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Calcium enhances the transfection potency of plasmid DNA–cationic liposome complexes

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Abstract

It is shown that calcium increases the in vitro transfection potency of plasmid DNA–cationic liposome complexes from 3- to 20-fold. The effect is Ca^{2+} specific as other cations, such as Mg^{2+} and Na^{+}, do not give rise to enhanced transfection and the effect can be inhibited by the presence of EGTA. It is shown that Ca^{2+} increases cellular uptake of the DNA–lipid complexes, indicating that increased transfection potency arises from increased intracellular delivery of both cationic lipid and plasmid DNA in the presence of Ca^{2+}. In particular, it is shown that the levels of intact intracellular plasmid DNA are significantly enhanced when Ca^{2+} is present. The generality of the Ca^{2+} effect for enhancing complex-mediated transfection is demonstrated for a number of different cell lines and different cationic lipid formulations. It is concluded that addition of Ca^{2+} represents a simple and useful protocol for enhancing in vitro transfection properties of plasmid DNA–cationic lipid complexes.

Keywords: Cationic liposome; Plasmid DNA–cationic lipid complex; Calcium; Transfection

1. Introduction

Plasmid DNA–cationic liposome 'complex' systems formed by incubation of plasmid with positively charged liposomes have been used widely as transfection agents in vitro and have promising potential for in vivo applications [1–3]. Studies on the mechanism of cationic liposome-mediated gene transfer indicate that complexes enter cells mainly by endocytosis [4–8] and that the plasmid escapes into the cytoplasm as a result of endosomal destabilization facilitated by a fusogenic 'helper' lipid, such as dioleoylphosphatidylethanolamine (DOPE) [9,10]. It has been suggested that anionic lipids in the endosomal membranes also play a role in facilitating plasmid DNA release by interacting with cationic lipid in the complexes [11,12]. The efficiency of cell transfection by plasmid DNA is greatly enhanced by the presence of the cationic liposomes; however, transfection by complexes remains a relatively inefficient process. Particular obstacles include escape from the endosome following endocytosis, intracellular nuclease degradation of plasmid [13], as well as entry of the plasmid DNA into the cell nucleus [7].

Efforts to improve liposomal gene transfer systems have focused primarily on designing novel cationic

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lipids and developing liposome formulations containing different 'helper' lipids [5,14–17]. Other approaches include the incorporation of fusogenic peptides, DNA–condensing agents or targeting ligands, such as nuclear localization signals to improve DNA transfer [18–23]. Relatively little work has focused on the effect of Ca^{2+} on the transfection potency of plasmid DNA–cationic liposome complexes. Traditionally, Ca^{2+} has been used to enhance in vitro transfection by the Ca^{2+}-phosphate precipitation method [24]. The uptake of DNA by cultured cells is believed to be facilitated by the formation of a Ca^{2+} phosphate-DNA co-precipitate, which enters the cell by endocytosis and subsequently is transferred into the nucleus after escape from the endosomal compartment [25,26]. Other studies have investigated the role of Ca^{2+} in increasing the rate of endocytosis and macro-molecular nuclear uptake [27–29]. Recently, a non-liposomal gene transfer system employing DNA-binding elements has shown enhanced transfection efficacy in the presence of Ca^{2+} [30,31]. It was suggested that Ca^{2+} could increase the rate of endocytosis [30] or facilitate endosomal release [31].

In this work, we have studied the effect of Ca^{2+} on cationic liposome-mediated cell transfection using a bovine hamster kidney (BHK) cell line. Experiments were conducted to demonstrate the specificity of Ca^{2+}-enhanced transfection and to examine the mechanism whereby Ca^{2+} improves transfection. The results indicate that the presence of Ca^{2+} during complex-mediated transfection facilitates improved intracellular delivery of cationic lipid and plasmid DNA, leading to enhancements in transgene expression of 3–20-fold in a number of different cell lines.

2. Materials and methods

2.1. Plasmids

The pCMVβ plasmid, which encodes the lacZ gene for the β-galactosidase (β-gal) protein, and the pCMVGFP plasmid, which expresses the green fluorescence protein (GFP), were both driven by a cytomegalovirus (CMV) promoter to assess expression. All plasmids were obtained from J. Thompson at Inex Pharmaceuticals Corporation (Burnaby, BC).

