours after transfection, the cells of two replicates were trypsinized for viability determination. The cells from two other replicates were assayed for β-galactosidase activity.

Incubation of Transfection Complexes in Serum—First, 1.2 μL of cationic lipid suspension (10 mg/mL) was added to 4 μL of plasmid solution (1 mg/mL). After 20 min at room temperature, the complex was incubated in 100 μL of fetal bovine serum (not heat inactivated) from 30 min to 2 h at 37 °C. Each cell sample of a 96-well plate was transfected with lipid−DNA complex containing 1.5 μg of cationic lipid and 0.5 μg of plasmid DNA (i.e., 13 μL of the serum−lipid−DNA incubation mixture). The measurement of transfection efficiency was performed as usual.

Assay for Protection of DNA in Transfection Complexes from DNase—A suspension of lipid−DNA complex (3:1, wt/wt) was prepared from EDOPC (10 mg/mL) and plasmid DNA (1 mg/mL) as already described. The EDOPC−DNA complex was then diluted in fetal bovine serum (not heat inactivated) or in D-PBS at a 1:20 ratio and incubated for 2 hs at 37 °C. As a control, plasmid DNA alone was incubated under identical conditions. The incubations were stopped by dilution into gel-loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol and by addition of sodium dodecyl sulfate to a final concentration of 1%, which also released the DNA from the cationic lipid. Afterward, the samples were electrophoresed through a 1% agarose gel and visualized by ethidium bromide staining.

To test the sensitivity of the complex to restriction endonuclease, plasmid DNA or EDOPC−DNA complex containing the same amount of DNA (4 mg) was treated with 12 units of Eco RI in a total volume of 6 μL of high salt restriction enzyme buffer. The samples were incubated for 2 h at 37 °C and diluted with D-PBS to a final volume of 100 μL. After addition of gel-loading buffer and sodium dodecyl sulfate, they were analyzed by electrophoresis on a 1% agarose gel as already described.

Fluorescence Microscopy of Lipid and DNA Uptake by Cells—BHK cells were grown in 35-mm culture dishes according to conditions already described. To generate fluorescent lipid mixtures, *N*-lissamine-rhodamine-B-phosphatidylethanolamine (RhPE) or ENBDPC was mixed with EDOPC in chloroform,

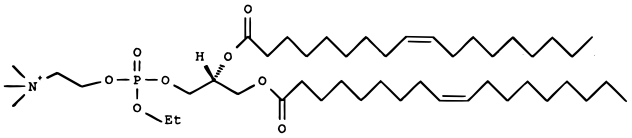


Figure 1—Structure of the cationic phospholipid EDOPC.

usually at 2.5−5 mol % fluorescent lipid. Mouse COT-1 DNA (50−300 bp) was used as is at 1 mg/mL in water or labeled with one of the high-affinity intercalating dyes, YOYO-1 or ethidium homodimer, at a ratio of 2 nanomol of dye per 10 μg of DNA. To verify that the pattern of DNA fluorescence seen in cells was not unique to COT-1 DNA labeled with an intercalating dye, we did additional experiments of this type using the standard plasmid DNA (pCMV-βgal) that was covalently labeled with Alexa maleimide dye with the FastTag Reagent (Vector Laboratories). For these experiments the lipid label was reduced to 0.5 mol % of RhPE to avoid dissimilar intensities of lipid and nucleic acid fluorescence.

Results

***O*-Alkylation of Phospholipids with Alkyl Trifluoromethanesulfonates (Triflates)**—Triflates react with phospholipids (e.g., DOPC) to generate *O*-alkyl derivatives (Figure 1). The reaction was complete in a few hours or less at room temperature, with yields up to 90%. The alkylated product migrates on silica gel plates with a R_f of 0.56 and was well separated from the reactant, which migrates with a R_f of 0.32 in a developing solvent of chloroform−methanol−water, 65:25:4. The structure of the product was verified by proton nuclear magnetic resonance (NMR) and MS analysis. The characteristic new absorption for the *O*-ethyl compound is a double quartet (one quartet for each molecule of the diastereomeric pair) at ~4.2 ppm. It may be mentioned that the reaction with ethyl triflate is not unique, and the reaction of phosphatidylcholines with other alkyl triflates having chain lengths as long as C_{18} (prepared from the corresponding alcohol and triflic anhydride[38]) has also been found to proceed smoothly.[39]

Cationic Phospholipid Stability and Metabolism—Although the experiments reported here were mostly done using material that was synthesized within the previous year, material was on hand from some early syntheses, having been stored in chloroform at −20° for nearly 5 years. Using current conditions for transfection, this material was found to have retained its ability to mediate transfection of DNA. According to TLC results, there was no detectable breakdown over that time period. As an aqueous dispersion, EDOPC can be stored for at least 3 months in the freezer without significant loss in transfection efficiency. This lipid is thus more than adequately stable for a wide variety of applications as a gene transfer agent.

