BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Gene transfer mediated by cationic lipids: lack of a correlation between lipid mixing and transfection

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Received 23 August 1996; revised 18 November 1996; accepted 20 November 1996

Abstract

Complexes of DNA with cationic lipids are used to transfect eukaryotic cells. The mechanism of transfection is unknown, but it has been suggested that the complexes are taken up into the cell by endocytosis, after which fusion of the cationic lipids with the membranes of intracellular vesicles would allow the DNA to escape into the cytoplasm. Here, we have compared transfection of CHO-K1 cells with lipid mixing measured by fluorescence assays, using liposomes or complexes with plasmid DNA of the cationic lipids 1,2 dioleolyl-3-*N*,*N*,*N*,-trimethylammonium-propane (DOTAP), *N*-[2,3-(dioleoyloxy)propyl]-*N*,*N*,*N*,-trimethylammonium (DOTMA), or combinations of these lipids with dioleoylphosphatidylethanolamine (DOPE), at various lipid/DNA charge ratios. Mixing of the lipids of the complexes or liposomes with cellular membranes occurred readily at 37°C, and was more efficient with liposomes than with complexes. Lipid mixing was inhibited at low temperatures (0–17°C), by the presence of NH_4Cl in the medium, and by low extracellular pH, indicating the involvement of the endocytic pathway in entry. In the absence of DOPE, there was no correlation between the efficiency of lipid mixing and the efficiency of transfection. Moreover, although DOPE, which is thought to promote membrane fusion, enhanced transfection, it did not always enhance lipid mixing. Neither the size nor the zeta potential of the complexes were clearly associated with transfection efficiency. Therefore, although fusion between the lipids of the complexes and cellular membranes takes place, a step at a later stage in the transfection process determines the efficiency of transfection.

Keywords: Gene transfer; Cationic lipid; Liposome

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; DOTAP, 1,2 dioleolyl-3-*N*,*N*,*N*,-trimethylammonium-propane; DOTMA, *N*-[2,3-(dioleoyloxy)propyl]-*N*,*N*,*N*,-trimethylammonium

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PII S0005-2736(96)00241-6

1. Introduction

Cationic lipids are useful reagents for the transfection of mammalian cells both in vitro and in vivo [1,2]. Little is known about the mechanism of transfection. It is clear that the lipids bind DNA, primarily by electrostatic interactions. When liposomes prepared from cationic lipids are added to DNA, membrane fusion occurs and the DNA collapses, leading to the formation of condensed structures which do not resemble classical liposomes [3–6]. These DNA/lipid complexes are taken up by cells, most likely by endocytosis, since plasmid DNA was consistently found in intracellular endosome-like vesicles during the transfection of cells with cationic amphiphiles [7–9]. Moreover, the uptake of cationic liposomes into cells [10] and the transfection of cells by cationic lipid/DNA complexes [11] were affected by substances known to affect intracellular vesicle trafficking.

How DNA escapes from the complexes within the vesicles to enter the cytoplasm is still poorly understood. Liposomes made of cationic lipids fuse with negatively charged liposomes [12–14] or cell membranes [10], in the absence of DNA. Therefore, it has been suggested that membrane fusion is involved in transfection. Furthermore, transfection is often more efficient if dioleoylphosphatidylethanolamine (DOPE) is added to the cationic lipid and is present in the transfecting complex, and this effect has been ascribed to the ability of DOPE to promote membrane fusion [15,16]. However, DOPE is not required for fusion, and the transfection efficiency of some cationic lipids is not enhanced by DOPE [17,18]. Alternatively, it was proposed that the complexes would destabilize the endosomal membrane such that negatively charged lipids from the cytoplasmic leaflet of the endosomal membrane would appear on the luminal face, where they would then displace the DNA from the cationic lipids [19]. Furthermore, complexes of cationic lipids with DNA were shown to produce pores and in membranes, and it was suggested that DNA would be translocated through these pores [20].