2.2. Cell lines

The 293 (transformed primary embryonal human kidney), BHK (bovine hamster kidney), CV-1 (African green monkey kidney), HS578T (human breast ductal carcinoma), and SK-OV3 (human ovary adenocarcinoma) cell lines were obtained from American Tissue Culture Collection (ATCC CCL-10, Rockville, MD). With the exception of HS578T cells which required an additional supplement of bovine insulin (10 μg/ml), all cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Pen-Strep). All cell lines were maintained as a monolayer at 37°C in a humidified atmosphere containing 5.0% CO_2.

2.3. Lipids and chemicals

N,N-Dioleyl-*N,N*-dimethylammonium chloride (DODAC), ^{14}C-labeled DODAC, and *N,N*-distearyl-*N,N*-dimethylammonium chloride (DSDAC) were obtained from Dr. S. Ansell at Inex Pharmaceuticals Corporation. *N*-[2,3-(Dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) was purchased from Gibco-BRL (Burlington, ON). 1,2-Dioleoyl-3-phosphatidylcholine (DOPC) and 1,2-dioleoyl-3-phosphatidylethanolamine (DOPE) were obtained from Northern lipids (Vancouver, BC). Dimethyl-dioctadecylammonium Bromide (DDAB) was purchased from Avanti Polar Lipids (Alabaster, AL). Calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), and sodium chloride (NaCl) were obtained from Fisher Scientific (Fair lawn, NJ). Ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was purchased from Sigma (St. Louis, MO).

2.4. Preparation of LUVs

All large unilamellar vesicles (LUVs) were prepared in a 1:1 molar ratio. Briefly, mixtures of lipids dispensed in chloroform were dried under stream of nitrogen gas and the residual solvent was removed under high vacuum for 2 h. The resulting lipid films were hydrated with dH_2O and LUVs were obtained by freeze–thawing and extruding [32]. The sizes of

LUVs were checked with a Nicomp Model 270 submicron particle sizer using quasielastic light scattering techniques. Phosphorus assays were used to quantify lipid concentrations [33].

2.5. In vitro transfection in the presence of Ca^{2+}

Prior to transfection with DNA–lipid complexes, cells were plated at a density of 2×10^4 cells per well of a 96-well plate overnight. $CaCl_2$ (1 M) stock solution was prepared in dH_2O and sterilized by filtering. Liposomes were first added to plasmid DNA and mixed briefly by pipetting up and down several times, after which the mixtures were immediately added to tubes containing appropriate [$CaCl_2$] and incubated at room temperature for 30 min before diluting to the final volume with DMEM culture media containing 10% FBS, which were then used to transfect cells. [$CaCl_2$] was calculated with respect to the final volume of the transfection medium applied to cells. Cells were incubated with the transfection complexes for 4 h unless otherwise noted, after which culture media was replaced and cells were further incubated at 37°C, 5% CO_2 overnight until assaying for gene expression.

2.6. Determination of transgene expression

β-Gal expression was assayed by using the substrate Chlorophenol red galactopyranoside (CPRG) (Boehringer Mannheim, Germany) at 1 mg/ml to induce color development, which was measured at 540 nm with a microplate EL-309 autoreader (Bio-Tek Instruments) as described previously [34]. Absorbance readings were calibrated according to a β-gal standard (Sigma). Total cellular protein was determined by using the Micro BCA Protein Assay Reagent Kit (Pierce, Illinois), which was subsequently used to normalize the β-gal activities of each transfection. For the fluorescence analysis of GFP expression, cells were plated at 5×10^5 cells per well of a 100×20 mm tissue culture dish the night prior to transfection. Cells expressing GFP were directly visualized and photographed at the indicated time point (24, 48, and 72 h) using a Zeiss (Axiovert S100) fluorescence microscope with an XF100 filter set (Ex = 475 nm, Em = 535 nm) from Omega Optical (Vermont, USA).