Although stable to hydrolysis in the absence of a catalyst, cationic phospholipids are degraded by some purified phospholipases. ENBDPC was treated with purified phospholipases from various sources by standard procedures. Hydrolysis was established by TLC of the reaction mixture. Reaction in the presence of phospholipase C (Clostridium) was too slow to be detected under our conditions. Phospholipase D (cabbage) produced slow degradation of ENBDPC. The product of this reaction has not been unambiguously identified, but based on its R_f, phosphatidylethanol appears to be the only possibility. Phospholipase A_2 (cobra and bee venom) catalyzed the release of the NBD fatty acid. This reaction was also investigated with EDPYPC, the reaction of which could be monitored in the fluorimeter.

Figure 2 shows the extent of phospholipase A_2-catalyzed hydrolysis of EDPYPC (*O*-ethyl cationic phospholipid with two pyrene butanoic acyl substituents in place of fatty acyl chains) as a function of time. The intact molecule exhibits

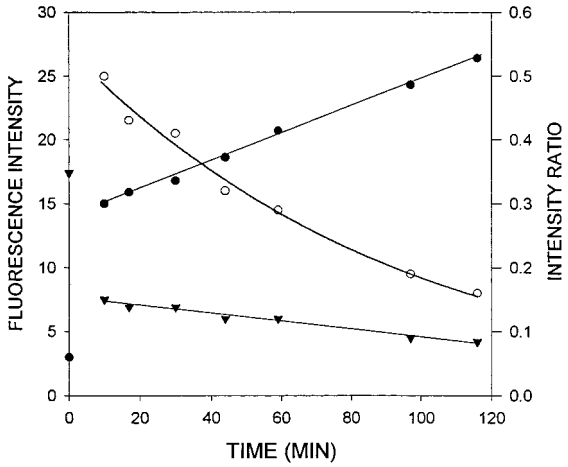


Figure 2—Time course of hydrolysis of dipyrenbutanoyl cationic phospholipid by phospholipase A_2. Hydrolysis of the pyrene-labeled fluorogenic phospholipase substrate is indicated by the increase in monomer emission (solid circles) and decrease in the excimer emission (triangles) as one of the pyrenebutanoic acid moieties is removed from the phospholipid. The substrate was dispersed in Tris buffer containing cholate, calcium ion and phospholipase A_2. The ratio of monomer to excimer emission intensity is given by the open circles.

excimer emission at 480 nm, whereas pyrene moieties that are not in contact with each other emit at 400 nm. Thus, hydrolysis can be quantified according to the rate of decrease in eximer emission and increase in monomer emission. The fluorescence intensity of the monomer (closed circles) increases markedly, but that of the excimer (triangles) falls modestly, as does the ratio of the former to the latter (open circles). This pattern shows that one of the pyrene acyl chains was removed from the molecule, for as hydrolysis occurs, the pyrene butanoic acid becomes diluted into detergent such that its emission as a monomer increases and concomitantly the excimer dissociates, leading to a reduction of its emission. Because there was initially little monomer and much excimer, the fractional change in monomer emission was large and that of the excimer was small.

The generation of product was linear for 2 h, as shown in Figure 2, however, abrupt changes in both monomer and excimer intensities are seen at very early times. This result could be due either to hydrolysis of the first molecules that bind to the enzyme followed by a steady-state rate dictated by a slower dissociation of the product from the enzyme or by a change in the conformation of the substrate—and thus in the probability of excimer formation—as the substrate binds to the enzyme (or perhaps other proteins in the solution). The average hydrolysis rate catalyzed by amounts of enzyme ranging from 40 to 160 units, as measured in experiments such as that of Figure 2, was 66 pmol/h/mg.

Comparison of the rate of hydrolysis of dipyrenebutanoylphosphatidylcholine with that of the cationic compound of Figure 2 showed that the cationic lipid was hydrolyzed at a rate ~40 X slower than that of the zwitterionic compound.

Using TLC, we determined the approximate relative rates of hydrolysis of dioleoylphosphatidylcholine and dipyrenebutanoylphosphatidylcholine. The procedure involved reducing the amount of enzyme used with the former substrate until the amount of product generated in a convenient reaction time (few hours) was approximately the same as that generated from the latter substrate. Based on this experiment, we estimate that pyrene butanoic acid was removed from phosphatidylcholine 1−2 orders of magnitude more slowly than was oleic acid. This result is not surprising, because others who have used the pyrene excimer assay have reported that when the pyrene group

