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Membrane perturbation and the mechanism of lipid-mediated transfer of DNA into cells

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Abstract

Mixtures of cationic lipids and unsaturated phosphatidylethanolamine are used extensively for the intracellular delivery of plasmids and antisense oligodeoxynucleotides (ODN) in vitro. However, the mechanism by which cytoplasmic delivery of these large molecules is achieved remains unclear. The common hypothesis is that phosphatidylethanolamine promotes fusion of lipid/DNA particles with endosomal membranes, but this is inconsistent with several reports that have failed to correlate the fusogenic activity of a wide variety of lipid/DNA particles, measured by lipid mixing techniques, with their transfection activity. To address this issue further we have conducted a detailed analysis of the lipid mixing and DNA transfer activity of two, physically similar but functionally different, lipid/DNA particles composed of equimolar dioleyldimethylammonium chloride (DODAC) and dioleoylphosphatidylethanolamine (DOPE) or dioleoylphosphatidylcholine (DOPC). In combination with DODAC both phospholipids form almost identical lipid/DNA particles, they are endocytosed by cells to the same extent and each undergoes equivalent lipid mixing with cell membranes after uptake. Despite this, DNA transfer is 10- to 100-fold more extensive for lipid/DNA particles containing DOPE. We conclude that lipid mixing between lipid-based delivery systems and endosomal membranes must occur for DNA transfer to occur. However, the potency of different lipid/DNA particles correlates better with the ability of the exogenous lipid to disrupt membrane integrity.

Keywords: Fusion; Cationic lipid; Transfection; Phosphatidylethanolamine; Drug delivery; Antisense

1. Introduction

The mechanism of lipid-mediated transfection (lipofection) has attracted a great deal of attention in recent years because of the need to develop non-viral, intracellular delivery systems for genes and antisense

Abbreviations: BSA, bovine serum albumin; CHE, cholesterol hexadecylether; DMEM, Dulbecco's modified Eagle's medium; DODAC, dioleyldimethylammonium chloride; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoyphosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; HBS, HEPES buffered saline; HBSS, Hanks buffer saline; HEPES, 4-(hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dioleoylphosphatidylethanolamine; ODN, oligodeoxynucleotide; PBS, phosphate buffered saline; Pyr-PC, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-glycero-3-phosphocholine; RBC, red blood cells; Rho-PE, 1,2-dioleoyl-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine β sulfonyl); TX-100, Triton X-100

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drugs [1,2]. Lipofection in vitro involves mixing preformed polycationic lipid vesicles with polyanionic DNA and incubating the resulting lipid/DNA particles, sometimes referred to as lipoplexes, with cells in culture. The efficiency of successful DNA delivery into the cytoplasm is dependent on the charge ratio (+/−) of the lipid/DNA particles and their lipid composition. The most active lipid/DNA particles usually contain unsaturated lipids, in particular DOPE [3–5]. Positively charged lipid/DNA particles avidly bind to cell surfaces and enter cells by endocytosis [6,7]. It is also apparent that active DNA transfer is dependent upon the subsequent disruption of the endosome and concomitant release of all or at least a proportion of the DNA from intracellular organelles. The molecular mechanism by which this is induced by lipids remains poorly characterized and a better understanding of the process is necessary for the rational design of non-viral delivery systems to enhance the clinical utility of gene drugs.

The synthetic, unsaturated phospholipid DOPE has been identified as a neutral lipid, which greatly enhances the efficiency of lipofection [8,9]. It is typically combined with equimolar cationic lipid and the mixture hydrated to form multilamellar liposomes, which are then reduced to unilamellar vesicles by sonication or extrusion prior to their being added to DNA. Unsaturated phosphatidylethanolamine (PE) is a common class of membrane lipid and extensive studies have linked it with membrane fusion events in vitro and in vivo [10]. It is unique amongst the common mammalian phospholipids because it prefers to adopt non-bilayer structures in isolation. When mixed with the appropriate co-lipids PE will adopt a stable bilayer configuration. However, specific conditions can be applied that induce PE to promote the formation of non-bilayer structures such as inverted micelles and membrane stalks, which have been proposed to act as intermediates that facilitate fusion [5,10].

Despite the strong link between DOPE and membrane fusion, a number of studies have failed to find a correlation between the inherent fusion activity of lipid/DNA particles in general and their transfection efficiency. For example, fusion is generally monitored by lipid mixing assays, but it has been observed that most cationic lipid vesicles undergo lipid mixing with anionic membrane targets whether they support transfection or not [11–14]. Furthermore, some cationic lipid formulations show relatively good activity in the absence of a neutral lipid [15–17]. In order to gain a better understanding of the role played by DOPE in DNA transfer we have conducted a detailed study into the comparative activity of two similar lipid/DNA particles, which differ only in the headgroup composition of the neutral lipid. In contrast to DOPE, DOPC prefers the bilayer configuration, is not fusogenic and does not promote transfection [3,5,9,18]. The extent to which the two particles interact with plasma and endosomal membranes was measured employing lipid mixing assays. Their capacity to transfer plasmid DNA into cells was determined by measuring the activity of β-galactosidase (β-gal) or luciferase following transfection. Intracellular delivery of oligonucleotide DNA was investigated by monitoring trafficking and nuclear delivery of fluorescent ODN and lipid as well as measuring the effect of an antisense ODN targeting human epidermal growth factor receptor (EGFR) on target mRNA levels.