2.7. Intracellular uptake studies employing ^{14}C-radiolabeled lipids

BHK cells were plated at 7.5×10^5 cells per 25 cm^2 cultured flasks the day before transfection. Trace amounts of ^{14}C-radiolabeled DODAC were incorporated into DODAC/DOPE liposomes. For each transfection, 2 μg pCMVβ was complexed to the liposomes at 1.5 charge ratio (mole of positive charges per mole of negative charges) in the presence or absence of 5 mM Ca^{2+}. Transfection media were removed from the cells at 1, 2, or 4 h time point. For the 24 h time point, transfection mixtures were allowed to incubate on cells for 4 h, after which complete media was replaced for the remaining time. Cells were detached by trypsinization and washed in isotonic buffer (250 mM sucrose, 3 mM $MgCl_2$, 50 mM HEPES, pH 7.2). One-quarter of the population was analyzed as the whole cell fraction, while the remaining cells were subjected to nuclear fractionation according to a previously described protocol with modifications [35]. Briefly, cells were treated with hypotonic buffer (10 mM HEPES pH 8, 10 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA) on ice for 15 min. Cells were then lysed by the addition of 0.6% Nonidet P40 (NP-40) (BDH Laboratory Supplies, UK) and mixed by gentle inversions. Nuclei were pelleted by centrifuging at $500\times g$ for 2 min, and pellets were washed using nuclei resuspension buffer (20 mM HEPES pH 8, 25% glycerol, 1.5 mM KCl, 0.2 mM EDTA). Both cell and nuclei pellets were lysed by the addition of 250 μl Sovable and digested for 2 h at 60°C. Solublized samples were subjected to scintillation counting in a Beckman LS3801 scintillation counter.

2.8. Dot blot and Southern blot analyses

Experimental protocols were the same as the ^{14}C-labeled lipid uptake experiments, except that all cells were trypsinized after incubation in the transfection mixtures for 4 h. The centrifuged pellets were washed with isotonic buffer and subsequently lysed by using the lysis buffer (10 mM Tris, pH 7.5, 0.5% SDS, 1 mM EDTA) containing Pronase E at 1 mg/ml (Sigma) overnight at 37°C. Genomic DNA and delivered plasmid DNA were isolated according to a published protocol [36]. Extracted DNA was resus-

pended in TE buffer. DNA recovery was determined by measuring the absorbance at 260 nm. 2 μg of total DNA from each sample was either dot blotted onto a nylon transfer membrane (Amersham) with a set of pCMVβ standards (0–10 pg) or loaded into a 1% agarose gel and size fractionated at 60 V for 2 h for the Southern analysis. Both blots were hybridized overnight at 68°C to a ^{32}P-labeled plasmid DNA probe, which was prepared with *Bam*HI cut-pCMVβ plasmid using the ^{T7}QuickPrime Kit (Pharmacia Biotech). Blots were washed three times with 2×SSC containing 0.1% SDS, and were then exposed on a PhosphoImager screen which was subsequently scanned (Molecular Dynamics-PhosphoImager SI).

3. Results

3.1. The transfection potency of complexes is increased in the presence of Ca^{2+}

In order to examine the effect of Ca^{2+} on complex-mediated transfection, BHK cells were transfected with plasmid DNA–cationic lipid complexes prepared in the presence of increasing concentrations of Ca^{2+} (0–100 mM). Plasmid DNA (pCMVβ) was complexed with DODAC/DOPE liposomes at a cationic lipid to plasmid DNA charge ratio of 1.5 (mol of positive charges/mol of negative charges). These complexes were immediately mixed with the appropriate concentration of Ca^{2+}, incubated for 30 min before diluting to the final volume with DMEM culture media, and then applied to cells. As shown in Fig. 1, no β-gal activity was detected when cells were transfected with Ca^{2+} and plasmid DNA alone. However, β-gal activities were clearly enhanced in cells that were transfected with complexes incubated in the presence of Ca^{2+}. In particular, up to 20-fold increases in transgene expression were detected at Ca^{2+} concentrations between 5 and 25 mM. Transfection potencies decreased for Ca^{2+} concentrations at 50 mM or greater, where more than 20% reductions in total cellular protein levels were observed, indicating cytotoxicity (data not shown). Cellular protein levels at Ca^{2+} concentrations below 50 mM were similar to those of cells transfected with lipid–DNA complexes prepared without Ca^{2+}. In order to avoid cell damage, Ca^{2+} concentrations of 10 mM or less were employed in subsequent experiments.