Transfection efficiencies vary greatly between different types of cationic lipids [1,2,21,22]. It is not known whether these differences are due to the differential ability of the lipids to form complexes with DNA, or rather reflect the efficiency with which they carry DNA into the cell.

Here, to investigate the involvement of membrane fusion in transfection, and to determine the correlation between fusion and transfection efficiency, we have measured the interaction between cationic lipid/DNA complexes and cells, using lipid mixing assays that are commonly used to study membrane fusion. The cationic lipids were either *N*-[2,3-(dioleoyloxy)propyl]-*N*,*N*,*N*,-trimethylammonium (DOTMA) [15] or 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [12], with or without DOPE. DOTMA and DOTAP possess an ether and an ester bond between the acyl chains and the cationic head group, respectively, and differ only by the nature of this linkage. It was found that lipid mixing between cationic lipid/DNA complexes and cells took place, but there was no correlation between the extent of membrane fusion and the transfection efficiency.

2. Materials and methods

2.1. Preparation of cationic lipid / DNA complexes

DOTAP and DOPE were purchased from Avanti Polar Lipids (Alabaster, AL). DOTMA was synthesized as previously described [15] and was at least 95% pure. Lipids were dried under vacuum and the lipid film was rehydrated with pure water, to a final lipid concentration of 1 mM. The preparation was then sonicated for 5 min. When DOPE was included in the preparation, the molar ratio of DOPE to cationic lipid was 1. The plasmid pGL3CMV contained the *Kpn*I–*Bgl*II promoter fragment from pT109luc [23], inserted between the *Kpn*I and *Bgl*II sites of pGL3basic (Promega Biosciences, Wallisellen, Switzerland). The *Xho*I–*Bgl*II TK promoter fragment of this construct was replaced by a CMV promoter, excised as a 788 bp *Xho*I–*Bam*HI fragment from pUHD10-1 [24]. The pCH110 plasmid containing a β-galactosidase gene under the control of an SV40 promoter was purchased from Promega. Plasmids were prepared according to standard protocols [25] and purified by affinity chromatography (Qiagen, Hilden, Germany). Ten μg of plasmid DNA were diluted in pure water in a sterile polystyrene

tube and an appropriate amount of cationic lipid suspension was added to a final volume of 200 μl. The charge ratio between cationic lipid (+) and DNA (−) was either 0.5, 1 or 2. DNA/lipid mixtures were allowed to stand at room temperature for 5–10 min prior to lipid mixing measurements, transfection, or size and zeta potential measurements. Size and zeta potential were measured using a zetasizer 4 (Malvern Instruments) after appropriate dilution in water.

2.2. Cells

Chinese hamster ovary (CHO-K1) cells were cultured as described before [26]. For kinetic measurements, cells were harvested by trypsinization of cultures growing in log phase, kept on ice and used within 3 h.

2.3. Cell transfection protocol

CHO-K1 cells were plated at a density of about $2 \cdot 10^6$ cells per 100 mm dish and grown for 24 h in medium containing 10% fetal calf serum (FCS, Gibco BRL, Basel, Switzerland). Transfection took place in Dulbeco's modified Eagle medium without FCS. Ten μg of plasmid DNA, encoding either luciferase or β-galactosidase, complexed with cationic lipids were applied per dish. Five h later the medium was removed and replaced by 10% FCS containing medium. Forty-three h later, luciferase activity was assayed as described previously [27]. β-Galactosidase activity was detected by cytochemical stain methods to evaluate the percentage of transfected cells [28].

