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The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer

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Abstract

In a reporter gene assay, cationic liposomes containing the cationic lipid 3β-(*N*-(*N'*,*N'*-dimethylaminoethane)carbamoyl)cholesterol (DC-Chol) and a neutral phospholipid dioleoylphosphatidylethanolamine (DOPE) showed high transfection activity. DNA/liposome complex which contained low amount of liposomes could bind to the cell surface but failed to transfect the cells. We have designed a two-step protocol to examine this phenomenon in more detail. A431 human cells were incubated on ice (pulse) with DNA complexed to a low level of cationic liposomes. The cells were washed and incubated at 37° C (chase) with or without free cationic liposomes of various composition (helper liposomes). Only liposomes enriched with DOPE showed helper activity; liposomes containing dioleoylphosphatidylcholine (DOPC), a structural analog of DOPE, had no helper activity. The delivery was inhibited by the lysosomotropic agent chloroquine and was optimal if the helper liposome chase was initiated immediately after the pulse. An endocytosis model of DNA delivery by cationic liposomes is proposed in which the principal function of the chase liposomes is to destabilize the endosome membrane and allow the release of DNA into the cytosol. This model is consistent with the known activity of DOPE to assume non-bilayer structures, hence destabilizing the endosome membrane.

Keywords: Transfection; Cationic liposome; Liposome; Gene therapy; Endocytosis

1. Introduction

Cationic liposomes are commonly used for mediating gene transfer, i.e. transfection, to mammalian cells (for recent reviews, see [1–4]). Their popularity is justified by their high transfection efficiency, low toxicity and commercial availability among others. In practice, they represent the simplest methodology known for DNA delivery. Simply, pure DNA of any size or shape is mixed with the cationic liposomes and incubated with cells for a few hours followed by a gene expression assay in one or two days.

One of the critical elements for efficient DNA delivery is the lipid composition of cationic liposomes [1,3]. The cationic lipid component is amphipathic and can vary in its chemical structure. Each cationic lipid may contain single or multiple cationic charges and the overall positive charge must be preserved. However, not all cationic lipids are capable of DNA delivery. Some cationic lipids with protein kinase C inhibition activity have no transfection activity [5].

Most cationic liposomes, with some exceptions [6,7], have a common neutral phospholipid component in addition to the cationic lipid component [1]. The phospholipid is needed for stabilizing most types of cationic lipids in a lipid bilayer and may provide the cell penetration function of cationic liposomes. A neutral phospholipid, dioleoylphosphatidylethanolamine (DOPE), serves as the second lipid component of cationic liposomes [1]. DOPE is critical for transfection because replacement of DOPE with another neutral phospholipid of the same acyl chain composition, dioleoylphosphatidylcholine (DOPC), abolish most of the transfection activity of cationic liposomes [5,8–10]. DOPC contains a choline head group instead of the ethanolamine head group on DOPE. This substitution changes many properties of the phospholipid [11]. One of the major differences between DOPE and DOPC is the high non-bilayer forming activity of DOPE and the absence of this activity with DOPC [11]. DOPE is a strong

Abbreviations: CAT, chloramphenicol acetyltransferase; DC-Chol, 3β-(*N*-(*N'*,*N'*-dimethylaminoethane)carbamoyl)cholesterol; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoyl phosphatidylethanolamine.

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destabilizer of lipid bilayers [11]. Recent work in our group have shown the effect of DOPE vs. DOPC in delivering DNA to the cytosol of cells transfected with cationic liposome complex [9]. Electron microscopy observations clearly showed the endosome destabilizing effect of DOPE-containing cationic liposomes. In comparison, DOPC-containing cationic liposomes showed no effect on endosomes [9].

This work is aimed at the elucidation of the role of DOPE in the cationic liposome mediated gene delivery. A new pulse-chase protocol has been designed to dissect the process of transfection and to allow more detailed examination of each step in the transfection. The studies presented here support a major role for endocytosis in the uptake of DNA/cationic liposome complex.