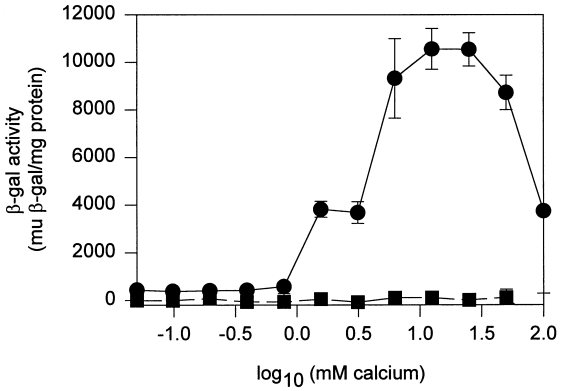

Fig. 1. Effect of increasing Ca^{2+} concentrations on the transfection activities of plasmid DNA–cationic liposome complexes. pCMVβ (0.25 μg) in the presence (●) or absence (■) of DODAC/DOPE liposomes was added to Ca^{2+} and incubated for 20 min at room temperature prior to transfecting BHK cells. DODAC/DOPE was complexed to pCMVβ at a charge ratio of 1.5 (mol/mol). Cells were exposed to the complexes for 4 h and then the transfection mixtures were replaced with complete DMEM media for a further 20 h incubation before assaying for β-gal expression, as outlined in Section 2. Experiments were performed in triplicate.

3.2. A higher proportion of cells are transfected in the presence of Ca^{2+}

Increased transfection in the presence of Ca^{2+} could be due to cells expressing a higher level of the delivered gene or a greater proportion of cells being transfected, or a combination of both. In order to examine the transfection efficiency in a population of cells, transfection was monitored employing fluorescent microscopy. BHK cells were transfected with pCMVGFP at a 0.75 cationic lipid-to-plasmid DNA charge ratio in the presence or absence of 2.5 mM Ca^{2+}. Complete DMEM medium was replaced at the end of the 4-h transfection period and cells were incubated in normal growing conditions for indicated periods of time. GFP expression was visualized at 24, 48, and 72 h post-transfection (Fig. 2). Since the plasmid DNA–cationic liposome complexes were prepared at a 0.75 charge ratio, only a small population of cells was expected to show expression of the GFP transgene (Fig. 2A). When transfected with complexes incubated in the presence of Ca^{2+}, a much higher proportion of cells expressing GFP

could be detected (Fig. 2B). As the delivered plasmid resulted in transient expression, the number of cells expressing GFP decreased as cells continued to grow and divide over the 72-h time period. Nevertheless, a significant number of cells transfected with the Ca^{2+}-enhanced complexes continued to express the transgene over the 72-h time period. These data demonstrate that the increase in transfection efficiency is due, at least in part, to a higher proportion of cells being transfected when Ca^{2+} is present in the transfection complex mixtures.

3.3. *Cation-dependent transfection enhancement is Ca^{2+} specific*

In order to show that Ca^{2+} specifically enhances transfection, the effect of $CaCl_2$, $MgCl_2$, and NaCl were compared. Increasing concentrations (0–50 mM) of $CaCl_2$, $MgCl_2$, and NaCl were incubated with pCMVβ-DOPE/DODAC complexes, which were subsequently used to transfect BHK cells. As shown in Fig. 3A, β-gal expression was enhanced only in the presence of Ca^{2+}. No significant increase in transgene expression was observed when transfection was performed in the presence of Mg^{2+}, and only a slight increase was detected at higher concentrations of Na^{+}. To further confirm the Ca^{2+} specific nature of this stimulatory effect, the ability of EGTA to inhibit Ca^{2+}-enhanced transfection was examined. EGTA was either pre-incubated with Ca^{2+} for 5 min prior to the addition of cationic liposomes and plasmid DNA, or it was added to the transfection com-