2.4. Lipid mixing measurements

The transfer of lipid from lipid/DNA complexes to cells was measured by a resonance energy transfer assay [29], or an assay based on excimer formation by pyrene-labeled lipids [30,31]. Labeled complexes contained 0.6 mol% (with respect to total lipid) each of *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine (*N*-Rh-PE) and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE), both from Avanti Polar Lipids, or they contained 5 mol% of 12-(1-pyrenedodecyl)phosphatidylcholine (Molecular Probes, Eugene, OR). For the *N*-Rh-PE/*N*-NBD-PE pair, fluorescence was recorded at excitation and emission wavelengths of 465 and 530 nm, respectively, with a 515 nm long-pass filter placed between cuvette and emission monochromator [32] on a SLM 8000 D spectrofluorometer with continuous stirring in a thermostated cuvette holder. All measurements were carried out in Hanks/Hepes buffer or low pH buffer as described before [33]. For calibration of the fluorescence scale, the initial residual fluorescence intensity was set to zero and the intensity at infinite probe dilution was set to 100%. The latter value was obtained by lysis of the liposomes with Triton X-100, and corrected for the effect of this detergent on the quantum yield of *N*-NBD-PE [29]. For pyrene, fluorescence was recorded at 397 nm, with excitation at 330 nm, and the fluorescence scale was calibrated in a similar fashion, as described previously [31]. Low pH buffer was prepared as described before [1,34].

3. Results

3.1. Lipid mixing during transfection with DOTAP

To investigate the interaction of DOTAP with cells, CHO-K1 cells growing in mid-log phase were harvested by trypsinization, and equilibrated for 5 min with Hanks/Hepes buffer (pH 7.4), in the cuvette of a fluorimeter at 37°C. Liposomes made of DOTAP and 0.6 mol% each of the fluorescent phospholipid analogues *N*-NBD-PE and *N*-Rh-PE were prepared in pure water as described in Section 2. Immediately after addition of the liposomes to cells, the fluorescence of *N*-NBD-PE increased (Fig. 1), indicating a decreasing resonance energy transfer between the probes. Thus, either the labeled lipids were degraded in the cells, or they were transferred from the liposomes to unlabeled lipids of cellular membranes [29]. No degradation of fluorescent label was observed by thin layer chromatographic analysis after 15 min of uptake (results not shown).

Transfer of lipids from labeled to unlabeled membranes could be result of fusion between liposomes and cellular membranes, or it could be caused by the molecular transfer of fluorescent molecules from liposomes to cellular membranes. To distinguish between these possibilities, lipid mixing was also measured by a second assay. Liposomes were prepared

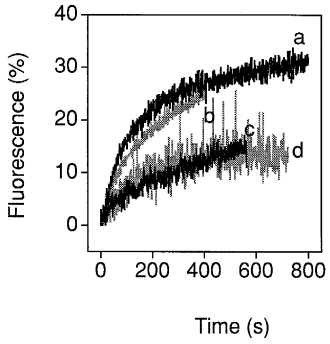

Fig. 1. Lipid mixing between DOTAP liposomes or DOTAP/DNA complexes and cells. 10^6 CHO-K1 cells, grown on dishes, were trypsinized and transferred to the cuvette of a fluorimeter containing 2 ml of Hanks/Hepes buffer at pH 7.4, 37°C. After equilibration, fusion between fluorescently labeled liposomes (a, b) or complexes (c, d) and cells was measured by the resonance energy transfer assay (a, c) or pyrene assay (b, d) as described in Section 2. The final concentration of cationic lipids in the cuvette was 15 μM, and the final concentration of plasmid DNA 2.5 μg/ml.

from DOTAP containing 5 mol% pyrene-labeled phosphatidylcholine. At this concentration, excited pyrene molecules can form excimers with pyrenes in the ground state, and these excimers fluoresce at a wavelength that differs from that of the monomers. Lipid mixing was then measured as an increase in the concentration of pyrene monomers [31]. It was found that lipid mixing measured with either the pyrene or *N*-NBD-PE/*N*-Rh-PE assay occurred with the same kinetics (Fig. 1). In contrast to *N*-NBD-PE and *N*-Rh-PE, which have their label attached to the phospholipid headgroup, the pyrene label resides on one acyl chain of the lipid. Since these chemically different probes, that were present at different concentrations, were transferred with the same kinetics, it seems very unlikely that the molecular transfer of individual phospholipids was the cause of the measured increase in fluorescence, and therefore the changes in fluorescence were caused by membrane fusion, corroborating the data reported by Wrobel et al. [10] for liposomes.