2. Materials and methods

2.1. Materials

The lipids 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) and 1,2-dioleoyl-*sn*-glycero-*sn*-3-phosphatidylcholine (DOPC), were purchased from Avanti Polar Lipids. 3β-(*N*-(*N'*,*N'*-Dimethylaminoethane)carbamoyl)cholesterol (DC-Chol) was synthesized as described by Gao and Huang [12]. Dulbecco's modified Eagle's medium (DMEM) was from Life Technologies. Fetal bovine serum was from Hyclone Laboratories. Acetyl-CoA and chloramphenicol were from Sigma. [^{3}H]Acetyl-CoA (3–6 Ci/mmol) was from Amersham. Betamax was from ICN Biomedical.

2.2. Plasmid DNA

Plasmid pUCCMVCAT contained the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the human cytomegalovirus immediate early promoter. The plasmid was constructed using standard molecular cloning techniques [13]. Briefly, the supercoiled plasmid vector pUCSV2CAT (a gift from Dr. T. Hazinski) was linearized with HindIII restriction enzyme to release the simian virus 40 promoter. A HindIII DNA fragment coding for the CMV promoter was ligated to the linearized vector and the correct orientation for gene expression was selected from transformed bacterial colonies. Supercoiled plasmid DNA was cloned in *Escherichia coli* and purified by alkaline lysis and cesium chloride gradient centrifugation as described [13]. Plasmid DNA was radioactively labeled with ^{32}P using a nick translation kit (Promega) and [^{32}P]dCTP.

2.3. Liposomes

Cationic liposomes containing DC-Chol and DOPE or DOPC were prepared by 5 min sonication, in a bath sonicator, of mixed and dried lipids in 20 mM Hepes buffer as described [12]. The liposomes, having an average diameter of 150 nm, were stored at 4° C. The lipid composition of liposomes is indicated by molar ratio or mol%.

2.4. Cell culture and transfection

The human epidermoid carcinoma cell line A431 (a gift from Dr. G. Carpenter, Vanderbilt University) was cultured in growth medium (DMEM with 10% fetal bovine serum, L-glutamine, high glucose, penicillin and streptomycin). 24-well plates were used for all the experiments. Cells were plated 2 days before transfection which was performed at 70–80% confluency. Two methods were used to transfect the cells with DNA. In one method plasmid DNA was complexed to cationic liposomes in DMEM at room temperature for 10–15 min and then the complex was incubated with cells for 4 h at 37° C. Cells were washed and cultured for another 20 h in growth medium at 37° C before the CAT assay. The second transfection method involved a two step procedure where the cells are initially incubated with DNA/liposome complex on ice (pulse) followed by an incubation with free liposomes at 37° C (chase). In the first step, DNA was mixed with cationic liposomes in ice-cold DMEM without serum and incubated on ice for 30 min to form a complex. Chilled complex was added to the washed cells and incubated on ice for 30 min (pulse). The cells were then washed three times with chilled DMEM followed by one wash with warm DMEM and a chase with free cationic liposomes in warm DMEM. The chasing liposomes added had different compositions and they were added at different time points after the pulse with the complex. The liposome chase was maintained for 4 h (unless indicated otherwise) at 37° C followed by washing with warm DMEM and incubation of cells with growth medium for another 20 h before lysis and CAT assay.

2.5. CAT assay

The CAT assay [12] was done with modifications. The cells were washed once with PBS and lysed with 0.1% Triton X-100 in 0.25 M Tris-HCl buffer (pH 7.8) at room temperature for 10–15 min. The samples were heat-inactivated at 65° C for 10 min and centrifuged in a microfuge at high speed at 4° C for 5 min. The CAT reaction was performed at 37° C for 1 h using 60 μg protein of cellular lysate (Tables 1 and 2 and Figs. 2–4) or for 15 min using 15 μg protein (Figs. 1 and 5) unless otherwise noted. The reaction conditions were as follows: 100 μl total volume containing 1 mM chloramphenicol, 0.1 mM acetyl-CoA, 0.1 μCi [^{3}H]acetyl-CoA and cell lysate in 0.25 M Tris-HCl, pH 7.8. The reaction was stopped by rapid freezing and products of CAT reaction were extracted with 600 μl toluene. 3 ml of organic liquid scintillation cocktail BetaMax was added to the organic phase of

the extract and counted in a Beckman liquid scintillation counter. CAT activity is expressed as % acetylation of chloramphenicol (% conversion per 60 or 15 μg protein as described in the CAT reaction conditions above). Proteins were quantitated using a Bio-Rad microprotein assay (Bio-Rad Laboratories) and bovine serum albumin was used as a standard.