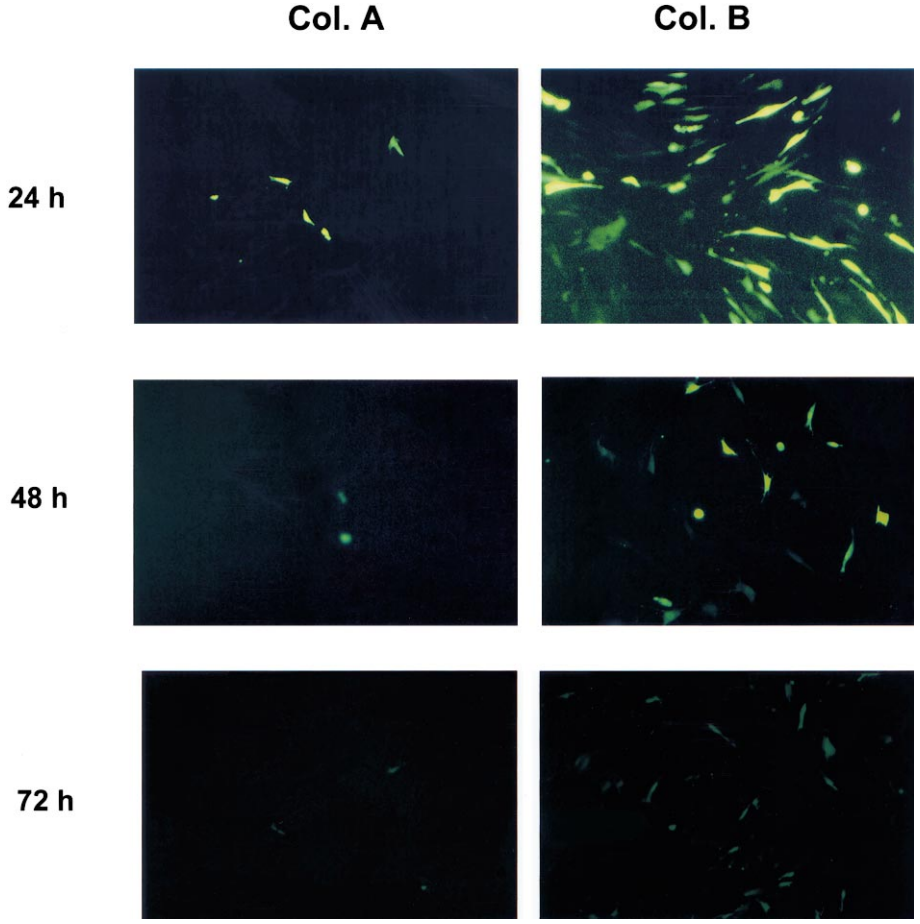

Fig. 2. Fluorescence microscopy of BHK cells transfected with pCMVGFP in the absence (column A) or presence (column B) of 5 mM Ca^{2+}. pCMVGFP (1 μg) was complexed to liposomes at a charge ratio of 0.75 and incubated on cells for 4 h. Cells were grown continuously on tissue cultured dishes with supplement of fresh DMEM media and splitting as required. Photographs of cells expressing GFP were taken at 24, 48, and 72 h post-transfection using a Zeiss fluorescence microscope with an XF100 filter set.