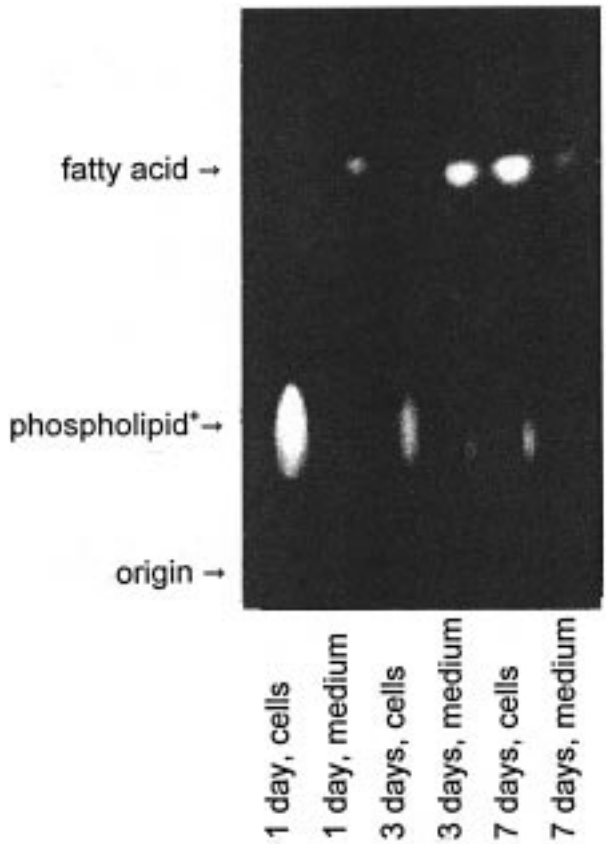


Figure 3—Cationic phospholipid degradation by cells. BHK cells were treated with a DNA–lipid complex in which the lipid was fluorescent (one acyl chain was NBD-dodecanoyl) and incubated with cells. Cells and medium were extracted with chloroform–methanol after 1, 3, and 7 days. The extracts were chromatographed, yielding the thin layer chromatogram shown, which was imaged under fluorescence illumination. Extent of degradation is indicated by growth of the upper spot and diminution of the lower spot.

is close to the ester bond, it reduces reactivity by about one order of magnitude.[40]

In addition to being susceptible to hydrolysis by isolated enzymes, PC+ is degraded by cells, as shown in Figure 3, which is the image of a TLC plate of extracts of ENBDPC that had been combined with DNA (1:1, by charge) and incubated with BHK cells for up to 7 days. Hydrolysis of ENBDPC (lower spot) to NBD−dodecanoic acid (upper spot) and lysoPC+ (not fluorescent) was detectable in the medium after 1 day, in cells after 3 days, and was very extensive in both cells and medium after 7 days. The amount of PC+ in the cells decreased drastically, whereas the fatty acid concentration in the cells rose considerably. In the same way, we also examined the hydrolysis of ENBDPC that had been presented to cells in the absence of DNA. Although there were some perceptible differences in the TLC pattern, essentially the same result was obtained, namely, the cationic phospholipid was largely metabolized within a week under culture conditions. To verify that hydrolysis of the cationic lipid was not limited to the NBD derivative, experiments identical to those of Figure 3 were done with EDPYPC and with 1-alkyl-2-pyrenebutanoyl-*sn*-glycero-3-ethylphosphocholine. These compounds were degraded to essentially the same extent as was the NBD-containing substrate. Furthermore, using the same methods, we found mouse L-cells also hydrolyze ENBDPC.

Transfection of DNA Mediated by EDOPC—To assess transfection efficiency of EDOPC, β-galactosidase and, to a lesser extent, green fluorescent protein were used as reporter genes. Cells used for routine assays were BHK,

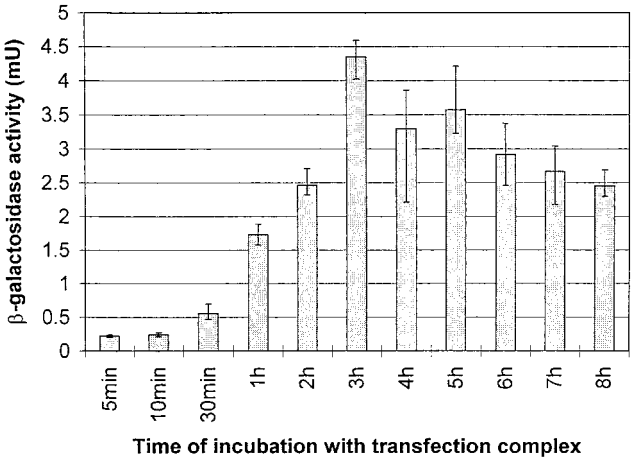
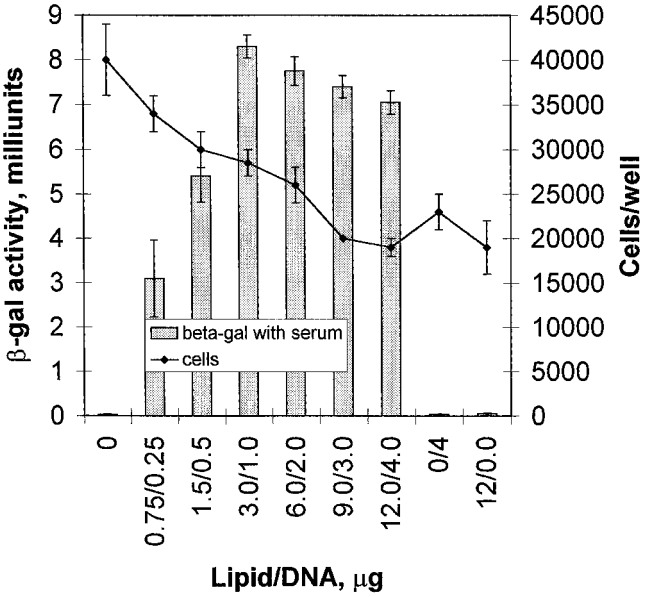


