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Effect of cationic cholesterol derivatives on gene transfer and protein kinase C activity

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Four different cationic derivatives of cholesterol were synthesized which contain either a tertiary or a quaternary amino head group, with and without a succinyl spacer-arm. Their ability to inhibit protein kinase C (PKC) activity was measured in a detergent mixed micellar solution. Derivatives containing a quaternary amino head group were effective inhibitors (K_i approx. 12 and 59 μM) of PKC and derivatives containing a tertiary amino head group were approx. 4–20-fold less inhibitory. Liposomes containing an equimolar mixture of dioleoylphosphatidylethanolamine (DOPE) and a cationic cholesterol derivative were tested for the DNA-mediated transfection activity in mouse L929 cells. Highest activity was found with the derivative with low PKC inhibitory activity and with a succinyl spacer-arm. The transfection activity of this tertiary amine derivative, *N*,*N*-dimethylethylenediaminyl succinyl cholesterol was dependent on DOPE as a helper lipid; liposomes containing dioleoylphosphatidylcholine and this derivative had little activity. The transfection protocol of this new cationic liposome reagent was optimized with respect to the ratio of liposome/DNA, dose of the complex and time of incubation with cells. Several adherent cell lines could be efficiently transfected with this liposome reagent without any apparent cytotoxicity. However, the transfection activity was strongly inhibited by the presence of serum components.

Introduction

DNA-mediated transfection has become an important tool in modern biology. Among the conventional reagents such as calcium phosphate, DEAE-dextran and other particulate reagents, liposomes have become increasingly acceptable as a convenient and reproducible reagent for DNA-mediated transfection. There are generally two classes of liposomal transfection reagents: those which are cationic [1–9] and those which are anionic [10–16]. Transfection with anionic liposomes generally requires that the DNA be entrapped in the internal aqueous space of the liposomes although one exception has been reported [17]. Because of the relatively time-consuming protocol for DNA entrapment, anionic liposomes are not widely used for transfection. Transfection with cationic liposomes, on the other hand, does not require DNA entrapment in the liposomes. Instead, complexes of DNA and liposomes can be easily prepared by simple mixing and reasonable transfection efficiency is reproducibly obtained for many different cell types. The most widely used cationic liposome reagent is lipofectin which is composed of an equimolar mixture of DOPE and DOTMA [1]. DOTMA is a double-chain amphiphile which contains a positively charged, quaternary amino head group [18]. Although lipofectin is effective in delivering DNA, RNA [1,19–22] and negatively charged proteins [22] into cells, its use is often limited by the toxicity to the treated cells and the relatively high cost of the reagent.

Cationic amphiphiles are often good stabilizer molecules for the Lα phase of unsaturated PE [3]. Amphiphiles containing a quaternary amino group show higher stabilization activities than those containing a tertiary amino group (Bottega, R. and Epand, R.M., unpublished data). This is related to the difference in the charge content of the amphiphile at neutral pH. Higher level of positive charge content of the quaternary amino group brings more interfacial hydration to stabilize the Lα phase of unsaturated PE (Bottega, R.

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Abbreviations: CAT, chloramphenicol acetyltransferase; DEAE, diethylaminoethyl; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*-trimethylammonium chloride; PE, phosphatidylethanolamine; PKC, protein kinase C; SUV, small unilamellar vesicles.

and Epand, R.M., unpublished data). On the other hand, amphiphiles containing quaternary amino groups are effective inhibitors of protein kinase C in addition to being better Lα phase stabilizers (Bottega, R. and Epand, R.M., unpublished data).

Here, we describe the protein kinase C inhibition activities of several cationic amphiphiles which contain a common hydrophobic moiety, i.e., cholesterol and compare their activities to promote the DNA-mediated transfection in mammalian cells. Furthermore, we have studied in detail the transfection activity of one of the cationic cholesterol derivatives containing a tertiary amino head group. The amphiphile, *N*,*N*-dimethyl ethylenediaminyl succinyl cholesterol, when mixed with DOPE to form liposomes, is highly effective in promoting the DNA-mediated transfection. Furthermore, the new transfection reagent is not toxic to the treated cells. The preparation and characterization of the new liposome reagent are described in this report.