To investigate the mode of interaction of DOTAP/DNA complexes with cells, labeled liposomes were gently mixed with plasmid DNA (1:1 charge ratio), as described in Section 2, allowed to stand for 5–10 min at room temperature, and then added to cells. An increase in fluorescence was also seen in this case (Fig. 1). Lipid mixing was slower and less extensive than in the absence of DNA. Again, both assays reported lipid mixing with the same kinetics, indicating that bulk transfer of lipids took place. The final extent of fluorescence increase, reached after about 10 min, was around 14% in this experiment (it varied between 12% and 22% for different preparations of cells), indicating that 14% of the lipid was transferred to the cells.

Spectra of the pyrene-phospholipid or the *N*-NBD-PE/*N*-Rh-PE pair indicated that in the lipid/DNA complex, before addition to cells, the probes were in a membrane-like environment (not shown). However, if the membrane-impermeant NBD reducing agent sodium dithionite [35,36] was given to DOTAP/*N*-NBD-PE/*N*-Rh-PE/DNA complexes, all NBD fluorescence was eliminated by dithionite reduction (not shown), indicating that the probes are either present in unusually permeable membranes or, more likely, in structures different from normal phospholipid bilayers. Therefore, the lipid mixing seen after addition of the complexes to cells is not 'membrane fusion' in the usual sense.

Lipid mixing was an active, and not a diffusion-mediated process, as indicated by the temperature dependence shown in Fig. 2. DOTAP liposomes or DOTAP/DNA complexes did not show lipid mixing with cells at 0°C, and very little at 17°C. Diffusion-mediated transfer would be only 10% slower at 0°C than at 37°C. Endocytosis should still occur below 20°C, but there is no transport from endosomes to

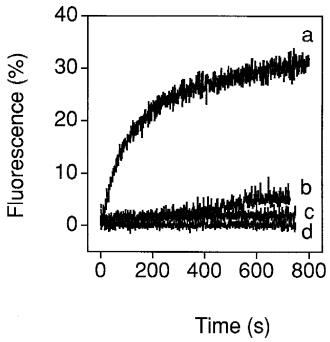

Fig. 2. Temperature dependence of lipid mixing between DOTAP liposomes or DOTAP/DNA complexes and cells. Cells were prepared as described for Fig. 1 and transferred to the cuvette of a fluorimeter containing 2 ml of Hanks/Hepes buffer at pH 7.4, 37°C (a), 17°C (b, c) or 0°C (d). After temperature equilibration, fusion between fluorescently labeled liposomes (a, b, d) or lipid/DNA complexes (c) and cells was measured by the resonance energy transfer assay. Other conditions as in Fig. 1.

lysosomes at 17°C [37], suggesting that the complexes were transported from endosomes to lysosomes prior to fusion. These results differ from those reported by Wrobel and Collins [10] for the interaction of DOTAP/DOPE liposomes in Hep G-2 cells; in that case, fusion occurred after endocytosis but the initial rate of fusion was almost as fast at 15°C as at 37°C, although a lower level of fusion was reached at 15°C.

To further investigate the involvement of endosomes and lysosomes in the internalization and fusion of DOTAP liposomes, lipid mixing was also measured in the presence of NH_4Cl, at 37°C. Under these conditions, lipid mixing was slower (Fig. 3), confirming earlier results for fusion in the presence of monensin [10]. NH_4Cl and monensin raise the pH of endosomes and lysosomes, and thus inhibition of fusion by these endosomo/lysosomotropic reagents is usually taken as evidence that fusion is induced by the low pH of these organelles, as has been described for a number of viruses [38]. However, in contrast to what has been reported for these viruses, adding the liposomes to cells at low pH did not induce the fusion between the liposomes and the plasma membrane of the cell. In fact, low extracellular pH was highly inhibitory to fusion (Fig. 3). An explanation for this surprising phenomenon is perhaps that endocytosis by itself is inhibited by low extracellular pH [39]. In any case, fusion is not driven by low pH, and