Figure 4—Time course of expression of β-galactosidase following delivery of EDOPC–DNA complex to BHK cells. EDOPC–DNA in amounts of 1.5 μg of EDOPC and 0.5 μg of DNA per well was incubated with BHK cells for various times (5 min to 8 h) as shown in the figure. The total volume of medium in each well was 100 μL. The medium with transfection complex was then aspirated and replaced with medium containing 10% fetal bovine serum. The efficiency of transfection was determined by the standard method. Bars show high and low values of three replicates.

but we also carefully examined the response of CHO cells and did less extensive examination of several other common cell lines. Transfected cells were visualized for expression of green fluorescent protein by fluorescence microscopy. For histochemical detection of β-galactosidase, the X-gal substrate was used. A large proportion of cells—up to 50% of the total—expressed β-galactosidase.

The quantitative assay for β-galactosidase used most frequently was microplate fluorimetry of fluorescein di-β-galactoside as the substrate. As shown in Figure 4, a 3−5 h incubation with EDOPC−β-galDNA complexes was sufficient for maximum expression of β-galactosidase in BHK cells. With both CHO and BHK cells, we found EDOPC gave slightly more transfection than did Lipofectamine at optimal dose and lipid−DNA ratio, although the optimal dose for EDOPC was typically somewhat larger than that for Lipofectamine. Transfection efficiency of EDOPC was rather weakly dependent on the ratio of cationic amphipath to DNA and on the total amount of complex added to cells. For a 3:1 ratio of lipid to DNA (3% excess positive charge), the activity was maximal at 3 and 1.5 μg of lipid per well. With increasing lipid-to-DNA ratio, the transfection efficiency gradually decreased. The underivatized DOPC was devoid of cell transfection activity; β-galactosidase expression in BHK cells treated with DOPC−DNA complexes was the same as background (data not shown).

Figure 5 depicts the relationship between dose of complex and transfection efficiency as well as cell viability, both in the presence and absence of serum. When the cells were cultured in the presence of serum for the entire time (upper panel), expression was higher than when serum was absent for the first 4 h of contact with the transfection complex, except at the lowest doses. This effect seems to be largely due to the better growth of cells in the presence of serum during the entire growth period, as is shown by the cell number data in Figure 5. The points connected by lines in the figure represent the number of live (trypan blue-excluding) BHK cells in wells that were treated with the same amount of transfection complex as those that were assayed for β-galactosidase. As the dose of lipid−DNA complex was increased beyond 1.5−3 μg/well (15−30 μg/mL of lipid or 5−10 μg/mL of DNA), enzyme expression tended to be maintained, even though there was some reduction in cell number, indicating a higher expression per cell.



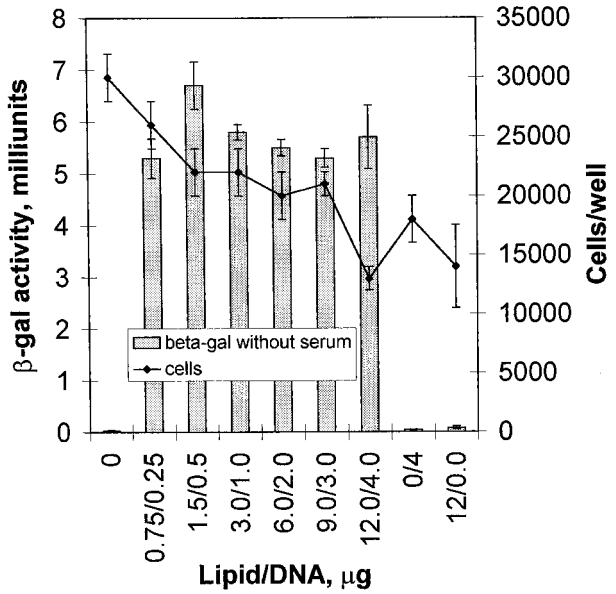


Figure 5—Transfection activity and toxicity of cationic phospholipid as a function of dose, with and without 10% serum. The figures show the level of expression of β-galactosidase (bars) and viable cells (line) for different amounts of lipid–DNA complex (in μg as shown on the horizontal axis) incubated with BHK cells in 96-well plates. The lipid:DNA ratio was 3:1 by weight in all cases, corresponding to a 3% excess of positive charge, except in the case of the control experiments corresponding to the last two bars. Volume per well was 100 μL. Upper panel: Serum present during entire incubation. Lower panel: Serum not present during first 4 h of incubation. Bars show high and low values of three replicates.

The cell number data of Figure 5 indicate that EDOPC has low toxicity because, although the number of viable cells was reduced to about half the initial number at doses of ~10 μg/well, this is a large dose, corresponding to ~2.5 mg of lipid in a 10-cm^2 Petri dish. By this test, EDOPC is ~30 times less toxic than Lipofectamine. The cell number counts in Figure 5 represent a lower limit, because it appears that cationic lipids reduce cell adhesion and some live cells are lost in washing procedures. The effects on cell viability of EDOPC and DNA separately were also examined and found not to differ greatly from that of the complex (two bars on far right of figure).