Materials and Methods

Materials. DOPE and DOPC were purchased from Avanti Polar Lipids (Alabaster, AL). DNAse I was purchased from Sigma. Lipofectin was purchased from Bethesda Research Laboratory.

Synthesis of cationic cholesterol derivatives. The synthesis of cholesteryl-3β-carboxyamidoethylenedimethylamine (IV), cholesteryl-3β-carboxyamidoethylene-trimethylammonium iodide (III), cholesteryl-3β-oxysuccinamidoethylenedimethylamine (II) and cholesteryl-3β-oxysuccinamidoethylenetrimethylammonium iodide (I) is to be described elsewhere (Bottega, R. and Epand, R.M., unpublished data). In that paper, compounds IX, VIII, IV and III refer to compounds I, II, III and IV in the present work.

Isolation of protein kinase C. Protein kinase C was purified from rat brain to near homogeneity by a modification of a published method [25]. 25 brains from Sprague-Dawley rats (150–200 g) were removed, washed, homogenized and applied to a DEAE-Sepharose, phenyl-Sepharose and polylysine agarose column. The uncontaminated fractions containing PKC were pooled and salts were removed using Amicon YM-10 ultrafiltration. The enzyme gave a specific activity of 200 nmol phosphate incorporated per min per mg of protein in a histone phosphorylation assay using the Triton mixed micelle assay with 6.5 mol% phosphatidylserine, 2.5 mol% DAG and 100 μM calcium present. Specific activities ranging from 30 nmol/min per mg [26] to 600 nmol/min per mg [27] have been observed for PKC using the Triton mixed micelle assay under the same conditions.

Mixed micelle assay for protein kinase C. The Triton X-100 assay previously described by Bell and coworkers was used to measure enzyme activity [26]. Phosphatidylserine and 1,2-diolein with and without additive were dissolved in a solution of chloroform/methanol (2:1, v/v). Solvent was evaporated with a stream of nitrogen and last traces removed using a vacuum desiccator at 40°C. The lipid films were then solubilized by the addition of 3% Triton X-100, vortexed vigorously for 30 s and then incubated at 30°C for 10 min to allow for equilibration. A 25 μl aliquot of this solution was used in a final assay volume of 250 μl, containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 200 μg/ml histone III-S, 100 μM $CaCl_2$, 10 μM adenosine 5'-tri[γ-^{32}P]phosphate, 2.75 mM Triton X-100, with 300 μM (6.5 mol%) phosphatidylserine and 107 μM (2.5 mol%) 1,2-diolein. For controls, 25 μl of 20 mM EGTA replaced the $CaCl_2$. To initiate the reaction, 150 ng of protein was added. After brief mixing, the tubes were incubated for 10 min at 30°C. The reaction was terminated by adding 1 ml of cold 0.5 mg/ml BSA and 1 ml of cold 25% trichloroacetic acid. This mixture was passed through a GF/C Whatman filter and washed five times with 2 ml of 25% trichloroacetic acid. After drying, the filters were counted with 6 ml ACS scintillation fluid.

Liposome preparation. DOPC or DOPE were combined with cationic cholesterol derivatives in chloroform and dried with N_2 gas to remove the chloroform solvent. The dried lipid film was vacuum desiccated for at least half an hour and suspended by vortexing in dH_2O. After this hydration step, the samples were vortexed briefly and sonicated in a bath type sonicator (Laboratory Supplies, Hicksville, NY) to generate small unilamellar vesicles. Vesicles were stored at 4°C at a total lipid concentration of 3.5 mM.

Cell culture. L929 mouse fibroblasts and Vero monkey kidney cells were routinely cultured in McCoy's 5A modified media (Gibco) supplemented with 10% bovine calf serum. HeLa cells were cultured in Eagle's MEM media with Earle's salts supplemented with 10% fetal calf serum (Hyclone). A431 human epidermoid carcinoma (from G. Carpenter, Vanderbilt University) and A375 human malignant melanoma were routinely cultured in DMEM with L-glutamine, 4.5 g/l glucose and 10% fetal calf serum. CCRF-CEM human T-lymphoblastoid cells (obtained from ATCC) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum. OKT4 hybridoma (obtained from ATCC) was cultured in Iscove's modified Dulbecco medium with 20% fetal calf serum. All media contained antibiotics: 100 units/ml penicillin, 100 μm/ml streptomycin and 10 μm/ml gentamycin. Cells were cultured in 5% CO_2 and 95% humidified air.