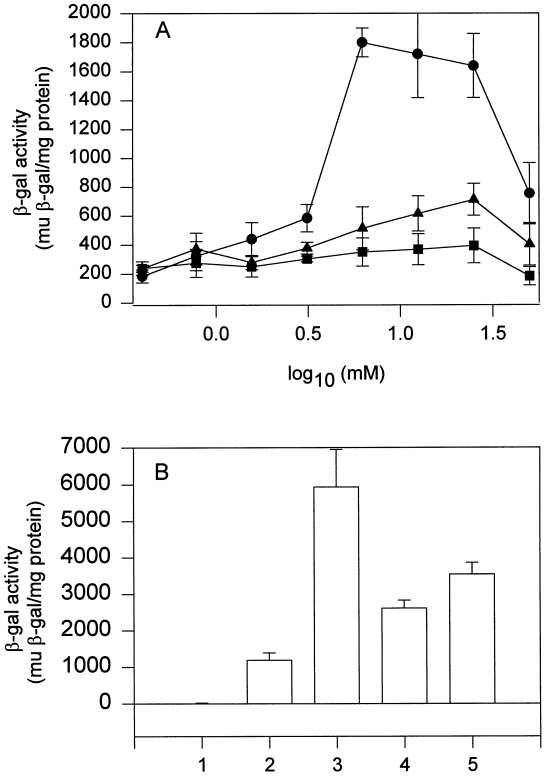

Fig. 3. Specificity of Ca^{2+} for enhancing cationic liposome-mediated transfection. (A) Ability of $CaCl_2$ (●), $MgCl_2$ (■), and NaCl (▲) to enhance transfection. pCMVβ (0.25 μg) was complexed with DODAC/DOPE (charge ratio 1.5) in the presence of increasing concentrations (0–50 mM) of $CaCl_2$, $MgCl_2$, or NaCl. (B) Effect of EGTA on Ca^{2+}-enhanced transfection. Equimolar concentrations of EGTA were added to Ca^{2+} (5 mM) either prior to the addition of liposomes and DNA, or after the formation of the Ca^{2+}-enhanced DNA–lipid complexes. 1, Untransfected cells; 2, DNA–lipid complexes; 3, Ca^{2+}-enhanced DNA–lipid complexes; 4, EGTA incubated with Ca^{2+} prior to addition of liposomes and plasmid DNA; 5, EGTA added after formation of Ca^{2+}-enhanced DNA–lipid complexes. Each experiment was performed in triplicate.

plexes after the three components had been incubated for 30 min at room temperature. Fig. 3B shows that the Ca^{2+}-enhanced transfection was significantly reduced in the presence of EGTA, whether it was added before or after the complex formation. Taken together, these results indicate that transfection was enhanced specifically by Ca^{2+}.

3.4. Ca^{2+} increases the rate of transfection by complexes

The effect of Ca^{2+} on transfection as a function of the time complexes were incubated with cells was investigated. Complexes prepared in the presence or absence of Ca^{2+} were incubated with cells for 30 min to 4 h. At the appropriate time points, transfection media was removed and replaced with complete media. Cells were further incubated overnight before assaying for β-gal expression. At the 1-h time point, only minimal transgene expression was detected in cells transfected with the control plasmid DNA–cationic lipid complexes (Fig. 4). Higher gene expression was observed at longer incubation periods, where maximum β-gal activity was achieved when the transfecting mixtures were incubated on cells for 3–4 h. On the other hand, exposure of cells for just 30 min to transfection mixtures prepared in the presence of Ca^{2+} resulted in transgene expression as high as the maximum transfection level achieved after a 4-h incubation of complexes prepared in the absence of Ca^{2+}.

3.5. Increased intracellular delivery of lipid is observed in the presence of Ca^{2+}

The shorter incubation period required for gene expression employing plasmid DNA–cationic lipid complexes prepared in the presence of Ca^{2+} suggests increased rates of intracellular delivery of complexes.

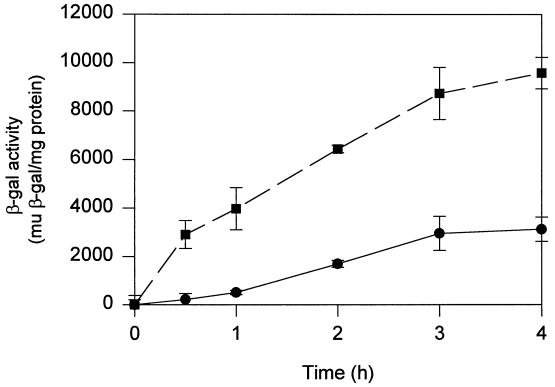

Fig. 4. Effect of incubation time on transfection employing control DNA–lipid and Ca^{2+}-enhanced DNA–lipid complexes. Transfection mixtures prepared in the presence (■) or absence (●) of Ca^{2+} were incubated with BHK cells for 0.5, 1, 2, 3, and 4 h. At the appropriate time points, complete media was replaced and cells were allowed to recover overnight and β-gal activity was assayed. Liposomes were complexed to 0.25 μg pCMVβ at 1.5 charge ratio. Each experiment was performed in triplicate.

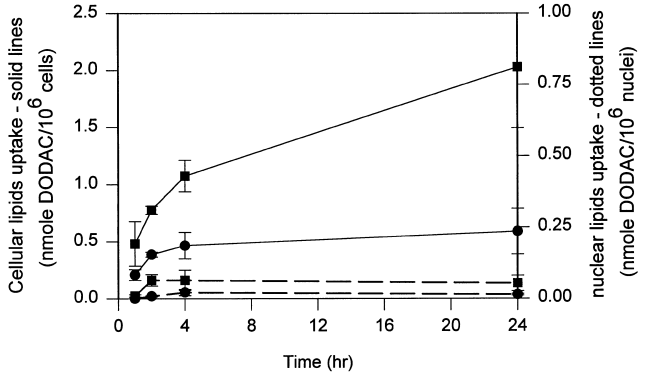

Fig. 5. Kinetic analysis of the intracellular delivery of lipids in the presence of Ca^{2+}. Uptake of ^{14}C-radiolabeled DODAC was examined over a 24-h time period for both whole cell (solid lines) or nuclear (dotted lines) fractions, prepared as described in Section 2. Cells transfected with DNA–lipid complexes prepared in the presence (■) or absence (●) of 5 mM Ca^{2+} were solubilized at the indicated time points (1, 2, 4, and 24 h) and assayed by scintillation counting to determine lipid uptake. Experiments were performed in triplicate.