Addition of DOPE and cholesterol to EDOPC, in amounts >10 mol % at a constant ratio of EDOPC to DNA, reduced transfection. The lack of stimulation by DOPE was surprising given that most cationic amphipaths are nearly inactive in the absence of DOPE, [20,41,42] although at 1:1, DOPE had the smallest inhibitory effect (25−50%) relative to higher or lower amounts.

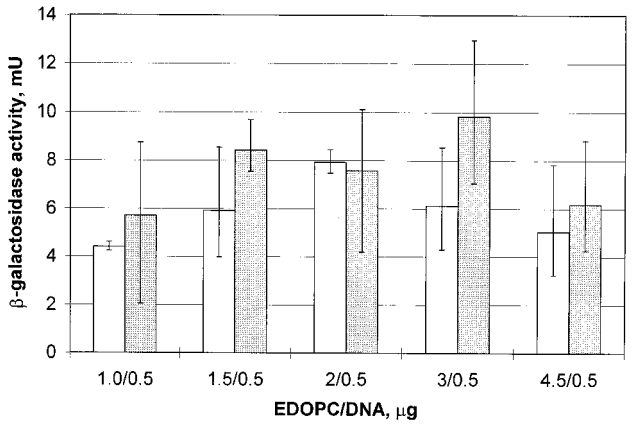


Figure 6—Effect of 10% serum on transfection efficiency as a function of the EDOPC:DNA ratio. EDOPC–DNA complexes were prepared according to the standard protocol using different lipid:DNA ratios. Transfection complexes with 2:1, 3:1, 4:1, 6:1, and 9:1 EDOPC:DNA weight ratios were then applied to BHK cells in medium without supplements (□) and to the cells in medium with 10% fetal bovine serum (■). Volume per well was 100 μL. Bars show high and low values of three replicates.

The EDOPC−DNA complex could be kept in the freezer or at room temperature for at least 3 days without loss of transfection efficiency. The ionic strength of the medium in which the complex was formed could be varied from the lowest value that was easily accessible, namely water, to a physiological value, namely D-PBS; however, this change in conditions had no significant effect on the transfection efficiency of the resultant complex.

Although not described in detail here, the phase state of the lipid appears to be important for transfection. We have examined EDPPC (the *O*-ethyl derivative of dipalmitoylphosphatidylcholine), a derivative that has a chain-melting phase transition temperature of ~40 °C. It was clear from the very low transfection efficiency of EDPPC that the phospholipid must be in the liquid phase to form an effective transfection complex.[43] In addition, extending the alkyl chain to a decyl group, which generates a lipid that resides in the hexagonal lyotropic phase, markedly altered the conditions for formation of transfection complexes with optimal activity.[39]

Another parameter likely to affect transfection efficiency is particle size. Based on dynamic light scattering, the number average particle size of the complex made under standard conditions is ~300 nm. The particle structure, according to both X-ray scattering and electron microscopy, is lamellar, consisting of DNA strands interspersed between lipid bilayers,[43] as are other complexes with low or no proportions of DOPE.[44,45]

To determine if DNA delivery to the nucleus by cationic phospholipid complexes depends on endocytosis and normal vesicle movement in the cell, we performed transfection with compounds that affect intracellular vesicle traffic. Preincubation of cells with chloroquine (60 μM), monensin (3 μM), or nocodazole (20 μM) inhibited transfection activity of EDOPC by >95%. A 2-h exposure to deoxyglucose (50 mM) reduced transfection by ~65%.

Stability of Transfection Complexes in Bovine Serum—Transfection efficiency usually improved in the presence of 10% serum over a wide range of EDOPC−DNA ratios (Figure 6). Given these data, it appeared possible that the EDOPC−DNA complex would survive incubation in a higher concentration of serum. To test for gross structural effects on the complex, which would be reflected

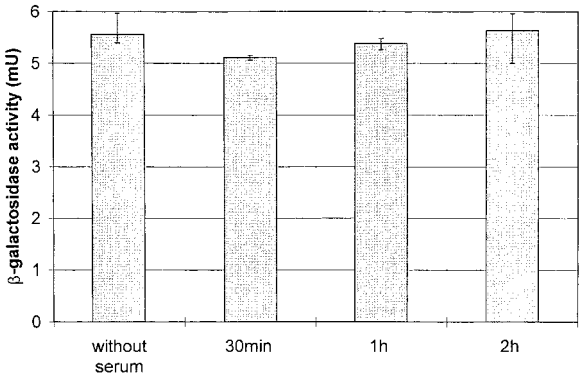


Figure 7—Transfection activity of cationic phospholipid is not affected by incubation with 95% serum. EDOPC–DNA complex was prepared from EDOPC and DNA at concentrations 10 times greater than usual (see *Methods, Standard Transfection Procedure*). The complex was incubated at a ratio of 1:20 with fetal bovine serum for 30 min and 1 and 2 h at 37 °C, and then used for transfection of BHK cells in 96-well plates in amounts corresponding to 1.5 μg of EDOPC and 0.5 μg of DNA in a total volume of 100 μL per well. Bars show high and low values of three replicates.