Transfection and CAT assay. Plasmid pUCSV2CAT (a gift from T. Hazinski) containing the *Escherichia coli* CAT gene driven by the SV40 virus early promoter was used in this study. Plasmid DNA was purified by standard method [29]. 5 μg plasmid DNA (1.75 mg/ml) in

1 × TE buffer was mixed gently with liposomes at room temperature in 1 ml serum-free McCoy's medium and incubated for 10 min before adding to cells. Cells at 50–80% confluency in 100-mm plastic plates were washed once with serum-free McCoy's medium after which 2 ml of the same medium was added per plate. DNA-liposome complex was added to the cells in a total incubation volume of 3 ml and was incubated at 37°C with 5% CO_2 for 5 h. Transfection media was removed and the cells were incubated in the growth medium containing serum for 48 h before the CAT assay which was performed as described [29]. Basically, 60 min reaction time, 0.1 μCi [^{14}C]chloramphenicol (53 mCi/mmol) and 4 mM acetylCoA were used per sample. Equal amounts of protein per sample were used for the assay as quantitated by the Bradford microprotein assay (Bio-Rad). Percent conversion of chloramphenicol to acetylated forms was normalized to mg proteins used per sample. The data in the figures represent an average of two experiments with duplicate samples per data point. The variability in CAT expression was reproducable under identical conditions and was eliminated when liposomes were used immediately after sonication. Variability is further decreased when the cells are transfected at the same degree of confluency.

Results

Cationic derivatives of cholesterol

We have synthesized four derivatives of cholesterol, all containing an amino head group (Fig. 1). Compounds I and II contain a succinyl spacer-arm between the substituted ethylene diamine and cholesterol,

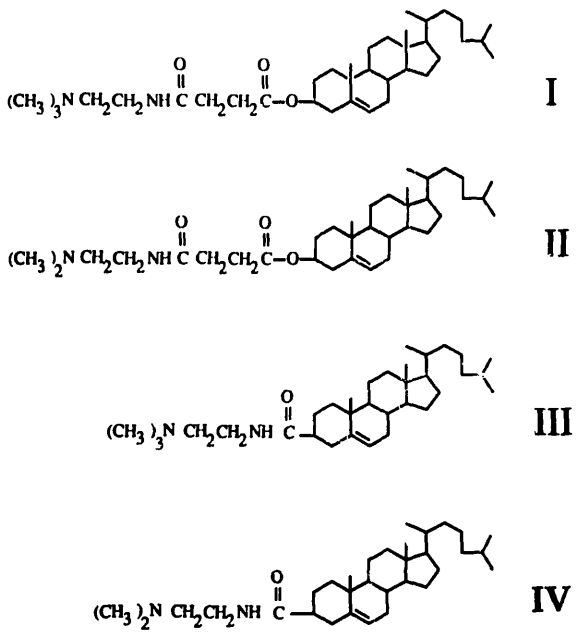

Fig. 1. The structure of the four cationic cholesterol derivatives.

TABLE I

Inhibition of PKC and promotion of DNA-mediated transfection activities of cationic cholesterol derivatives

Derivative	PKC inhibition K_i (μM)	CAT activity [a] (mU/mg protein)	Cellular toxicity [b] (% viable cells)	
			70 μM	93 μM
I	59	5	85.0 ± 3.6	74.3 ± 2.5
II	191	143	94.7 ± 3.1	90.0 ± 2.6
III	12	4	78.3 ± 5.0	69.0 ± 5.0
IV	258	56	92.0 ± 3.6	86.0 ± 3.0
DOTMA	95	40	63.7 ± 7.6	44.0 ± 6.6

[a] 23 μM liposomes (70 nmol lipid) containing an equimolar mixture of DOPE and cationic derivative were mixed with pUCSV2CAT DNA (5 μg) and added to L929 cells. After 5 h incubation at 37°C, cells were washed and incubated in fresh medium containing 10% serum for 2 days before assayed for CAT activity which is shown as the average of three different experiments.