3. Results

In order to understand the mechanism of DNA delivery by cationic liposomes we have studied the effects of liposome concentration, lipid composition, and other parameters on the transfection activity and cellular uptake of DNA.

3.1. Effect of cationic liposome concentration on transfection activity and DNA uptake by cells

To verify the effect of different concentrations of cationic liposomes (DC-Chol/DOPE, 1:4 mole ratio) on the cellular uptake of DNA, a range of concentrations of the liposomes were used for delivering a constant amount of ^{32}P-labeled plasmid DNA (pUCCMVCAT). The complexes were incubated with cells for 4 h at 37° C to allow continuous cellular uptake of DNA. The results shown in Fig. 1 indicate that cell associated DNA increased with increasing amount of liposomes, saturating at 10 nmol total lipids (40 μM) per 1 μg DNA. Any increase in liposome concentration beyond the optimal concentration did not result in further increase in DNA uptake by the cells. To verify the effect of liposome concentration on functional DNA delivery (transfection activity) we repeated the same experiment shown in Fig. 1 using unlabeled DNA for delivery. In addition, the cells were incubated for an additional 20 h at 37° C in growth medium to allow for expression of the reporter gene (quantitated by a CAT assay) delivered by the liposomes. The results of this experiment are also shown in Fig. 1. There was a close correlation between the transfection activity and total cellular uptake of DNA. The ratio of 10 nmol lipids (40 μM) per 1 μg DNA allowed both maximum transfection and maximum cellular uptake of DNA. This result suggests that the efficiency of transfection is determined by the total of DNA uptake.

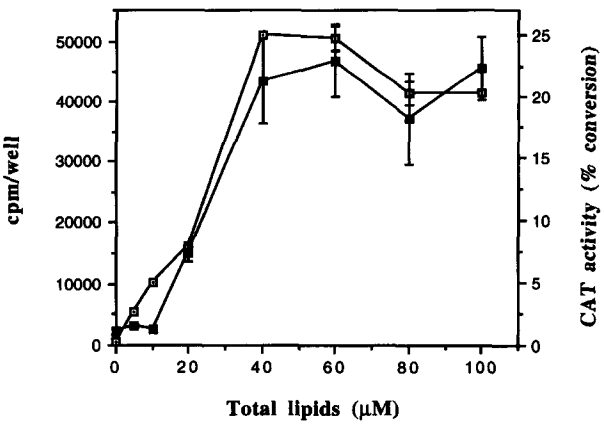

Fig. 1. Effect of cationic liposome concentration on transfection and DNA uptake by cells. 1 μg ^{32}P-labeled (□) or unlabeled (■) pUCCMV-CAT plasmid DNA was complexed to different amounts of cationic liposomes (DC-Chol/DOPE, 3:2 mole ratio) and incubated with A431 cells for 4 h at 37° C. Thereafter, cells were harvested for counting (□) or incubated for 20 h before the CAT activity (■) was assayed. Total volume of incubation was 250 μl/well.