in the amount of light scattered by the complex, we measured the apparent absorbance of EDOPC−DNA particles at the wavelength of 450 nm (where there is no significant true absorbance) over the course of a 2-h incubation in serum at 37 °C. The compositions of the complexes were 3:1 and 6:1 by weight, prepared by mixing lipid at 1 mg/mL with DNA at 0.1 mg/mL. One volume of serum ($A_{450} = 0.32$) was mixed with one volume of complex dispersion (A_{450} were 0.62 and 0.72 for 3:1 and 6:1 weight ratio EDOPC−DNA complexes, respectively). Immediately after mixing, the absorbance was slightly higher than the sum of the absorbances of the components measured separately. We presume this difference is due to a small amount of aggregation occurring immediately, which amounted to 8% for the EDOPC−DNA complex at a 3:1 weight ratio and ~21% for the complex at a 6:1 weight ratio. Subsequently, there was a slight increase in absorbance with time: 8%/h for the complex with a 3:1 EDOPC−DNA weight ratio and 5.5%/h for the complex with a 6:1 weight ratio.

Given this indication of stability, we therefore examined the effect on transfection of 95% (the highest we could conveniently use) serum. Transfection of BHK cells was performed with lipid−DNA complexes preincubated in fetal bovine serum for 30 min, or 1 or 2 h (Figure 7). For this experiment, EDOPC and DNA were used at concentrations 10 times more than usual, which allowed us to add serum and transfection complex simultaneously to cells. As seen from the figure, the EDOPC−DNA complex retained its transfection efficiency during incubation in serum.

The effect of serum on DNA in the EDOPC−DNA complex was examined using a gel-electrophoresis-based protection assay. Figure 8 shows that after a short incubation of naked DNA in serum, the supercoiled form of plasmid was converted to linear and open circular forms. Although significant conversion to linear form also occurred in the EDOPC−DNA complex, the lipid clearly afforded a considerable amount of protection against nuclease activity. Similar results were obtained after treatment of DNA and EDOPC−DNA complex with the restriction enzyme Eco RI. In that case, the supercoiled plasmid, as well as all of the open circular plasmid present in the preparation, was completely converted to linear form. Again, EDOPC partially protected DNA from the action of restriction enzyme; that is, ~50% of the DNA remained supercoiled over the time course of the experiment.

Uptake by Cultured Cells of Cationic Phospholipids and Their DNA Complexes—Intracellular transport of transfection particles was investigated using separate labels for EDOPC and DNA. Figure 9 shows BHK cells that had been treated with EDOPC doped with RhPE, alone or in combination with DNA. When examined the next day, the pattern of labeling was quite dramatic and differed quite significantly, depending on whether DNA was delivered simultaneously with the lipid. When treated with lipid alone (upper panel), most cells exhibit localized fluorescence in large compartments adjacent to the nuclei. Although not definitively identified, the latter compartments are very similar to those labeled when cells are exposed to FITC−dextran and examined a day later, hence are suspected to be lysosomes.[46] Labeling of cellular membranes outside the perinuclear region was usually relatively weak. When cells were treated with lipid−DNA complexes, the typical lipid fluorescence pattern was significantly different (lower panel). Some fluorescence was seen around the nucleus, but the bulk of it was distributed throughout the cell in a punctate pattern, with extensive labeling of the surface and internal membranes.

When the DNA of the complex contained the tightly bound fluorophore, YOYO-1, the cytoplasm and the nuclei (with the exception of the nucleoli) of some of the cells were also fluorescent with the green fluorescence of intercalated YOYO-1. Our images of cells treated with lipid plus DNA were similar to those of CHO cells obtained by Hui et al.,[47] who introduced this combination of dyes in an earlier study of the transfection agent, 1,2-dioleoyl-3-(trimethylammonio)propane (DOTAP). We obtained the same results when plasmid DNA was covalently labeled with Alexa 488 C_5 maleimide dye as when we used the intercalating dye YOYO-1, indicating that the fluorescence observed was not due to migration of the YOYO-1 from delivered DNA to cellular DNA.

Discussion

We found that it is straightforward to change a zwitterionic phospholipid to a cationic phospholipid that is a highly effective transfection agent. The reaction is one step, involves readily available precursors, and purification is straightforward. The combination of efficacy and low toxicity makes the cationic phospholipids attractive candidates for agents to deliver nucleic acids and other pharmacological and physiological agents[17] to cells. Because the precursors are readily available and inexpensive, these compounds should also be cost-effective options for such applications.