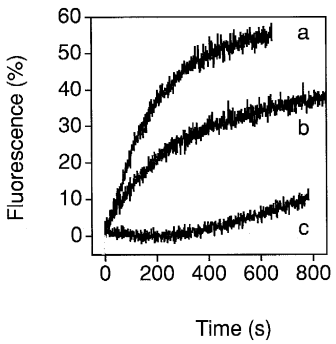

Fig. 3. Influence of NH_4Cl or low extracellular pH on lipid mixing between DOTAP liposomes and cells. $1.2 \cdot 10^6$ CHO-K1 cells were prepared as described for Fig. 1 and transferred to the cuvette of a fluorimeter containing 2 ml of Hanks/Hepes buffer at pH 7.4, 37°C (a), or Hanks/Hepes buffer containing 20 mM NH_4Cl (b), or 2 ml of low pH buffer (see Section 2) at pH 5, 37°C (c). Fusion between fluorescently labeled DOTAP liposomes (15 μM) was measured by the resonance energy transfer assay. Other conditions as in Fig. 1.

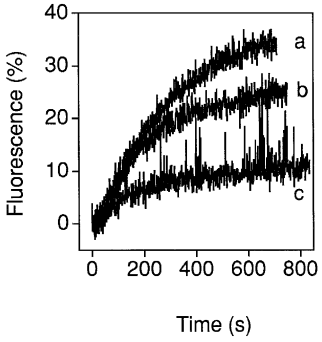

Fig. 4. Lipid mixing between DOTAP/DNA complexes and cells at various charge ratios. Lipid mixing between 10^6 CHO-K1 cells and DOTAP/DNA complexes at a charge ratio of 2 (a), 1 (b), 0.5 (c) at pH 7.4, 37°C was measured by the resonance energy transfer assay as described in Fig. 1. The final concentration of cationic lipids in the cuvette was kept constant at 15 μM.

it is therefore difficult to understand how NH_4Cl and monensin would act to inhibit fusion.

Although NH_4Cl inhibited lipid mixing, it is known to enhance cationic lipid-mediated transfection in some cell lines [2,7,11]. Furthermore, in the above experiments, lipid mixing reached a maximum in minutes, but transfection reaches a maximum after several hours [15]. Thus, transfection did not seem to correlate with the amount of lipid mixing. To investigate this in more detail, we varied the DOTAP/DNA ratio, and first measured lipid mixing. The ratio of positive to negative charges is known to be an important factor in transfection [15,40]. Kinetic measurements with cells in suspension showed that the rate and extent of lipid mixing increased with increasing DOTAP/DNA ratio (Fig. 4).

Transfection experiments are usually carried out with cells that are growing on tissue culture dishes, rather than with cells in suspension as were used in the above experiments. Therefore, we next transfected cells on dishes with *N*-NBD-PE/*N*-Rh-PE labeled DNA/lipid complexes at different charge ratios and compared transfection efficiencies and the corresponding amount of lipid mixing directly. Two different plasmids were used; pGL3-CMV, containing the firefly luciferase reporter gene, allowing measurement of the efficiency of transfection by quantitation of the luciferase transgene [27], and pCH110, a plasmid encoding β-galactosidase, allowing determination of the number of transfected cells. Besides DOTAP and mixtures of this lipids with DOPE, the