2. Materials and methods

2.1. Materials

DOPE and DOPC were purchased from Northern Lipids (Vancouver, B.C., Canada). Dr. S. Ansell synthesized DODAC at Inex Pharmaceuticals (Burnaby, B.C., Canada). ^{14}C-CHE and ^{3}H-CHE were from Amersham Corp. (Arlington Heights, IL, USA) while ^{3}H-inulin was from Dupont NEN (Boston, MA, USA). Rho-PE and NBD-PE were from Avanti Polar Lipids (Alabaster, AL, USA) and Pyr-PCPC was from Molecular Probes (Eugene, OR, USA). Influenza virus was obtained from the laboratory of Dr Jan Wilschut (University of Groningen, The Netherlands). Virus particles were grown in and subsequently harvested from the allantoic fluid of embryonic chicken eggs as described previously [19]. Bio-Beads (SM2) were obtained from Fluka (Buchs, Switzerland). A 15-mer, phosphorothioate antisense ODN against human EGFR with the sequence 5′-CCGTGGTCATGCTCC-3′ [20] and corresponding

scrambled control sequence 5′-CGCGATTCCGT-CCGT-3′ were synthesized by the Oligonucleotide Synthesis Laboratory at the University of British Columbia (Vancouver, B.C., Canada). The plasmids used, pCMVβ-gal and pCMVLuc, were constructed by Dr. Patrick Tam (Inex Pharmaceuticals Corp., Vancouver, B.C., Canada) and contain the reporter genes β-galactosidase and luciferase respectively, under the control of the murine CMV immediate early promoter. The plasmids were purified using the alkali lysis method followed by two rounds of CsCl/ethidium bromide equilibrium centrifugation. DMEM was from Stem Cell Technologies Inc. (Vancouver, B.C., Canada) and serum was from Intergen Company (Purchase, NY, USA). Mouse anti-human EGFR and phycoerythrin conjugated sheep anti-mouse IgG antibodies were from Pharmingen (Los Angeles, CA, USA) and Sigma (Oakville, Ont., Canada) respectively.

2.2. Vesicles

Equimolar quantities of the cationic lipid DODAC and neutral lipid DOPE or DOPC were mixed in chloroform and dried under a stream of nitrogen gas. The thin lipid film was exposed to high vacuum for approximately 2 h to remove residual solvent before hydration with distilled water to form multilamellar vesicles, typically at a concentration of 40 mM total lipid. The vesicles were freeze-thawed five times utilizing liquid nitrogen and warm water cycles and then extruded through 0.1 μm pore-sized polycarbonate filters using an Extruder (Lipex Biomembranes, Vancouver, B.C., Canada) to generate large unilamellar vesicles [21].

2.3. Formation of lipid/DNA particles

Cationic vesicles were mixed with equal volumes of plasmid DNA or ODN (0.01 mg/ml final concentration) in distilled water and incubated at room temperature for 10–20 min before use. The vesicle to nucleotide ratios are expressed in terms of a +/− charge ratio calculated by assuming mean molecular weights for phosphodiester and phosphorothioate nucleotide sodium salts of 325 and 341 respectively. One nucleotide base carries one negative charge while DODAC has one positive charge.

2.4. Transfection of BHK cells

BHK-21 cells were plated the night before in 96-well plates at 4×10^4 cells/well in 5% FBS–DMEM. pCMVβ-gal or pCMVLuc plasmid DNA (0.5 μg/well, 50 μl) complexed to various amounts of DODAC/DOPE or DODAC/DOPC vesicles (+/− charge ratio range of 0.8 to 2.0) were added to the cells in 0.2 ml of 5% FBS–DMEM. After 20 h, the cells were gently rinsed with PBS before being lysed with 50 μl PBS containing 0.1% v/v TX-100 and two freeze-thaw cycles. An aliquot (20 μl) of the cell lysate was taken for protein determination using a BCA protein assay. The remaining cell lysate was assayed for β-galactosidase activity using a photometric method [3] or luciferase activity using a commercial kit (Promega, WI, USA).

2.5. Virosome preparation

Reconstituted influenza virus envelopes (virosomes) were made following the method of Stegmann et al. [22]. The viral envelope of influenza virus was solubilized in the detergent octaethylene glycol *n*-dodecyl monoether in the presence of 10 mol% Pyr-PC relative to total viral phospholipid, which was determined by lipid extraction [23] and phosphate assay [24]. The viral nucleocapsid was removed by ultracentrifugation at $85\,000\times g$ for 30 min at 4°C. The supernatant, containing the viral envelope proteins, lipids and Pyr-PC, was shaken with Bio-Beads SM2 to remove the detergent. Subsequently, the virosome preparation was centrifuged on a 10–40% (w/v) discontinuous sucrose density gradient at $13\,000\times g$ for 90 min and virosomes collected from the interface between the sucrose layers.

2.6. Virosome fusion with BHK cells

A million BHK cells were suspended in 2 ml of HBSS containing 0.1% w/v BSA at 37°C. Pyr-PC labeled virosomes (0.2 μM final phospholipid concentration) were added to the cells and Pyr-PC excimer fluorescence monitored employing an Aminco Bowman Series 2 fluorimeter (SLM Aminco, Urbana, IL, USA) equipped with a magnetic stirrer and a temperature regulated water jacket. The fluorescence for infinite dilution of Pyr-PC was deter-

mined by the addition of 1 M octyl glucopyranoside to give a final concentration of 50 mM. For fusion measurements in the presence of NH_4Cl, an aliquot of a concentrated stock of NH_4Cl was added to the cell suspension to give a final concentration of 50 mM 15 min prior to the addition of virosomes and was present throughout the experiment. The pyrene fluorophore forms fluorescent excimers, consisting of dimers of one molecule in the ground state and the other in the excited state [25]. The formation of pyrene excimers is proportional to the membrane density of pyrene labeled lipids. A decrease in the excimer fluorescence is indicative of fusion with unlabeled membrane as exogenous lipids dilute the Pyr-PC. Fusion was calculated as follows:

$$\%\text{fusion} = 100\times[((F_o - F'_o)-(F-F'_o))/((F_o-F'_o) - 1.05(F_\infty - F'_\infty))]$$

where: F_o = initial fluorescence of the cell and virosome suspension, F'_o = fluorescence (light scattering) measured in the presence of cells only, F = fluorescence at a given time point, F_∞ = fluorescence of cells and virosomes after addition of detergent, F'_∞ = fluorescence (light scattering) of cells in the presence of detergent, 1.05 = dilution factor to account for the volume of added detergent.