3.2. Effect of cationic liposome concentration on DNA binding to the cell surface

There are many steps involved in the process of DNA uptake. To examine the initial step of uptake, i.e., the binding of DNA to the cell surface, we have incubated cells with DNA/liposome complex at 0° C to avoid the subsequent step of internalization. DC-Chol/DOPE (3:2) liposomes of various concentrations were mixed with a fixed amount of ^{32}P-labeled DNA and the complexes were incubated with cells on ice for 30 min followed by washing. The short incubation time was necessary to insure high viability of cells which do not tolerate cold temperature for an extended period of time. Preliminary experiments showed that 30 min was sufficient to allow for maximal binding of DNA/liposome complex to cells (data not shown). To compare the DNA binding with the transfection activity after the low temperature incubation we have also used unlabeled DNA in a separate experiment for the initial period of 0° C incubation for 30 min. After washing, the cells were incubated with growth medium at 37° C for an additional 20 h before the CAT assay was done. As can be seen in Fig. 2, DNA binding to the cell surface was not well correlated with the transfection activity of the bound DNA. Maximal DNA binding was observed at liposome concentration of 10 μM or higher; whereas the maximal transfection activity required at least 4-fold more liposomes, i.e., 40 μM total lipid. Apparently, the DNA bound to the cell surface via low amounts of liposomes (< 40 μM lipid) was not available for gene expression. Only the DNA/liposome complexes containing more liposomes were active in both cell binding and transfection. Therefore, the data indicate that at low liposome concentration maximal DNA binding to cells is allowed but one or more of the subsequent events, such as internalization or release of DNA into the cytosolic compartment, might be inhibited.

3.3. Effect of helper liposome on DNA delivery

The results from Fig. 2 clearly established the requirement for a minimal dose of cationic liposomes for optimal DNA delivery. We raised the question whether the addition of an extra dose of free cationic liposomes would help

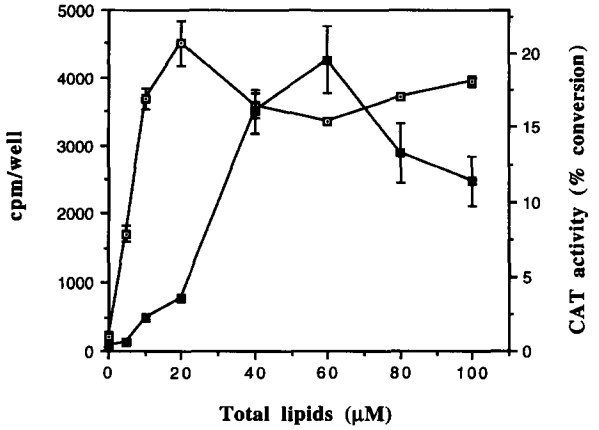

Fig. 2. Effect of cationic liposome concentration on transfection and cell binding. 1 μg ^{32}P-labeled (□) or unlabeled (■) pUCCMVCAT plasmid DNA was complexed to different concentrations of cationic liposomes (DC-Chol/DOPE, 3:2 mole ratio) and incubated with A431 cells for 0.5 h on ice. Thereafter, cells were washed and harvested for radioactivity counting (□) or incubated in growth medium at 37° C for 20 h before the CAT activity (■) was assayed. Total volume of incubation was 250 μl/well.

DNA delivery after DNA was already bound to cell surface using a low concentration of complexing cationic liposomes at low temperature. Adding free liposomes to deliver DNA may allow us to study the effect of phospholipid composition and mole ratio on the delivery of the DNA which is already bound to cell surface. Thus, DNA/liposome complex containing a relatively low amount (20 μM) of liposomes composed of DC-Chol/DOPE (3:2) or DC-Chol/DOPC (3:2) was incubated with cells on ice for 30 min (pulse) followed by washing and warming to 37° C with or without the addition of free liposomes (chase). Free cationic liposomes containing different ratios of DC-Chol to DOPE or DOPC were used in the chase to study the helper (free) liposome activity. It is clear from Table 1 that only DOPE-containing liposomes were capable of helping DNA delivery when DNA was complexed to either DOPE or DOPC complexing liposomes. DOPC-containing free liposomes had essentially no detectable activity in this assay system. Helper activity of DOPE liposomes increased with increasing DOPE content in the liposomes with 80% DOPE content being optimal (8.4-fold enhancement in activity). Interestingly, liposome containing excess DOPE (i.e., 90% DOPE) showed no helper function at all (Table 1). This is probably related to the instability of these liposomes due to their high content of DOPE, a phospholipid with high tendency to form non-bilayer structures [11]. If 10% of DOPE was replaced with DOPC to improve the liposome stability, the helper function of the liposomes was again maximal. Further degree of DOPE substitution with DOPC (20–30% DOPC) had resulted in decreased helper activity, indicating that the helper function is closely associated with the DOPE content. Other liposome compositions were also tested and the results (not shown) indicate that as little as 5% DC-Chol in the liposomes enriched with DOPE was sufficient for a significant helper activity. These data, taken together, indicate that the most important lipid ingredient in the helper liposomes is DOPE; only minimal amount of cationic lipid is needed probably to provide a means for the binding of liposomes to the negatively charged cell surface.