cationic lipid DOTMA, with or without added DOPE was also tested. In these experiments, the amount of DNA given to the cells was kept constant, and the amount of lipid varied. Cells were transfected for 5 h, and then washed to remove unbound complexes, after which they were either grown for a further 43 h for the measurement of transfection, or they were trypsinized, and rapidly cooled to 4°C. Lipid mixing and the amount of fluorescent lipid remaining associated with the cells were then measured at this temperature, by taking emission spectra between 480 and 600 nm with excitation at 465 nm, before and after the addition of Triton X-100. The percentage of cell-associated lipid that had mixed with cellular lipid was calculated from the emissions at 530 nm before and after addition of the detergent as outlined in materials and methods [29]. It was found to vary between 36.7% for DOTMA/PE at a 1:1 lipid/DNA ratio and 17.8% for DOTMA at a 1:1 lipid/DNA ratio. The amount of cell-associated lipid was calculated from the emission maximum of *N*-NBD-PE after addition of the detergent, and corrected for the contribution of light scattering at this wavelength using a sample of cells without added liposomes as a blank. It varied from 0.76 nmol for DOTMA/PE at a 1:2 lipid/DNA ratio to 4.12 nmol for DOTAP/PE at a 1:1 lipid/DNA ratio. The amount of mixed lipid (Table 1, right-hand column) was then calculated as the product of the percentage of fused liposomes and the amount of associated lipid.

For DOTAP, the number of transfected cells nearly doubled, and the luciferase activity increased 100-fold going from the lowest lipid/DNA ratio to the highest, but the amount of mixed lipid at the two ratios was the same, reaching an optimum at a 1:1 charge ratio (Table 1). 0.25–1% of the lipids of the complexes that were initially added to the cells finally ended up being mixed with cellular lipids. Thus, these results, obtained with adherent cells after 5 h of incubation were quite different from those obtained for lipid mixing with cells in suspension at short times after the initiation of fusion (cf. Fig. 4); with adherent cells, much less lipid mixing was observed. DOTAP/DOPE mixtures gave rise to more lipid mixing than pure DOTAP, and it increased with increasing lipid/DNA ratio. The luciferase activity increased only about sixfold going from the lowest to the highest ratio, and the number of transfected cells remained constant. Therefore, for DOTAP with or without added DOPE, there was no quantitative correlation between lipid mixing and transfection.

Likewise, for DOTMA, it was found that the

Table 1
Comparison of physical characteristics, transfection and lipid mixing for different DNA/positively charged lipid complexes

Liposome type and lipid/DNA charge ratio	Size (nm)	Zeta potential (mv)	Transfected cells (%) [a]	Luciferase activity [b]	Mixed lipid (nmol) [c]
DOTAP 0.5	163 ± 31	−18	9 ± 1	$3.8 \cdot 10^5$	0.30
DOTAP 1	**	−3	11 ± 4	$1.7 \cdot 10^6$	0.50
DOTAP 2	186 ± 29	+35	17 ± 2	$3.2 \cdot 10^7$	0.32
DOTAP/DOPE 0.5	235 ± 15	−52	15 ± 5	$7.4 \cdot 10^6$	0.44
DOTAP/DOPE 1	**	+7	22 ± 6	$2.3 \cdot 10^7$	0.76
DOTAP/DOPE 2	335 ± 43	+27	16 ± 4	$4.3 \cdot 10^7$	0.97
DOTMA 0.5	124 ± 16	−27	8 ± 1	$5.3 \cdot 10^4$	0.52
DOTMA 1	**	+2	10 ± 3	$2.7 \cdot 10^6$	0.40
DOTMA 2	171 ± 5	+35	13 ± 3	$5.2 \cdot 10^6$	0.60
DOTMA/DOPE 0.5	264 ± 20	−16	16 ± 3	$1.0 \cdot 10^7$	0.33
DOTMA/DOPE 1	**	−1	15 ± 4	$4.0 \cdot 10^7$	0.49
DOTMA/DOPE 2	897 ± 184	+23	15 ± 6	$9.4 \cdot 10^6$	0.50

[a] As determined by cytochemical staining 48 h after transfection with the pCH110 plasmid (pSV40-βgal). Three fields of cells were counted, containing between 100 and 200 cells per field.
[b] Expressed as light units/mg of cell protein. Measured 48 h after transfection with the pGL3-CMV plasmid (pCMV-luc).
[c] Product of the cell-associated amount of lipid and the percentage of associated lipid that mixed with the cellular lipid, as described in the text. Variations in these data from experiment to experiment were on the order of 10%.
** At a 1:1 charge ratio, precipitates are present which make size determination impossible.

number of transfected cells and the luciferase activity increased with increasing lipid/DNA ratio, but lipid mixing changed little. DOTMA/DOPE complexes gave rise to the same extent of lipid mixing at lipid/DNA ratios of 1 and 2, and similar transfection efficiencies at all ratios.