[b] Liposome/DNA complexes were prepared as described in footnote a. After 5 h incubation of L929 cells with the complex, cell viability was determined by the Trypan blue assay. % viable cells is shown as mean ± S.D. of three determinations. Control cells which received no complex showed 98% viability.

whereas compounds III and IV do not. Compounds I and III are quaternary amines and compounds II and IV are tertiary amines.

Inhibition of PKC

PKC activity was assayed in Triton mixed micelles containing various amounts of the cationic cholesterol derivatives. As can be seen in Table I, derivatives I and III, both containing a quaternary amino group, were effective inhibitors. K_i values of these compounds were in the range of a few tens of μM. On the other hand, derivatives II and IV containing a tertiary amino group were weak inhibitors. Their K_i values were about 4–20-fold higher than those of derivatives I and III.

Liposome preparations

Cationic cholesterol derivatives did not form a stable dispersion by themselves at physiological pH. It was necessary to add at least 10% phospholipid, either DOPE or DOPC, to obtain a stable liposome dispersion by sonication. We routinely prepare liposomes with an equimolar mixture of a cationic cholesterol derivative and DOPE. Furthermore, the liposomes were prepared in deionized water or TE buffer to avoid aggregation due to the high ionic strength of the medium. The average size of the liposomes was 147 ± 5 nm as measured by dynamic light scattering.

Transfection activity and cellular toxicity of cationic cholesterol derivatives

Liposomes containing an equimolar mixture of DOPE and a cationic cholesterol derivative were tested

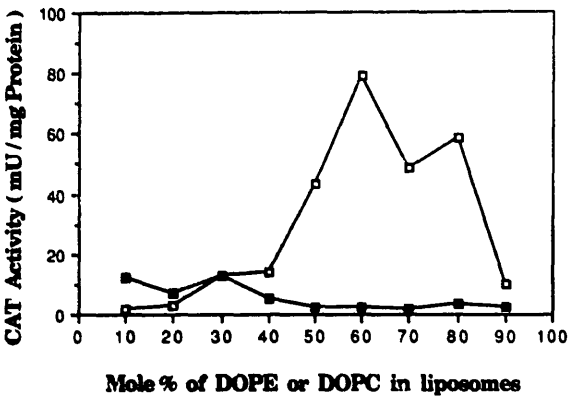

Fig. 2. The effect of phospholipid composition of cationic liposomes on the transfection activity. Liposomes were prepared with derivative II and various % mol amounts of DOPE (□) or DOPC (■). Liposomes (23 μM total lipids) were mixed with 5 μg DNA and added to L929 cells in serum-free McCoy's media. Transfection was allowed for 5 h after which the cells were washed and incubated at 37°C in serum-containing media for 2 days before CAT activity was measured.

for the transfection activity in mouse L929 cells. Plasmid DNA was mixed with liposomes and the CAT activity of the cell extracts were determined by a standard protocol. As can be seen in Table I, derivatives II and IV showed positive transfection activity with the activity of II much higher than that of IV. Derivatives I and III, both quaternary amines, did not show any appreciable transfection activity. Furthermore, the toxicity of the liposome/DNA complex to L929 cells was also measured by Trypan blue exclusion assay. It is clear from the data in Table I that complexes containing quaternary amine derivatives (I and III) were more toxic to the treated cells, whereas those containing the tertiary amine derivatives (II and IV) were less toxic. These results indicate that liposomes containing the tertiary amine derivatives warrant further studies. We have decided to concentrate on the derivative II and to characterize in detail the transfection activity of the liposomes containing this derivative.

Transfection activity as a function of phospholipid composition

Liposomes containing mixtures of II and DOPE, or II and DOPC were tested for transfection activity on the mouse L929 cells. The relative proportion of the phospholipid, either DOPE or DOPC in the liposomes was varied from 10 to 90%. Fig. 2 shows the result of this experiment. Obviously, liposomes containing 20–50% II in DOPE induced very high levels of CAT activity in the treated cells, whereas liposomes containing either too little (10%) or too much (> 60%) II in DOPE did not. Also shown in Fig. 2 is the observation that liposomes containing II in DOPC were not active in transfection, regardless of the concentration of the cationic amphiphile in liposomes. Thus, the transfection activity of II depended on the nature of the helper phospholipid in the liposomes. Furthermore, optimal activity of the liposomes was in those containing 20–50% II in DOPE. The transfection activity of liposomes containing equimolar mixture of DOPE and II were used for further studies, because these liposomes appeared homogenous in size. However, the transfection activity of these liposomes decayed with storage (4°C) with an estimated half-life of only 2 days.