3.4. The effect of helper liposome dose on DNA delivery

The effective dose of helper liposomes needed for DNA delivery was determined (Fig. 3). Cells were pulsed with DNA/DC-Chol/DOPE (3:2) liposome complex and chased with increasing concentrations of free helper liposomes containing 20% DC-Chol and 80% DOPE or DOPC. DOPE liposomes were found to perform a detectable helper function at concentrations as low as 1 μM and optimal delivery at 20–40 μM. Free DOPC-containing

Table 1
Effect of lipid composition of helper liposome on DNA delivery [a]

Helper liposome (mole ratio)			CAT activity (% conversion)	
DC-Chol	DOPE	DOPC	complexing liposome:	
			DC-Chol/DOPE (3:2 mole ratio)	DC-Chol/DOPC (3:2 mole ratio)
–	–	–	2.5 ± 0.6	0.2 ± 0.1
6	–	4	4.2 ± 0.3	0.2 ± 0.1
2	–	8	2.4 ± 1.4	0.3 ± 0.2
6	4	–	8.2 ± 0.6	1.8 ± 0.2
4	6	–	12.8 ± 0.4	2.8 ± 0.4
2	8	–	21.0 ± 0.5	6.0 ± 0.5
1	9	–	2.8 ± 0.5	0.3 ± 0.1
1	8	1	24.7 ± 2.0	–
1	7	2	14.5 ± 2.5	–
1	6	3	10.6 ± 2.3	–

[a] A431 cells were incubated (pulse) for 30 min on ice with pUCCMVCAT DNA (1 μg) complexed to DC-Chol/DOPE (3:2) or DC-Chol/DOPC (3:2) complexing liposomes (20 μM), washed, warmed, and incubated (chase) for 4 h at 37° C with free helper liposomes (40 μM) containing different lipid composition as shown. After the chase, cells were washed and cultured in growth medium at for 20 h at 37° C before the CAT assay.

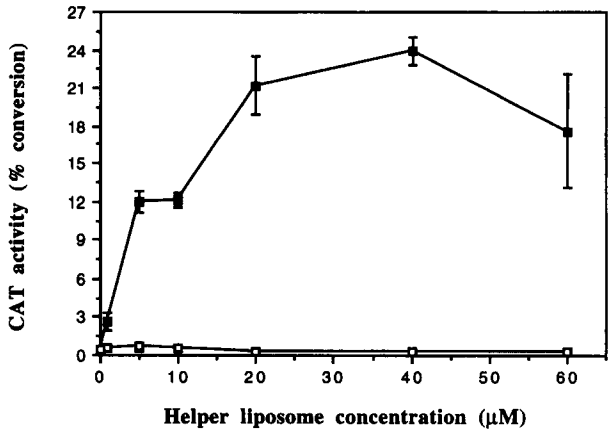

Fig. 3. Effect of helper liposome concentration on DNA delivery. A431 cells were pulsed with pUCCMVCAT DNA (1 μg) complexed to DC-Chol/DOPE (3:2) liposomes and chased with increasing concentrations of DC-Chol/DOPE (1:4) (■) or DC-Chol/DOPC (1:4) (□) liposomes. The cells were then washed and incubated at 37° C in growth medium for 20 h before harvested for CAT assay.

cationic liposomes showed no detectable helper activity at all concentrations used.