These differences between the transfection and lipid mixing activities of the complexes could result from differences in the efficiency by which the complexes were taken up by the cells or differences in the ability of the lipids to complex DNA. In order to investigate these possibilities, the size and zeta potential of the complexes were measured (Table 1). Before complex formation with DNA, all cationic liposomes had diameters between 80 and 90 nm, except for DOTAP/DOPE liposomes, which were considerably larger, around 260 nm. DOPE and DOTAP did not mix well, and a translucent liposome preparation could not be obtained, even after 5 min of sonication, confirming earlier reports [41]. The DNA/lipid complexes were larger than the liposomes from which they were produced, and the diameters varied, depending on the presence of DOPE and the charge ratio (Table 1). For complexes prepared at a 1:1 charge ratio, a precipitate formed shortly after preparation, regardless of the presence of DOPE or the type of cationic lipid used. In that case, the size of the complexes could not be established reliably, as shown previously [4].

Measurements of the zeta potential were in good agreement with theoretical cationic lipid/DNA charge ratios (Table 1), indicating that complexation of DNA with the different kinds of lipids was similar. At a 1:1 charge ratio, zeta potentials were either slightly negative or positive. Therefore, surprisingly, there was no obvious correlation between the size of the complexes and transfection efficiency or lipid mixing. For DOPE-containing complexes, there was little correlation between transfection efficiency and zeta potential of the transfecting particles. For example, DOTMA/DOPE/DNA complexes showed similar transfection efficiencies at cationic lipid/DNA charge ratios of 0.5 and 2.0 while the zeta potential of the particles varied from -16 mV to $+23$ mV. A possible explanation is that at low cationic lipid/DNA ratios, only part of the DNA is complexed by the cationic liposomes and exists in an active form [2]. While these active, positively charged complexes would only contribute moderately to the overall zeta potential of the preparation, they could mediate efficient transfection of cells. However, this was not true for DOPE-free formulations, and at a lipid/DNA charge ratio of 2, transfection was always two orders of magnitude higher than at a ratio of 0.5, both for DOTMA and DOTAP.

4. Discussion

The mechanism whereby cationic lipids mediate the transfection of mammalian cells is unknown. Wrobel and Collins [10] demonstrated that, in the absence of DNA, cationic liposomes enter cells in suspension by endocytosis and fuse with cellular membranes as measured by a lipid mixing assay, and thus it was concluded that membrane fusion could play a role in the transfection process. However, a number of physicochemical changes takes place upon the addition of DNA to such liposomes, resulting in the formation of lipid/DNA complexes that are unlike liposomes [3–6]. Here, using liposomes or cationic lipid/DNA complexes we demonstrate that, although cationic lipids mix with the lipids of suspension and adherent cells, and data obtained with cells in suspension are compatible with endocytosis as their route of entry into cells, there was no correlation between lipid mixing and transfection for either DOTAP or DOTMA at various lipid/DNA charge ratios. Cationic lipid/DNA complexes always gave rise to less lipid mixing than the corresponding liposomes without DNA, as was found for the fusion of cationic liposomes or lipid/DNA complexes with negatively charged liposomes [14]. The tight association between lipid and DNA, the unusual membrane structure of the transfecting complexes and their large size may contribute to this effect.