Effect of lipid concentration on the transfection activity

Liposomes composed of an equimolar mixture of II and DOPE were used to study the lipid concentration dependence of the transfection activity. In the experiment described in Fig. 3, 5 μg DNA was mixed with various amounts of liposomes and added to the L929 cells. The transfection activity showed a sudden increase at about 60 nmol total lipid above which maximal levels of CAT activity were found in the treated cells. Also shown in Fig. 3 is the comparison of transfection activity of a well-established liposome reagent, lipofectin, also using 5 μg DNA for the assay. It is clear that lipofectin had a higher activity than the cationic liposomes described here when a sub-optimal lipid concentration was used. However, the activity of II/DOPE liposomes was much higher than that of lipofectin if higher lipid concentrations were used. Lipofectin-mediated transfection at concentrations greater than 40 nmol is not shown due to observed cytotoxicity of the treated cells.

Effect of DNA concentration on transfection activity

In an experiment in which the total lipid concentration was kept at 70 nmol and the amount of DNA varied from 0 to 20 μg, maximal transfection activity of

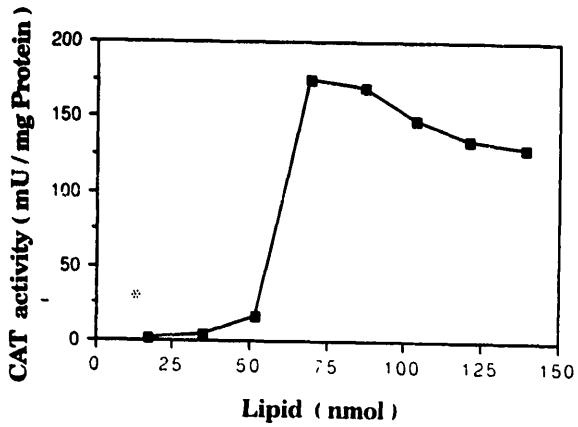

Fig. 3. The effect of total lipid concentration on transfection activity. Different amounts of liposome (composed of an equimolar mixture of derivative II and DOPE) (■) were mixed with 5 μg DNA and used for transfection of L929 cells as described in the legend to Fig. 2. Lipofectin (*) was also used for comparison of transfection activity under conditions identical to those used for the cationic liposomes.

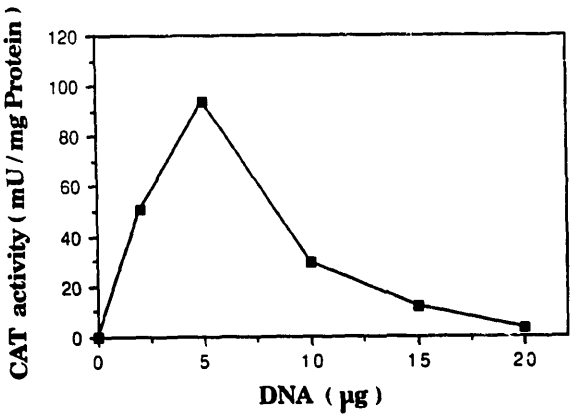

Fig. 4. The effect of DNA concentration on transfection activity. 0–20 μg DNA was mixed with liposomes composed of equimolar mixture of II and DOPE (70 nmol) and transfection of L929 cells was carried out as described in the legend to Fig. 2.