3.5. Time-course of DNA delivery by helper liposomes

Chasing the bound DNA with helper liposomes is critically dependent on the initiation time of the chase after the pulse with DNA/liposome complex. Helper liposomes were added at different time points after the initiation of the chase (Fig. 4). Complexing liposomes were DC-Chol/DOPE (3:2), and helper liposomes were either DC-Chol/DOPE (3:2) or DC-Chol/DOPE (1:4). Data in Fig. 4 showed that the DNA delivery activity decreased gradually as the addition of helper liposomes was delayed, with half of the original activity found when the helper liposomes were added 70–80 min after the initiation of the chase. Similar to the results in Table 1, Fig. 4 shows that free cationic liposomes with higher DOPE content (80%) performed better DNA delivery function than free cationic liposomes with lower DOPE content (40%). In this experiment, the chase incubation period lasted for 4 h for every time point listed. The next experiment was to investigate the length of chase period required for optimal DNA delivery when all chases were initiated immediately after the pulse. The results showed that even 15 min of total chase time (followed by washing off the helper liposomes) was sufficient to achieve a substantial level of helper activity (Table 2).

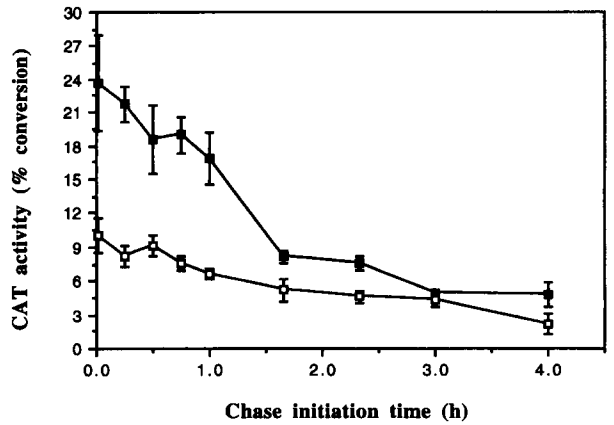

Fig. 4. Time-course of helper liposome chase. A431 cells were pulsed with pUCCMVCAT DNA (1 μg) complexed to 20 μM of DC-Chol/DOPE (3:2) liposomes and chased with 40 μM of DC-Chol/DOPE (1:4) (■) or DC-Chol/DOPE (3:2) (□) liposomes at the indicated time points after the completion of the pulse. The cells were then washed and incubated at 37° C in growth medium for 20 h before harvested for CAT assay.

Table 2
Effect of chase incubation time on DNA delivery by helper liposomes [a]

Helper liposome (μM)	Chase incubation time (h)	CAT activity (% conversion)
0	4	2.9±0.17
40	0.25	17.7±1.2
40	0.5	16.0±0.6
40	1	18.9±3.4
40	2	19.4±0.9
40	3	20.3±1.2
40	4	16.2±1.6
40	5	14.9±0.7

[a] A431 cells were incubated (pulse) for 30 min on ice with pUCCMV-CAT DNA (1 μg) complexed to DC-Chol/DOPE (3:2) complexing liposomes (20 μM), washed, warmed, and incubated (chase) for different length of time at 37° C with DC-Chol/DOPE (1:4) free helper liposomes (40 μM). After the chase, cells were washed and cultured in growth medium at for 20 h at 37° C before the CAT assay.

3.6. Effect of chloroquine on DNA delivery

Chloroquine is a lysosomotropic agent known to interfere with endocytosis by raising (neutralizing) endosomal and lysosomal pH and by inhibiting maturation of endosomes [14]. Therefore, we decided to test the effects of

Table 3
Effect of chloroquine on DNA delivery

Chloroquine treatment [a] (μM)	CAT activity (% conversion)		
	pulse/chase [b]	transfection [c]	
		DOPE liposomes	DOPC liposomes
0	21.9±2.0	17.0±1.1	4.4±0.2
200	0.8±0.1	0.3±0.0	0.2±0.0

[a] Chloroquine was added during incubation at 37° C.
[b] A431 cells were incubated (pulse) with pUCCMVCAT (1 μg) DNA/DC-Chol/DOPE (3:2) liposome (20 μM) complex on ice for 30 min, washed, and incubated (chase) with 40 μM of free helper liposomes (DC-Chol/DOPE (1:4) at 37° C for 4 h. Cells were then washed and incubated at 37° C in growth medium for 20 h before the CAT assay.
[c] A431 cells were transfected with pUCCMVCAT (1 μg) DNA/DC-Chol/DOPE (3:2) or DNA/DC-Chol/DOPC (3:2) liposome (40 μM) complexes at 37° C for 4 h. Cells were then washed and incubated at 37° C in growth medium for 20 h before the CAT assay.