Transfection Activity of Cationic Phospholipids—EDOPC exhibits transfection activity that is similar to that of other available agents, like Lipofectamine. Cationic phospholipids are, however, unaffected by serum, a property that distinguishes them from a number of common cationic amphipaths that are significantly inhibited by even 10% serum. Because it is not completely understood why those compounds are inhibited by serum, we cannot be sure why the cationic phospholipid escapes that fate. Current evidence points to interactions with serum proteins as an important cause for inhibition,[26] and it was found that inhibition could be avoided by changing the ratio of lipid to DNA,[23] the size of the complex,[22] or the concentrations at which it was formed.[25] In the case of EDOPC, because resistance to serum was observed over a wide range of PC+:DNA ratios (Figure 6), it is unlikely that the stability of the complex can be attributed to a particular surface charge density of the particle surface. It therefore would seem to be a property of the complex itself and evidently depends on a characteristic of the cationic phospholipids. The results of protection assays showed that when incubated in serum, supercoiled plasmid DNA complexed with

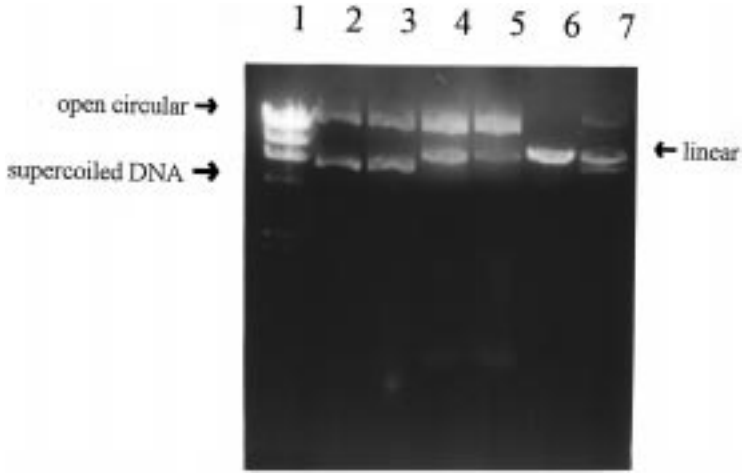


Figure 8—Agarose gel electrophoresis. DNA protection assay. EDOPC–DNA complexes were formed at concentrations 10 times higher than usual and incubated in fetal bovine serum or with the restriction endonuclease EcoRI (see *Methods* for additional details): Lane 1, in λ\HindIII; lane 2, DNA in D-PBS; lane 3, EDOPC–DNA in D-PBS; lane 4, DNA in serum; lane 5, EDOPC–DNA in serum; lane 6, DNA after treatment with EcoRI; lane 7, EDOPC–DNA after treatment with EcoRI.

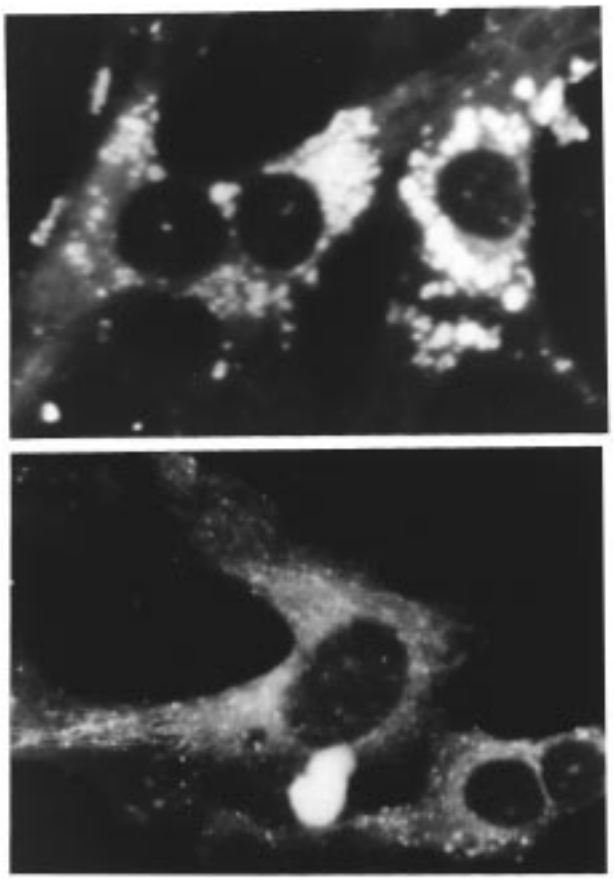


Figure 9—Cell uptake of cationic lipids and DNA–cationic lipid complexes. BHK cell cultures were treated with EDOPC containing 2.5 mol % RhPE either alone (upper panel) or complexed with DNA (lower panel). After incubation for 6 h in serum-free medium and an additional 14–18 h in serum-containing medium, the cells were fixed, mounted, and subsequently photographed. When lipid alone was added to the cells, it accumulated in large cellular vesicles in the region of the nucleus. When cells were treated with lipid–DNA complex, the surface and intracellular membranes often became fluorescent. Punctate fluorescence is also seen in these cells, but is usually distributed throughout the cell in much smaller vesicles than those seen in cells treated with lipid alone. The dark ovals outlined by surrounding fluorescence are the nuclei.

EDOPC was converted mainly to the open circular form instead of the linear form. This protection characteristic of EDOPC could be due to the structure of the polar group region, or to the fact that there is a single type of lipid present (which would exclude the possibility that even small incompatibilities between the two components could lead to some form of disproportionation under the influence of serum). Whether or not this means that cationic phospholipid complexes with DNA bind appreciably less serum protein than other transfection complexes remains to be determined. In any case, the ability to withstand extended contact with serum is an essential characteristic of a transfection complex if it is to be useful in gene therapy, and it is hence a potentially important characteristic of cationic phospholipids.