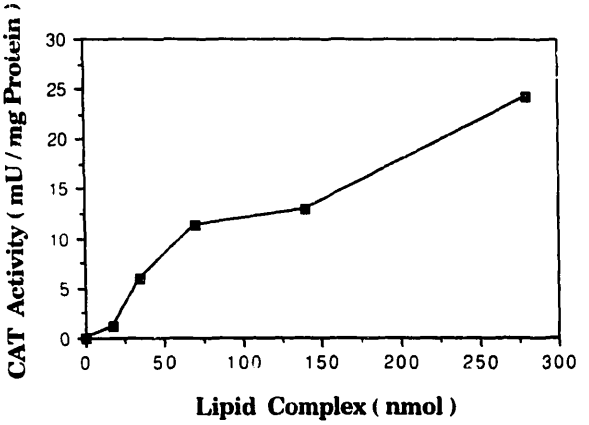

Fig. 5. The effect of dose of the DNA/liposome complex on transfection activity. A ratio of 70 nmol lipid (an equimolar mixture of II and DOPE) and 5 μg DNA was maintained for transfection at different doses of complex. 0, 1.25, 2.5, 5, 10 and 20 μg DNA was used with 0, 17.5, 35, 70, 140 and 280 nmol lipids, respectively. Transfection conditions are the same as described in the legend to Fig. 2.

the treated L929 cells was found to be at 5 μg DNA (Fig. 4). Higher or lower DNA concentrations resulted in lower activities, producing a bell-shaped curve shown in the figure. From the results of this experiment and those of Fig. 3, it was decided that a ratio of 70 nmol total lipids to 5 μg DNA gave optimal transfection activity for liposomes containing an equimolar mixture of II and DOPE. The ratio of positive to negative charges of the complex at this ratio can be calculated to be approx. 2.2.

Electrophoretic characterization of liposome / DNA complexes

We have characterized the liposome/DNA complexes by agarose gel electrophoresis (data not shown). Incubation mixtures containing increasing amounts of liposomes showed decreasing intensities of free DNA bands and a concomitant increase in the amount of DNA on the top of the gel which did not enter the gel during electrophoresis. Furthermore, all of the uncomplexed, free DNA could be digested by DNAse, but only a small portion of the complexed DNA was digested. At the optimal liposome/DNA ratio for transfection, all DNA was complexed with liposomes.

Effect of the complex dose on transfection activity

Complex of liposomes and DNA was prepared at the optimal ratio of 70 nmol lipid and 5 μg DNA. Various amounts of the complex were added to L929 cells to study the dose effect on transfection activity. The result shown in Fig. 5 clearly indicates that the transfection activity linearly increased with the dose until 280 nmol total lipid and 20 μg DNA was added. Higher doses did not produce any significant increase in transfection activity (data not shown). Minimal cytotoxicity of the treated cells was observed even at the highest dose used. CAT activities shown in the figure were lower than normal because the liposomes used were not freshly prepared.

Effect of serum in transfection activity of the complex

The transfection activity of the liposome/DNA complex was sensitive to the presence of serum (Fig. 6). As little as 5% of calf bovine serum inhibited transfection activity by approx. 85%. No CAT activity could be detected in the cell extract if 20% serum was included in the incubation medium. Thus, the optimal condition for transfection with this novel liposome reagent should not include serum in the incubation medium.

Effect of incubation time course of liposome / DNA complex on transfection activity

Using optimal complex (70 nmol lipid and 5 μg DNA), we have incubated the L929 cells with complex in the absence of serum for different periods of time.

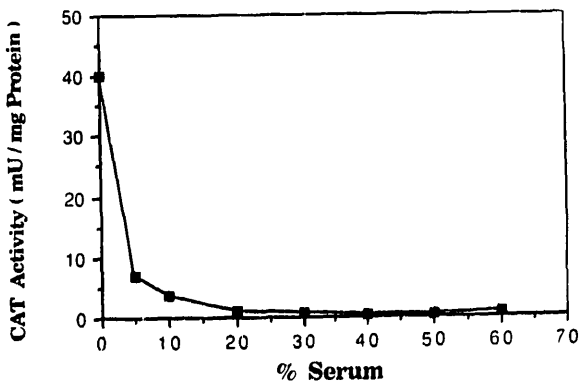

Fig. 6. The effect of serum on transfection activity. 0–60% calf serum was added to the transfection media. 5 μg DNA and 70 nmol lipid were used. Other conditions were the same as described in the legend to Fig. 2.