chloroquine on transfection and on helper liposome activity in the pulse/chase experiments shown above. Cells were transfected with DNA/DC-Chol/DOPE (3:2) liposomes or DNA/DC-Chol/DOPC (3:2) liposomes at 37° C for 4 h with or without 200 μM chloroquine (Table 3). Alternatively, cells were pulsed with DNA/DC-Chol/DOPE (3:2) liposomes on ice for 30 min, washed and chased with free DC-Chol/DOPE (1:4) liposomes with or without 200 μM chloroquine at 37° C (Table 3). Chloroquine addition inhibited over 95% of the transfection activity associated with both transfection and helper liposome activity. Regardless of the type of phospholipid used in the liposome formulation (DOPE or DOPC), DNA delivery was abolished by the chloroquine treatment (Table 3).

3.7. Ratio of cationic lipid to phospholipid in cationic liposomes for transfection

DNA delivery by cationic liposomes is largely affected by the critical ratio of cationic lipid to phospholipid present in the liposome formulation [5,9,12]. The ability to complex DNA and to bind with the cell surface, which is negatively charged, is dependent on the liposome content of cationic charges. To establish the optimal ratio of phospholipid to cationic lipid (DC-Chol) for optimal transfection, cells were transfected with a complex of cationic liposomes and DNA for 4 h at 37° C. The liposomes contained different ratios of DC-Chol to DOPE or DOPC. The results of this experiment are shown in Fig. 5. At all lipid ratios used, DOPE cationic liposomes were superior in DNA delivery when compared to DOPC cationic liposomes. DOPC liposomes had detectable transfection activity at 30–50% DOPC content but had none above 50%. This is in contrast to the easily detectable DNA delivery at 30–80% DOPE. The optimal DC-Chol content in the DOPE liposomes was about 50–60%.

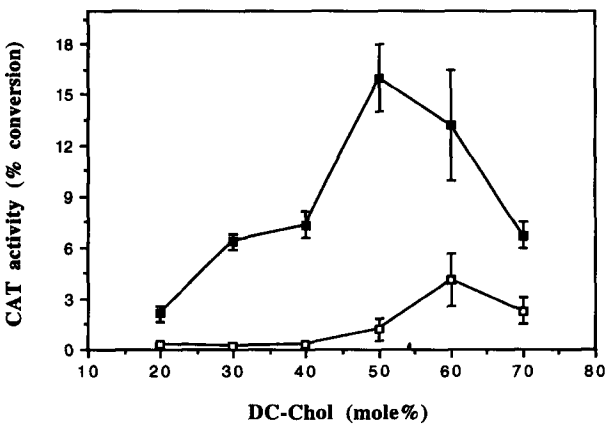

Fig. 5. Effect of lipid composition of liposomes on transfection. A431 cells were transfected for 4 h at 37° C with 1 μg pUCCMVCAT plasmid DNA complexed to 40 μM total lipids of liposomes containing different mol% of DC-Chol in DOPE (■) or DOPC (□) liposomes. Cells were lysed and assayed for CAT activity 20 h after transfection.