Mixtures of cationic lipids with DOPE usually give rise to more efficient transfection than the corresponding cationic lipid alone [2], and it has been suggested that DOPE has this effect on transfection because it promotes membrane fusion [15,16]. Membrane fusion requires the formation of non-bilayer structures, at least temporarily and locally at the site of fusion [42]. DOPE, which is capable of forming non-bilayer structures like inverted hexagonal phases is thought to facilitate bilayer/non-bilayer transitions

involved in membrane fusion [43]. However, transfection of cells by some cationic lipids is not enhanced by DOPE [17,18]. Moreover, enhancement by DOPE seems to depend upon the type of cells used and some cells, such as primary hepatocytes, are even transfected more efficiently in the absence of DOPE [44].

In this study though, DOPE-containing formulations were always the most efficient in transfecting CHO-K1 cells, particularly at a cationic lipid/DNA ratio of 0.5. At this ratio, DOPE increased the luciferase activity for DOTMA or DOTAP 200- or 20-fold, respectively (Table 1). However, whereas DOPE promoted lipid mixing induced by DOTAP at that ratio, it did the opposite for DOTMA. Therefore, in our hands, although DOPE promoted transfection, the promotion did not correlate with enhanced lipid mixing.

Since lipid mixing was inhibited by NH_4Cl, low extracellular pH, and at low temperatures (Fig. 3), it is likely that it took place after endocytosis, confirming earlier observations with cationic liposomes [10], and in line with studies showing that endocytosis is the major route of entry of cationic lipid/DNA complexes into cells [8,9,11]. With suspension cells, lipid mixing from within these endosomal compartments was rapid, reaching a maximum in less than 15 min, in agreement with the observations of Friend et al. [9], showing cationic/lipid DNA complexes in endosomal compartments after only 5 min of incubation. Although some cell lines show maximal transfection after only a 15 min to 1 h incubation with the transfecting complexes [45], and most of the uptake of cationic lipid/DNA complexes by cells occurs within the first hour of incubation [8,9,11], maximal transfection usually requires an incubation time of several hours [15]. Therefore, it is clear that transfection is not concomitant with, but takes place long after, lipid mixing.

Together, these observations indicate that lipid mixing does occur after uptake of cationic lipid/DNA complexes in endosomes, but that the efficiency of transfection is determined by a later step in the transfection process. It could be that lipid mixing takes place in endosomal-like vesicles but does not correlate with the escape of DNA from these vesicles. Zabner et al. [8] observed sequestration of material with a highly ordered tubular structure in intracellular vesicles after transfection with cationic lipid/DNA complexes. Much of the DNA was found to be aggregated in this vesicular compartment [8]. A thickening of intracellular membranes was observed after the uptake of cationic lipids, which could reflect an interaction, perhaps the mixing, between cationic lipids and intracellular membranes [9]. These results suggests that lipid mixing from within an intracellular vesicle does not necessarily allow DNA to escape from the vesicle. Xu and Szoka [19] have suggested that, after destabilization of the endosomal membrane by cationic lipids, negatively charged lipids from the cytoplasmic leaflet of endosomes would flip-flop to the interior leaflet, and displace the DNA from the positively charged lipids. In that case one would expect a correlation between DNA release and lipid mixing. Although they have demonstrated that negatively charged substances can do this in vitro, it remains to be investigated whether such a mechanism exists in vivo. But even if lipid mixing at the endosomal level would lead to the transfer of DNA into the cytoplasm, other barriers such as the translocation of the DNA into the nucleus may be the main barrier to the transfection process.

Besides the above observations showing that the efficiency of transfection is not determined by the efficiency of membrane fusion or lipid mixing induced by a given cationic lipid, a surprising observation was that there was also very little correlation between basic physicochemical parameters such as the zeta potential or the size of the complexes and transfection (Table 1). A mixed population of particles, with different physicochemical and transfection potential, may be present in the various preparations and measurement of the zeta potential or size only reflects their overall properties, and not necessarily those of the transfecting population. Therefore, at present, the transfection efficiencies of cationic lipids cannot be predicted from these physicochemical properties or their membrane fusion potential.

Acknowledgements

This study was supported by Grant 3100-042953.95/1 of the Swiss National Science Foundation.

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