2.7. Lipid mixing between lipid/DNA particles and BHK cells in buffer

Following the method reported by Wrobels and Collins [18], 40 nmol of vesicle total lipid complexed to DNA (+/− charged ratio = 1) were incubated with 2×10^5 BHK cells in 1 ml of ice-cold PBS. After 15 min, unbound lipid/DNA particles were removed by centrifugation of the cells at 4°C. The cell pellet was resuspended in 1 ml of ice-cold PBS and centrifuged a second time. The cells were resuspended in 0.1 ml of ice-cold PBS and transferred into a cuvette containing 3 ml of PBS at 37°C. Pyrene fluorescence was monitored as described above.

2.8. Lipid mixing between lipid/DNA particles and BHK cells in the presence of serum

Lipid/DNA particles were made in distilled water using vesicles labeled with 10 mol% Pyr-PC and 2.3 μCi ^{3}H-CHE/μmol lipid at a charge ratio (+/−) of 1.0. The particles (3 μg DNA in 300 μl) were added to BHK cells plated the night before at 5×10^5 cells/well (30 mm diameter) in the presence of 2 ml 5% FBS–DMEM at 4°C for 1 h to allow cell binding to occur. After 1 h the particles were removed, cells washed with PBS and either assayed immediately or incubated for a further 3 h at 37°C in 5% FBS–DMEM. To assay for lipid mixing, the cells were trypsinized, pelleted and resuspended in PBS. Pyrene excimer fluorescence was measured as described above and normalized to the total amount of lipid bound as determined by radioactivity. Light scattering corrections were made for the cells in suspension by comparing the cell concentration, as measured using a hemocytometer, to a standard curve constructed using untreated cells.

2.9. Downregulation of EGFR mRNA by antisense ODN

KB cells were plated the night before at 3.5×10^5 cells/well (30 mm diameter) in DMEM containing 10% FBS. Lipid/ODN particles (0.5 ml) were prepared as described above and added to the cells in 2 ml of 10% FBS–DMEM at a final concentration of 400 nM ODN. After 5 h, total RNA was extracted from the cells using a Qiagen Rneasy kit (Qiagen Inc., Santa Clarita, CA, USA). The purified RNA samples were resolved on a 1% agarose gel containing 6.7% formaldehyde and transferred to nylon membranes by capillary elution. The blots were hybridized with EGFR and G3PDH cDNA probes radiolabeled with [α-^{32}P]dCTP by random primer labeling using a commercial T7 polymerase kit (Promega, WI, USA). Probes hybridized to mRNA transcripts were visualized and quantified employing a PhosphorImager (Molecular Dynamics, CA, USA).

2.10. FITC fluorescence measurements in KB cells

KB cells, plated the day before at 3.5×10^5 cells/well (30 mm diameter), were exposed to 0.5% Rho-PE labeled lipid/FITC–ODN particles at a final concentration of 800 nM phosphorothioate ODN in 8% FBS–DMEM at 4°C. Note that fluorescence microscopy indicated that whilst at 4°C, Rho-PE fluorescence remained punctate at the cell surface, which

supports the assumption that little if any lipid mixing or endocytosis occurs at this temperature. In contrast, when the cells were raised to 37°C diffuse Rho-PE fluorescence was observed associated with the plasma membrane and punctate fluorescence with endocytic vesicles. After 1 h, unbound lipid/DNA particles were removed, fresh media added and the cells incubated at 37°C. At various times the cells were lifted from the plate with 2 mM EDTA in PBS, pelleted and then resuspended in PBS. FITC fluorescence of the cell suspension was measured at excitation and emission wavelengths of 482 nm and 520 nm respectively. Total FITC fluorescence was obtained by diluting the cell suspension three times with PBS in the presence of 0.5% v/v TX-100. Light scattering corrections for the cell suspensions were made as described above.

2.11. Preparation of red blood cell ghost membranes

An aliquot (2 ml) of packed, freshly collected human blood was lysed by rapid dilution in 300 ml of ice-cold lysis buffer (5 mM EDTA, 5 mM HEPES, pH 7.4). The lysed red blood cells (RBC) were then pelleted by centrifugation at $27\,000 \times g$ for 40 min at 4°C. The pellet was resuspended in lysis buffer and pelleted again three times or until the RBC membrane preparation was white. The resulting RBC ghosts were resealed with HBS containing 5 mM $MgCl_2$ and incubated at 37°C for 1 h. They were then isolated again by centrifugation at $27\,000 \times g$, suspended in HBS and stored in small aliquots at −20°C until required.

2.12. Lipid mixing with RBC ghosts

A fluorescent assay based on resonance energy transfer between NBD-PE (the energy donor) and Rho-PE (the energy acceptor) was used to measure the extent of lipid mixing [26]. Lipid/DNA particles, made with vesicles containing 1 mol% Rho-PE and 1 mol% NBD-PE, were added to RBC ghosts in 10 mM HEPES, pH 7.4 at 25°C. Fluorescence from NBD-PE was monitored at excitation and emission wavelengths of 460 nm and 530 nm respectively, using a fluorimeter. Total fluorescence was determined following the addition of TX-100 (1% v/v final concentration) which represents maximum dilution of the probes and therefore maximum NBD-PE fluorescence.

2.13. Hemolysis measurements

Human blood, freshly collected in EDTA-vacutainer tubes from healthy volunteers, was washed three times with cold HBS and pelleted by centrifugation at 2000 rpm for 10 min. The pellet of packed cells was resuspended in 10 volumes of HBS, an aliquot was subjected to chloroform/methanol extraction [23] and the chloroform phase analyzed for phosphorus content [24] to determine the phospholipid concentration (approximately 0.25 μM cell phospholipid) and kept on ice until use. Aliquots of fresh RBC, pre-washed with HBS, were added to cationic lipid vesicles or lipid/DNA particles (+/− charge ratio = 1) in HBS or HBS containing 5% FBS and incubated for 30 min at 37°C. Unlysed RBC were pelleted by centrifugation at 2000 rpm for 10 min and the hemoglobin content in the supernatant measured at an absorbance of 405 nm using a Biolumin960 plate reader (Molecular Dynamics, Sunnyvale, CA, USA). Total hemolysis was measured by lysing an aliquot of cells in excess distilled water.