The cationic phospholipid exhibits relatively low toxicity to cells in culture, for which two possible explanations are apparent. One possibility is simply that the compounds may be sufficiently similar to cellular lipids that they have less disruptive effects on normal membrane function. A second possibility is that the lipids are degraded by cells to species that may either diffuse out of cells or be metabolized further. The rate of degradation was low, with a half time of days for the fluorescent substrates we tested, but if the intracellular phospholipase A_2(s) is similar to the venom enzymes we tested in the test tube, the unlabeled lipid could be degraded an order of magnitude faster. We doubt, however, that this potential advantage is realized under cellular conditions. Rather, we suspect that lipid degradation is limited by accessibility; the very mass of the lipid—both in the lipid by itself and the lipid–DNA complex—is likely to present a significant barrier to access by intracellular lipases. This interpretation is strongly supported by the punctate disposition of fluorescent lipids in the cell; large fluorescent masses appear within cells shortly after administration of lipid or complex and they remain sizable for at least a day.

Unlike many amphipathic cations used as transfection agents, EDOPC does not require DOPE for effectiveness.[41,42] We found that no mixtures of DOPE and EDOPC were as effective as pure EDOPC; however, the 1:1 mixture (the common ratio used with other cationic lipidic transfection agents) was nearly as effective as pure EDOPC. (We do not yet know what phase these mixtures reside in, and we will publish more extensive transfection results on DOPE-containing complexes when their phase structures have been determined.) It has been suggested that DOPE—a hexagonal phase-preferring lipid—may destabilize the endosomal membrane to facilitate escape of the DNA.[21] It may be that this adjunct activity is not required by PC+

because PC+ possesses membrane-destabilizing characteristics on its own; we have observed that cationic phospholipid vesicles fuse with anionic lipid vesicles,[48] EDOPC−DNA particles fuse with the bilayer of phosphatidylglycerol vesicles,[49] and mixtures of EDOPC and dioleoylphosphatidylglycerol form a bicontinuous cubic phase.[50]

Differential Uptake of Lipid and Lipid−DNA Complex by Cells—In the absence of DNA, the lipid fluorophore that was presented to cells accumulated in the perinuclear region. Presented in complex with DNA, the fluorophore was distributed in many small compartments throughout the cell. There are two obvious differences in the physical properties of the lipid and the lipid−DNA complex. One is buffer capacity. The complex could accept protons, because the DNA is present in the complex as a salt. The cationic phospholipid, being a quaternary amine, has no buffer capacity. Endosomal compartments may thus become more slowly acidified if DNA is present along with cationic phospholipid. The lipid is, in fact, delivered to compartments that resemble lysosomes morphologically, as would be expected if there were no significant interference with the normal acidification sequence in cellular vesicle traffic patterns. These results are also consistent with the cell fractionation results reported by Wattiaux et al. for liver cells treated with cationic amphipaths, in which the compounds accumulated in lysosomes.[51] Others have argued that cationic transfection agents function in part by neutralizing endosomal acid, with the result that lysosomal nucleases are inhibited.[52] If this result is true, then perhaps the reason we have noticed a difference between delivery of the lipid and that of the complex is because in our case only the complex would have buffer capacity.

The other obvious difference between the lipid and the complex is in osmolarity. The lipid has an osmolarity that is nearly the same as the concentration of its counteranions. The complex, in contrast, has an osmolarity that is essentially zero, for at the most effective composition for transfection, the charges on both lipid and DNA are almost completely neutralized. But the complex does have a latent high osmolarity, for if the DNA becomes protonated or if hydrophobic cellular anions diffuse into the complex, the complex is no longer a single particle in the osmotic sense. Thus, residence in an endosome could lead to changes in the lipid−DNA complex that could both buffer the endosome and lead to osmotic swelling of the compartment, both of which would delay transit to lysosomes. Generally attention has been focused on the cationic component as instrumental for DNA delivery, but it should not be overlooked that DNA, in complex with a cationic amphipath, acquires new properties that could play a role in its own delivery to the nucleus.

A number of cell lines were transfected by EDOPC, including CHO, mouse 3T3, Niemann-Pick, Rcho-1, L, K562 (erythroleukemia), and quail fibroblasts, although conditions were not optimized except for the first and second of these. Human vascular endothelial cells, primary cells of umbilical cord origin, are also efficiently transfected by EDOPC, however, in this case, not in the presence of serum.[53] In the case of CHO and 3T3 cells, the optimal conditions were not greatly different from those for BHK. Even without optimization, however, it was clear that EDOPC is effective with a wide variety of cells. An important advantage of the relatively low toxicity of EDOPC is that high doses may be used with recalcitrant cell lines. It remains to be determined to what extent cationic amphipaths will acquire clinical importance as gene therapy agents; however, because cationic phospholipids can be synthesized with a considerable variation in structure, there is reason to believe that a variety of these molecules could be constructed as effective delivery agents with minimal toxic effects.

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