4. Discussion

It has been shown by us [9] and others [8,10] that endocytosis of DNA/liposome complex is the major route of DNA uptake by cells during transfection. The complex first adsorbs to cell surface by charge interaction. The surface bound complex is then internalized by endocytosis into endosomes and lysosomes. A small portion of the endocytosed DNA is released into the cytosol from which the DNA must enter into the nucleus for transcription. The majority of the internalized DNA stays in the endocytic compartments and is eventually degraded. This is a complicated mechanism which involves multiple steps. The fact that the transfection activity of DC-Chol/DOPE liposomes closely correlates with the total cellular uptake of DNA (Fig. 1) strongly suggests that one or more of the above mentioned steps may control the amount of DNA arriving at the final transcription compartment which in turn determines the transfection activity of the liposomes. Ideally, it is important to dissect the overall uptake process into individual steps and analyze the contribution and control of each step. As the first step in this approach, we have used a pulse/chase protocol and examined the relationship between the amount of DNA bound to the cell surface and the final activity of transfection. Data presented in Fig. 2 indicate that only the complexes containing sufficient amount of liposomes could eventually arrive at the final destination for transfection. Those DNA/liposome complexes with suboptimal amount of liposomes, although bound to cells efficiently, were not active in transfection. This observation gave us an opportunity to examine if additional liposomes added at the onset of chase incubation could improve the transfection activity of the DNA/liposome complex already bound to the cell surface. Our hypothesis was that these complexes are internalized into the endosomes but not released into the cytosol. Co-internalization of helper liposomes of proper composition may enhance the release of DNA into the cytosol and increase the transfection activity.

This hypothesis is supported by several lines of observation. First, only liposomes enriched with DOPE showed strong helper activity (Table 1). DOPE is a phospholipid which exhibits a high tendency to form inverted hexagonal (H_{II}) phase particularly at acidic pH [11]. DOPC, a structural analog of DOPE, has no activity to form H_{II} phase under physiological conditions [11]. Cationic liposomes containing DOPC showed no helper activity (Table 1). Zhou and Huang [9] have previously shown by transmission electron microscopy that only DNA/liposome complex containing DOPE can destabilize the endosome membrane and escape into the cytosol; complex containing DOPC has no such activity. Apparently, in our present

study complexes containing a low level of DOPE could not destabilize the endosome membrane. Only when additional helper liposomes enriched with DOPE are co-localized into the same endosome, the complex could then be released into the cytosol after the endosome membrane is destabilized. That the action of the helper liposomes is in an intracellular compartment is also supported by the data in Fig. 4. Delayed addition of helper liposomes would not result in the co-localization with DNA/liposome complex in the same endosome, thus showing reduced or diminished helper activity. Furthermore, the lysosomotropic agent, chloroquine, completely inhibited the activity of helper liposomes (Table 3), indicating that endocytosis is a necessary element for the helper liposome activity. The proposed mechanism of action is also consistent with the data in Table 2 which showed that cells only needed to be exposed to the helper liposomes for a short period of time to exhibit an enhanced transfection activity. Only the helper liposomes initially bound to the cell surface would have a chance to be co-internalized together with the DNA/liposome complex. Prolonged incubation with the helper liposomes would not significantly enhance the transfection because the helper liposomes bound to the cell surface at the later time could not be co-localized with the complex in the same endosome. Taken together, the data presented here and published by others [8–10] strongly support the role of DOPE in the cationic liposome mediated gene transfer as that of an endosomolytic agent, similar to that of the adenovirus [15–19] and fusion peptides [20] used to enhance the transfection activity of molecular conjugates.

If the optimal helper liposomes are those enriched with DOPE, then why the optimal transfection liposomes were those relatively enriched with DC-Chol as shown in Fig. 5? Obviously, there are steps in the transfection process other than the release of DNA from endosome in which DC-Chol plays an important role. For example, liposomes with sufficient amount of DC-Chol may be required for the formation of proper complex with DNA. It is likely that the negatively charged DNA is coated with a layer or more of positively charged lipids such that the net charge of the complex is neutral or positive. Preliminary results from this lab (Sorgi et al., unpublished data) indicate that the optimal transfection complex contain a collection of heterogeneous structure including tubes and fused liposomes. The role of DC-Chol, or other cationic lipids, in the formation of transfection complex is presently unknown, but must be important in the determination of the final transfection activity. Obviously, more work is needed to answer this question.

Finally, liposomes containing DC-Chol and DOPC, although weak in activity, were active in transfection (Fig. 5). Since the activity was inhibitable by chloroquine, endocytosis must also be involved in the mechanism of action of these liposomes (Table 3). This and other cationic lipid formulations [6,7], which are completely devoid of DOPE, obviously rely on other unknown mechanism(s) to escape the endosome or lysosome. This interesting aspect of liposome mediated gene transfer will be an important subject for future studies.

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