2.14. Vesicle binding to RBC

Lipid/DNA particles were prepared containing trace amounts of ^{14}C-CHE. RBC were exposed to the particles for 5 min at 4°C in HBS spiked with 0.13 μCi/ml ^{3}H-inulin. Unlysed cells were pelleted at 2000 rpm using a centrifuge pre-cooled to 4°C and an aliquot of the supernatant was removed to determine the degree of hemolysis. The amount of vesicles bound to cells was determined from the level of ^{14}C-CHE associated with the RBC pellet. ^{3}H-inulin was used to calculate the extracellular volume so that the pellet radioactivity could be corrected for unbound vesicles. Controls conducted in the absence of RBC indicated that vesicles did not pellet under the centrifugation conditions employed.

2.15. Miscellaneous analytical procedures

Protein concentrations were measured using a standard BCA protein assay. Vesicle sizes were de-

termined by quasi-elastic light scattering (QELS) using a Nicomp 370 submicron particle sizer (Nicomp Instruments, Goleta, CA, USA) operating at 632.8 nm and 5 mW. Radioactivity was measured by liquid scintillation counting using a Beckman LS3801 instrument. Fluorescence micrographs were prepared employing a Nikon Diaphot 300 epifluorescence microscope, equipped with a 450–490 nm wavelength excitation filter, 510 nm dichromatic mirror and a 520 nm barrier filter.

3. Results

3.1. Lipid/DNA particles and gene transfer

The cationic lipid employed in these studies (DODAC) is a dimethylammonium chloride salt with two 18-carbon monounsaturated (di-18:1) alkyl chains covalently linked directly to the nitrogen (Fig. 1). In combination with DOPE, the di-18:1 derivative exhibits the highest level of gene transfer activity when compared to other lipids in the series in which the alkyl chains are varied [5]. The characteristics of DODAC are similar to those observed for other univalent cationic lipids capable of supporting lipid-mediated gene transfer [27]. When equimolar with DOPE it will stabilize the bilayer configuration as determined by ^{31}P-NMR (data not shown). And as a component of small unilamellar lipid vesicles it will charge neutralize DNA causing the nucleic acid to condense into lipid/DNA particles in which the nucleic acid is no longer accessible to ethidium bromide or other intercalating dyes [5]. Compaction of plasmid DNA occurs as a result of charge neutralization by polycations and when induced by cationic lipid vesicles the process appears to be independent of the nature of the lipids employed. Consequently, lipid/DNA particles formed using DODAC/DOPC or DODAC/DOPE vesicles appear to be indistinguishable [5]. Transfection, however, is very much lipid dependent and cationic lipid/DNA particles containing DOPE show greatly enhanced activity compared to the same particles prepared with DOPC (Fig. 2).

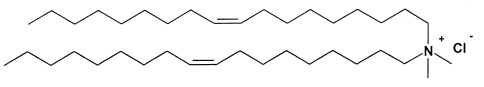

Fig. 1. Structure of DODAC.

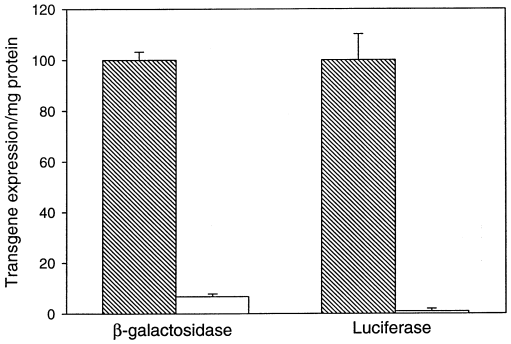

Fig. 2. Transfection of BHK cells mediated by DODAC/DOPE and DODAC/DOPC vesicles. BHK cells, plated the night before at 4×10^4 cells/0.32 cm^2, were exposed to 0.5 μg of pCMVβ-gal or pCMVLuc plasmid complexed to various amounts of DODAC/DOPE (hatched bars) or DODAC/DOPC (open bars) vesicles in the presence of 5% FBS–DMEM. After 20 h, β-galactosidase or luciferase protein expression was assayed as outlined in Section 2. The data plotted are at the maximum transfection efficiency observed over a charge ratio range of 0.8 to 2.0, which for β-galactosidase and luciferase were at +/− charge ratios of 1.3 and 1.2, respectively. The data have been normalized to the transfection efficiency obtained with DODAC:PE and represent an average of three samples ± one S.D.

3.2. Lipid mixing and transfection activity

In order to compare the lipid mixing activities of the low transfection (DOPC-containing) and high transfection (DOPE-containing) lipid/DNA particles we employed a Pyr-PC assay that has been used extensively to characterize the fusion of reconstituted viral particles (virosomes) with cells growing in culture [22,28]. In order to validate this assay we repeated the work described by Stegmann et al. [22] employing virosomes that contain the influenza fusion protein hemagglutinin (HA). Virosomes containing 10 mol% Pyr-PC were prepared as outlined in Section 2, and a time course of their fusion with BHK cells at 37°C is shown in Fig. 3 (circles). Two features indicate that fusion between virosomes and endosomal membranes occurs as described previously [22,28]. First, Pyr-PC dilution and concomitant decrease in excimer fluorescence occur only after a 10–20 min lag period. This reflects the time required for endocytosis to take place followed by acidification of the endosomal compartment. At pH $<$ 5.5 HA undergoes a conformational change that activates the fusion process. Second, the presence of

50 mM NH_4Cl inhibits the lipid mixing because it prevents acidification of the endosome and therefore activation of the viral fusion protein.