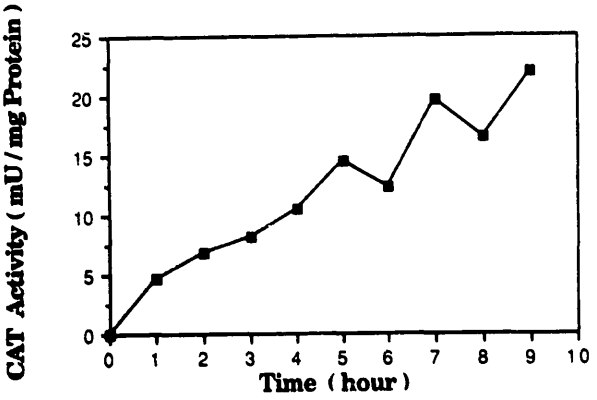

Fig. 7. The effect of transfection incubation time on transfection activity. 5 μg DNA was mixed with 70 nmol total lipid composed of equimolar mix of derivative II and DOPE and added to cells as described in the legend to Fig. 2.

Data in Fig. 7 show that the degree of transfection increased almost linearly with time for up to 9 h. Longer incubation times resulted in even higher levels of CAT activity in the treated cells (data not shown). However, since the incubation was carried out in the absence of serum, we have chosen 5 h as the standard condition to assure good cell viability during the incubation. The cells were approx. 95% viable by the Trypan blue exclusion test under these conditions (Table I).

Test of the transfection activity of the complex on other cell lines

Several cell lines, both adherent and in suspension, were tested for transfection with the complex using the conditions optimized for the L929 cells (Table II). It is clear that three other cell lines, i.e., A431 epidermal cells, Vero kidney cells and HeLa, could be transfected at efficiencies more than 2-fold higher than that of L929 cells. However, not all adherent cells showed high transfection activity, because FOA hepatoma cells could only be moderately transfected. Two suspension cell lines, CEM and OKT4, were hardly transfected at all. These results indicate that there is a large difference between the transfectability among different cell types and cell lines using the liposomes composed of II and DOPE. It may be possible to obtain better transfection results if the conditions were optimized for the cell line of interest.

TABLE II

Transfection efficiency of the DNA / liposomes complex on different cell lines

5 μg DNA and 70 nmol total lipids (liposomes composed of equimolar mixture of II and DOPE) were used. Transfection incubation time was 5 h. CEM and OKT4 cells are suspension cells and the rest are adherent cells.

Cell line	CAT activity (mU/mg protein)
HeLa	61.2
Vero	43.5
A431	30.9
L929	14.8
FOA	3.3
CEM	0.2
OKT4	0.1

Discussion

It is clear from the data shown in Table I that cationic cholesterol derivatives are inhibitors of PKC, with quaternary amino derivatives showing stronger inhibitory activities than the tertiary amino derivatives. This observation is consistent with the previous conclusions that cationic amphiphiles are generally inhibitors of PKC (Bottega, R. and Epand, R.M., unpublished data), probably as analogs of sphingosine, an endogenous negative effector of the enzyme [31]. Also shown in Table I is the gene-transfer activity of these derivatives when mixed with DOPE to form cationic liposomes. Tertiary amino derivatives (II and IV) showed much stronger activities than the corresponding quaternary amino derivatives (I and III) on a per mg protein basis. This is likely related to the cellular PKC activity which may be crucial for the mechanism with which the plasmid DNA is delivered to cells via the cationic liposomes. Furthermore, the expression of the reporter gene as controlled by the SV40 early promoter is stimulated by an activation of the PKC activity [32]. When PKC is inhibited by the quaternary amino derivatives, some yet unidentified step(s) in the delivery and expression of the reporter gene may be inhibited. This is supported by the preliminary results which showed that the transfection activity of liposomes containing DOPE and derivative II can be enhanced by including phorbol myristate acetate in the liposomes (data not shown). Recently, it has been shown that transfection by the calcium phosphate method can also be significantly enhanced by PKC activators [33].

Liposome/DNA complexes containing derivatives I and III were more toxic to the treated cells than those containing II and IV (Table I). This is probably also related to the fact that I and III are stronger inhibitors of PKC than II and IV. The amounts of I and III used in the transfection experiments were close to their K_i values, thus bringing the strong possibility that the cellular PKC was inhibited throughout the period of incubation. Of the two tertiary amino derivatives, II was more effective than IV in the transfection activity (Table I). This is probably due to the fact that II contains a longer spacer-arm between the amino group and the hydrophobic anchor (Fig. 1) which would provide a better binding to the positively charged amino group with the negatively charged DNA liposomes containing ligands in the longer spacer-arm bind to

receptors more readily than those with a shorter arm [34]. Since liposomes containing derivative II showed little or no toxicity to the cells and facilitated a strong transfection activity, detailed studies were done with this derivative.