In contrast to the controlled, pH-dependent fusion of the virosomes, lipid/DNA particles under the same conditions undergo extensive lipid mixing immediately upon contact with the plasma membrane at 37°C in PBS (Fig. 3, squares) or PBS containing 0.1% w/v BSA (data not shown). Moreover, despite the fact that DOPE-containing particles support transfection to a greater extent than particles containing DOPC there was no difference in their ability to support lipid mixing with the plasma and endosomal membranes of BHK cells. Fluorescence microscopy also revealed the presence of fluorescent plasma membranes after BHK cells were incubated with Rho-PE labeled lipid/DNA particles, supporting the observation that significant lipid mixing occurs prior to and during the uptake process. Furthermore,

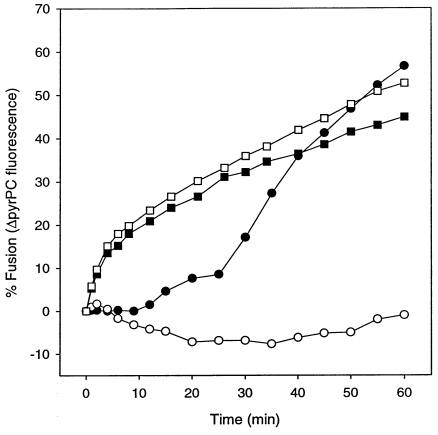

Fig. 3. Lipid mixing of virosomes and lipid/DNA particles with BHK cells measured by Pyr-PC. Virosomes were incubated with BHK cells in HBSS-BSA buffer in the absence (●) or presence (○) of 50 mM NH_4Cl at 37°C as previously described [22]. In the latter case, the cells were pre-incubated with 50 mM NH_4Cl for 15 min prior to the addition of virosomes. Alternatively, following the method of Wrobel and Collins [18], DODAC/DOPE (■) or DODAC/DOPC (□) vesicles complexed to DNA (+/− charge ratio = 1) were pre-bound to BHK cells in 1 ml of PBS at 4°C. After 15 min, unbound vesicles were removed by pelleting the cells. The cell pellet was resuspended in 0.1 ml of ice-cold PBS prior to being added to a cuvette containing 3 ml of PBS at 37°C (time zero). Pyrene excimer fluorescence was monitored with continuous stirring at excitation and emission wavelengths of 345 and 480 nm, respectively. Final fluorescence was obtained by addition of OGP (50 mM final) and % fusion calculated as outlined in Section 2.

50 mM NH_4Cl had no effect on the fluorescence profiles shown in Fig. 3 for lipid/DNA particles (data not shown) indicating that lipid mixing was not pH dependent, as it is for the virosome, but occurs spontaneously following lipid/DNA binding to the cell surface.

3.3. The effect of serum on lipid mixing

The mixing data described above were obtained in the presence of buffer or low concentrations of BSA according to the virosome method being followed and not the presence of 5–10% serum typically used for transfection. However, it has been noted previously that in vitro serum generally inhibits the transfection activity of lipid/DNA particles [29]. Transfection by DODAC/DOPE vesicles is reduced but not eliminated by serum and the difference in activity between DOPE- and DOPC-containing lipid/DNA particles remains similar to that shown in Fig. 2. To compare lipid mixing for the two DNA delivery systems in the presence of serum, lipid vesicles were labeled with 10% Pyr-PC and ^{3}H-CHE. Lipid/DNA particles were prepared at a +/− charge ratio of 1.0 (previously optimized to give maximum transfection for the plasmid) and added to plated BHK cells in the presence of 5% FBS–DMEM at 4°C. After 1 h unbound material was removed and replaced with fresh media then cells were incubated for a further 3 h at 37°C. Pyrene fluorescence at 1 h (bound) was then compared to fluorescence at 4 h (bound and endocytosed) after normalizing to the total amount of ^{3}H-lipid associated with the cells. A significant drop ($P < 0.001$) in excimer fluorescence occurs for both DOPC- and DOPE-containing particles during the 3 h incubation at 37°C (Fig. 4, hatched bars), indicating extensive lipid mixing has taken place. In this series of experiments a 60 ± 30% decrease in fluorescence was observed for DOPE-containing particles compared to 30+30% for those containing DOPC, however the difference is not statistically significant ($n = 7$). The amount of lipid bound to the cells for both DOPC- and DOPE-containing particles was similar, approximately 30 pmol lipid per 1 million cells. These data support the observations made above and indicate that lipid mixing alone does not explain the large differences in transfection activity between the two delivery systems.

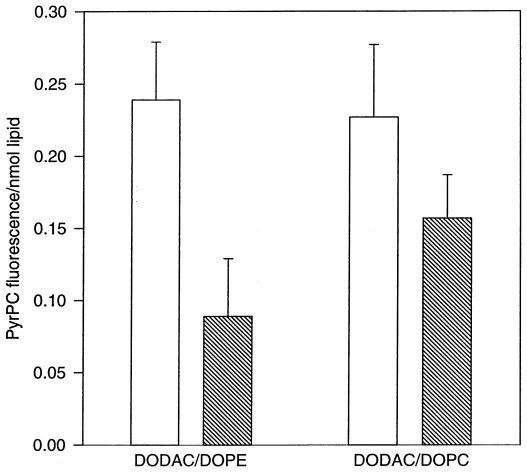

Fig. 4. Lipid mixing of lipid/DNA complexes with BHK cells in the presence of serum. Lipid/DNA complexes were made with DODAC/DOPE or DODAC/DOPC vesicles labeled with 10 mol% Pyr-PC and ^{3}H-CHE and added to plated sub-confluent BHK cells in the presence of 5% FBS–DMEM at 4°C. After 1 h, unbound complexes were removed, cells washed with PBS and either assayed (open bars) or incubated for a further 3 h at 37°C (hatched bars) in 5% FBS–DMEM. To assay for lipid mixing, the cells were detached from the plate with 2 mM EDTA, pelleted and resuspended in PBS. Pyrene excimer fluorescence was measured as outlined in Section 2 and the results expressed as pyrene excimer fluorescence normalized to the amount of lipid bound as determined by ^{3}H-CHE. The data represent the average of seven samples ± one S.D.

3.4. *Intracellular delivery of antisense oligodeoxynucleotides*

Lipids used for gene transfer can also be applied to antisense ODN [4]. Successful intracellular delivery of nuclease resistant phosphorothioate ODN can be observed not only by monitoring the downregulation of target mRNA and protein but also by the appearance of fluorescent nuclei when FITC-tagged ODN are incorporated into the lipid/ODN particles [30,31]. When FITC labeled phosphorothioate ODN are injected directly into the cytoplasm they rapidly diffuse into the nucleus [32], consequently, they make excellent markers for intracellular delivery and endosomal release.

For the studies reported here we employed a 15-mer phosphorothioate ODN complementary to the human EGFR stop codon region [20]. Following delivery by DODAC/DOPE vesicles into human KB cells the antisense reduced levels of EGFR mRNA by as much as 80% after 24 h compared to glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA, whereas a scrambled control and lipid alone had no effect (Fig. 5A). Replacing DOPE with DOPC eliminated the antisense activity (Fig. 5B), even though the extent of ODN uptake was the same for both lipid/ODN particles, approximately 0.3 ng ODN/mg cell protein. The different activity of the two phospholipids is also clearly illustrated in Fig. 6, where the intracellular fate of lipid/ODN particles labeled with Rho-PE and FITC–ODN is shown. Approximately 4 h after uptake the nuclei of KB cells treated with lipid/ODN particles containing DOPE appear green due to the accumulation of FITC–ODN. The orange fluorescence of Rho-PE is clearly visible in the perinuclear region, indicating

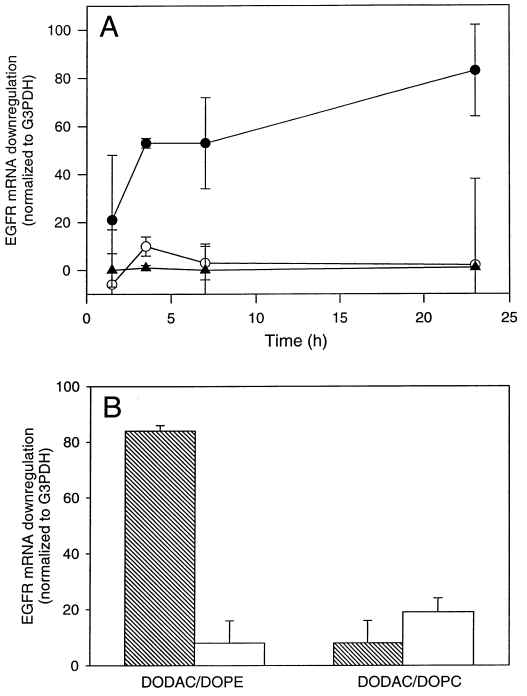

Fig. 5. Antisense activity mediated by lipid/ODN complexes in KB cells. (A) DODAC/DOPE vesicles were mixed with the active EGFR (●) or scrambled (○) antisense ODN in distilled water and added to KB cells at a concentration of 400 nM in 8% FBS–DMEM. Control cells were exposed to an equivalent amount of distilled water (▲). At various times, total RNA was isolated from the cells. A Northern blot of the extracted RNA was probed for EGFR and G3PDH mRNA and quantitated using a phosphoimager. (B) DODAC/DOPE or DODAC/DOPC vesicles were mixed with the active EGFR (hatched bars) or scrambled (open bars) ODN and added to KB cells as outlined above. After 5 h, the cells were assayed for EGFR mRNA content. The data represent an average of three samples ± one S.D.