Essentially, the transfection activity of II was observed only when it is mixed with DOPE but not DOPC (Fig. 2). Other cationic lipids also show a higher transfection activity when they are used to prepare liposomes with DOPE than with DOPC [1]. Unsaturated PE has a high propensity to form the inverted hexagonal (H_{II}) phase at the physiological conditions. Although stable bilayer liposomes can be obtained when DOPE was mixed with an appropriate amount of II, aggregates of liposomes with DNA could contain some non-bilayer structures due to phase separation. The presence of these non-bilayer structures, which would be absent in liposomes containing DOPC, could be important for the entry of the complex into cells. Although fusion of the liposomes with cell membrane has been proposed as the mechanism of DNA delivery by the cationic liposomes [17], it is more likely that the liposome/DNA complex enters the cells by endocytosis [3]. Formation of the H_{II} nonbilayer phase in the acidic endosomes/lysosomes would probably destabilize the membrane of these endocytic vesicles, resulting in the release of the DNA into the cytoplasm. This mechanism, has been demonstrated for the action of another cationic liposome formulation containing lipopolylysines (Zhou and Huang, unpublished data) as well as some other PE-containing liposomes [35,36].

The transfection protocol was optimized with respect to both the liposome and DNA concentrations (Figs. 3 and 4). Optimal concentrations were found such that the positive-to-negative charge ratio of the complex was approx. 2.2, i.e., there was an apparent excess of liposomes. However, it is unlikely that all negative and positive charges in the complex are involved in the ionic interactions because both the supercoiled DNA and liposomes are bulky enough to provide steric hindrance preventing close contact of each other. The excess positive charge content of the complex will certainly allow an efficient binding and perhaps internalization, of the complex by the cells. Preliminary results using cells incubated with a fluorescentlylabeled complex have confirmed this hypothesis.

When the incubation conditions were optimized with respect to the concentration of the liposome/DNA complex (Fig. 5) and the incubation time (Fig. 7), the transfection activity of the DOPE/II liposomes was about 3-fold greater than that of lipofectin using L929 cells and 23 μM total lipids (Table I). The principal factor which contributed to this difference is the fact that the DOPE/II liposomes are not toxic to cells when complexed with DNA, whereas the complexes with lipofectin at the similar concentrations showed a high level of cytotoxicity to the treated cells (Table I). The PKC inhibitory activity of the cationic lipid (DOTMA) in lipofectin showed a relatively lower value of K_i in comparison to that of II probably due to the presence of a quaternary amino group in DOTMA [1]. The lack of toxicity and the excellent transfection activity on many different cell lines (Table II) of the cationic liposomes composed of DOPE and II have made this liposome composition highly desirable as a reagent for in vitro transfection of mammalian cells. In addition, this liposome composition may be useful for stable transfection efficiency of cultured cells. A stable transfection efficiency of $3 \cdot 10^{-4}$ was observed in LMTK$^-$ cells using HSVTK plasmid DNA and following the transient transfection protocol. However, the in vivo use of the reagent will be limited due to the fact that the transfection activity is strongly inhibited by the presence of serum (Fig. 6). Large amounts of negatively charged serum proteins probably also complex with the liposomes and interfere with the binding with cells. In this regard, the behavior of the new transfection reagent is similar to what has been reported for lipofectin [1]. Another drawback of the new liposome reagent is that it is relatively unstable. This is probably due to the fact that II contains a ester bond which can be readily hydrolysed. The instability of liposomes containing II is reflected in the variation in CAT expression in the figures presented. The older the liposomes are, the less efficient they are in delivering DNA to the cells. These liposomes are most efficient if used within a few hours after sonication. We have recently described a cationic cholesterol derivative (DC-chol) of similar structure but containing a more stable carbamoyl bond. Liposomes containing DC-chol are indeed much more stable than those containing II [6].

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