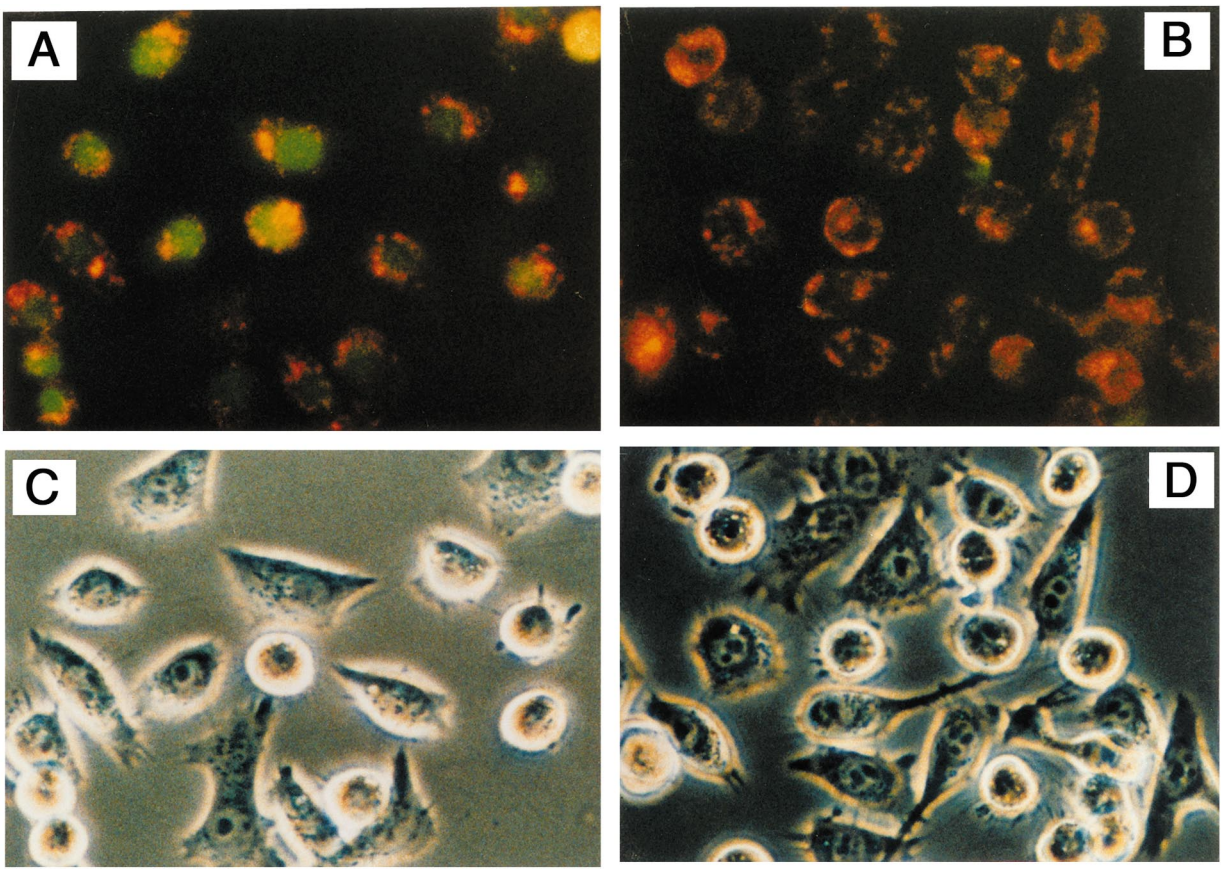

Fig. 6. Fluorescent microscopy of KB cells exposed to Rho-PE labeled lipid/FITC–ODN particles. DODAC/DOPE (panels A and C) or DODAC/DOPC (panels B and D) vesicles labeled with 0.5 mol% Rho-PE were complexed to FITC–ODN and added to KB cells at a concentration of 410 nM ODN in 8% FBS–DMEM and incubated at 37°C for 4 h.

that the lipid remains associated with intracellular membranes and does not cross the nuclear envelope (Fig. 6A). Marcusson et al. [31] have made similar observations during the lipid-based delivery of antisense ODN targeting protein kinase C-alpha, these authors also demonstrated that target mRNA did not start to decline until nuclear fluorescence could be observed.

In contrast, lipid/ODN particles containing DOPC remain trapped within the endosome/lysosome pathway (Fig. 6B), where the FITC fluorescence is quenched by a combination of the low pH and close proximity to Rho-PE [33]. Confocal microscopy also confirmed that both types of lipid/ODN particles were endocytosed (data not shown). The increase in FITC–ODN fluorescence as it is released from quenching conditions and accumulates in the nucleus can also be used to quantitatively monitor intracellular release of ODN, as demonstrated in Fig. 7. Lipid/ODN particles made with FITC–ODN were pre-bound to KB cells at 4°C for 1 h, then unbound particles were removed and the cells incubated with fresh media (time zero). At various times, cellular FITC fluorescence was measured as outlined in Section 2. The data show that as DOPE-containing lipid/ODN particles are endocytosed and processed further, FITC fluorescence increases rapidly during the first 4 h, which correlates with the appearance of fluorescent nuclei. On the other hand, the fluorescence measured from DOPC-containing particles gradually decreases, which is consistent with the increased quenching expected as the endosome compartment acidifies.

3.5. Lipid mixing and membrane perturbation

We have demonstrated that lipid/DNA particles containing equimolar DODAC and the phospholip-

ids DOPC or DOPE undergo lipid mixing with cell membranes to a similar extent, yet only DOPE supports extensive DNA (plasmid or phosphorothioate ODN) transfer into the cytoplasm or nucleus. To investigate this further we examined the effects of lipid mixing on the integrity of the red blood cell (RBC) plasma membrane, which was used as a model representative of a typical biological membrane. In the first series of experiments we employed fluorescence energy transfer to confirm that lipid/DNA (plasmid) particles (+/− charge ratio 1.0) containing Rho-PE and NBD-PE mix rapidly with RBC membranes at 37°C, behavior similar to that described above for BHK cells. Red blood cell ghosts were used to avoid any interference with the assay from hemoglobin. Extensive lipid mixing was observed after only 10 min for both DOPE- and DOPC-containing particles, 98 ± 6% and 58 ± 5% of maximum probe dilution respectively.

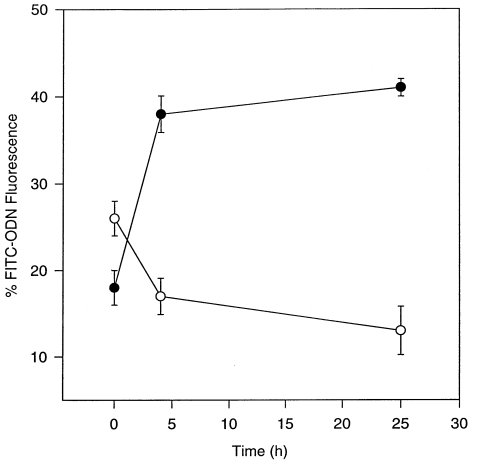

Fig. 7. Quantitation of endosomal release of FITC–ODN in KB cells after exposure to lipid/FITC–ODN particles. DODAC/DOPE (■) or DODAC/DOPC (□) vesicles labeled with 0.5 mol% Rho-PE were complexed to FITC–ODN and added to KB cells at a concentration of 820 nM ODN in 8% FBS–DMEM at 4°C. After 1 h, unbound complexes were removed, fresh media added and the cells incubated at 37°C (time zero). At various times, cellular FITC fluorescence was determined by detaching the cells from the plate with 2 mM EDTA. The cells were pelleted, resuspended in PBS and FITC fluorescence measured using an SLM Aminco fluorimeter at excitation and emission wavelengths of 482 nm and 520 nm, respectively. Total FITC fluorescence was determined after addition of 0.5% TX-100. Corrections were made for light scattering as outlined in Section 2. The data represent an average of three samples ± one S.D.

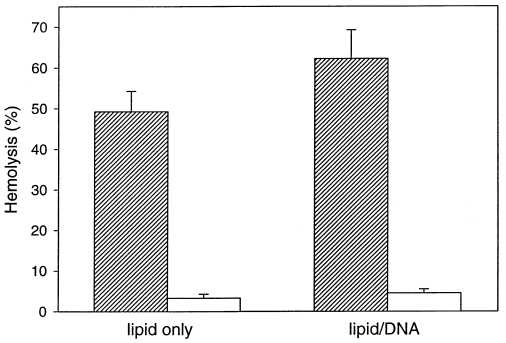

Fig. 8. Hemolysis of RBC by lipid vesicles and lipid/DNA particles. Fresh RBC (25 nmol phospholipid) were added to 10 nmol DODAC/DOPE (hatched bars) or DODAC/DOPC (open bars) vesicles and hemolysis measured after a 30 min incubation at 37°C as outlined in Section 2. Vesicles were also complexed to DNA (charge ratio = 1) prior to adding to RBC. The data represent an average of three samples ± one S.D.

In order to determine what effect lipid mixing with the plasma membrane has on membrane integrity, lipid vesicles and lipid/DNA particles were incubated with intact RBC and hemolysis measured as an indicator of membrane perturbation. At ratios of vesicle lipid to RBC membrane lipid of approximately 0.5:1 the data show that both vesicles alone and lipid/DNA (plasmid) particles containing DOPE are much more hemolytic than those containing DOPC (Fig. 8). Binding studies demonstrated that the total amount of cell-associated lipid is the same for both DOPE- and DOPC-containing systems. Consequently, taken together with the lipid mixing results, these data suggest that the RBC plasma membrane is destabilized to a greater extent by an influx of DOPE than it is by an equivalent influx of DOPC.

4. Discussion

The focus of this work was to measure the membrane interactions of two closely related cationic lipid vesicles that differ greatly in their capacity to transfer DNA into cells in vitro. Since the first report by Felgner et al. [8] demonstrating that cationic liposomes containing DOPE can efficiently deliver functional plasmid into mammalian cells, there have been many attempts to correlate the fusion activity of lipid/DNA particles with their ability to transfect. This is because it is widely accepted that a non-viral gene

delivery system, that can mimic the DNA delivery mechanisms of viruses by fusing with plasma or endosomal membranes to release a nucleic acid payload directly into the cytoplasm, will speed the clinical development of gene therapy [1,2]. Given the well-known relationship between unsaturated phosphatidylethanolamine, non-bilayer lipid structures and membrane fusion [10] it is reasonable to speculate that DOPE is responsible for enabling lipid/DNA particles to fuse with either the plasma membrane or endosomal membrane after phagocytosis [9].

However, despite many attempts to confirm this hypothesis a correlation between lipid mixing (commonly assumed to reflect fusion processes) of non-viral, lipid-based delivery systems and transfection has not been clearly established [11–14]. Here we demonstrate that lipid/DNA particles containing DOPC exhibit levels of transfection that are 10- to 100-fold less than can be achieved with DOPE-containing particles (Fig. 2), yet both systems readily undergo lipid mixing with cell membranes (Figs. 3 and 4). Similar behavior is seen for antisense delivered by cationic lipid vesicles. In the presence of DOPE a sequence specific antisense effect on mRNA and protein levels can be measured, indicating that ODN has accessed the cell cytoplasm and nucleus. No such effects, however, are observed when antisense is delivered using vesicles in which DOPC has been substituted for DOPE (Fig. 5). Fluorescence microscopy shows that in the presence of DOPC the ODN cannot escape from the endosomal pathway (Fig. 6), whereas in the presence of DOPE, ODN accumulates in nuclei and appears to separate from lipid, which remains associated with intracellular organelles.

From these data we conclude that fusion, hemifusion or close apposition resulting in extensive lipid mixing between plasma or endosomal membranes and bound cationic lipid/DNA particles, is not necessarily sufficient to induce the release of DNA into the cytoplasm. We propose that the nature of the exogenous lipid being inserted into the biological membrane is more relevant than the lipid mixing event itself. It is known, for example, that RBC can tolerate larger amounts of exogenous phosphatidylcholine in the outer monolayer of the plasma membrane if the fatty acid composition reflects that of the endogenous lipid [34], an incompatible fatty acid composition results in hemolysis. One explanation for the different DNA delivery activities of DODAC/DOPE and DODAC/DOPC we observe, despite similar binding, cell uptake and lipid mixing behavior, is that the plasma and endosomal membranes are more tolerant of exogenous DOPC than DOPE. The differential hemolysis data presented in Fig. 8 support this hypothesis. The molecular shape and non-bilayer structural preferences of DOPE likely destabilize the membrane resulting in lysis through pore formation [35] or other types of perturbation [5]. Moreover, the degree of destabilization induced by excess DOPE, but not DOPC, may also be expected to induce transbilayer flip-flop of anionic membrane lipids capable of displacing cationic lipid from the DNA thus enhancing the release of nucleic acid into the cytoplasm [7].

Both types of lipid/DNA particles employed here undergo lipid mixing with the plasma membrane of BHK cells without any evidence of a lag period and independent of endosomal acidification (Fig. 2). Similar behavior has recently been described for 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP), another cationic lipid commonly used for transfection in combination with DOPE [14]. These data suggest that lipid mixing is initiated by close apposition between lipid/DNA particles and the cell surface. In contrast vesicles containing reconstituted HA proteins require an acidic environment for fusion to proceed (Fig. 2). It is interesting to note that we (data not shown) and others have observed that serum tends to inhibit lipid mixing but does not significantly affect the binding of lipid/DNA particles to cells [14,29]. The exchange of lipids between lipid/DNA particles coated with serum proteins and cell membranes may be restricted by the steric and hydrophilic barrier presented by the bound protein. Therefore transfection in the presence of serum may require at least partial enzymatic digestion of bound material whilst lipid/DNA particles are in endosome/lysosome compartments.

Phosphatidylethanolamine is not the only lipid capable of perturbing membrane structure when present in excess. However, membrane disruption is only part of the role played by the neutral lipid component of cationic lipid/DNA particles. It must also form bilayer vesicles in combination with a cationic lipid and subsequently form a protective barrier

around the nucleic acid to prevent nuclease degradation by serum nucleases prior to cell uptake. In some instances multivalent cationic lipids alone have been demonstrated to successfully deliver DNA into cells [17] and cholesterol can be substituted for DOPE with some monovalent cationic lipids [36]. From the studies presented here we conclude that measuring lipid mixing phenomena alone is not sufficient to predict the transfection potency of lipid-based non-viral delivery systems. More important is the ability of the lipids undergoing mixing, either alone or in combination with endogenous lipids, to cause sufficient perturbation to enable the nucleic acid to gain access to the cytoplasm.

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