The kinetics of cationic lipid uptake were therefore examined. DODAC/DOPE liposomes labeled with a trace amount of ^{14}C-radiolabeled DODAC were used to prepare complexes in the presence or absence of Ca^{2+}. Lipid uptake was monitored over a 24-h time period. In the absence of Ca^{2+}, cellular lipid uptake increased up to the 4-h time point and remained stable for the remaining time (Fig. 5). Enhanced lipid uptake was observed for cells transfected with complexes prepared in the presence of Ca^{2+}. At the 1-h time point, cellular lipid uptake similar to the maximal level of complexes prepared without Ca^{2+} was achieved. This was equivalent to a more than 2-fold increase in intracellular lipid uptake when compared to the control complexes at the same time point. This greater than 2-fold increase was maintained as the transfection process continued through the 4-h incubation period. It is interesting to note that lipid uptake continued to increase over the 24-h time period (after removal of the complexes at 4 h), suggesting continued uptake of complexes absorbed to the cell surface. Examination of the nuclear fraction reflected that the level of nuclear associated lipid remained low even in the presence of Ca^{2+}.

3.6. Increased intracellular delivery of intact plasmid DNA is observed in the presence of Ca^{2+}

Intracellular delivery of plasmid DNA was determined using the dot blot assay, and the integrity of the plasmid was examined employing the Southern blot analysis. Cells were exposed to complexes prepared in the presence or absence of calcium. The levels of plasmid DNA uptake for the different sys-

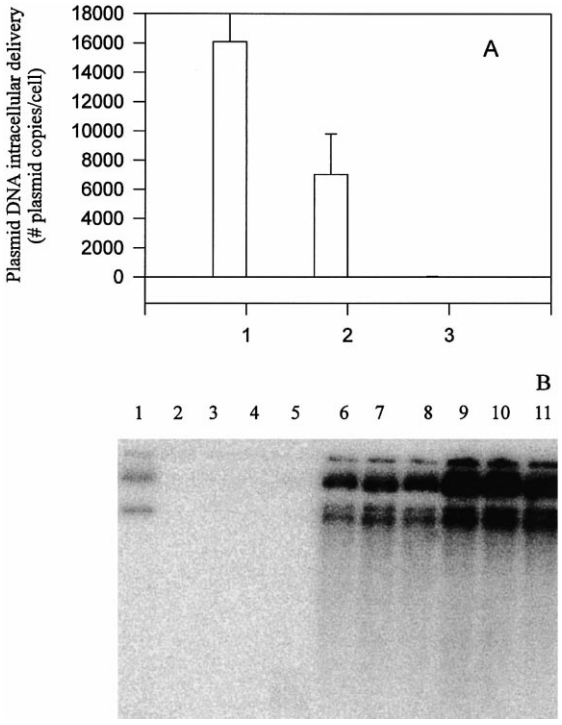

Fig. 6. Analysis of plasmid DNA uptake using dot blot and intracellular integrity using Southern blot. (A) Intracellular delivery of plasmid DNA determined by the dot blot assay. Plasmid DNA delivered by: 1, DODAC/DOPE with Ca^{2+}; 2, DODAC/DOPE; and 3, untransfected cells. (B) Southern blot analysis of intracellular plasmid DNA. Lanes: 1, pCMVβ standard; 2, TE buffer; 3–5, untransfected cells; Lanes 6–8, plasmid delivered by DODAC/DOPE; and 9–11, plasmid delivered by DODAC/DOPE prepared with Ca^{2+}. pCMVβ (2 μg) complexed to liposomes at 1.5 charge ratio. Cells were transfected for 4 h, in the presence or absence of 5 mM Ca^{2+}. Genomic and plasmid DNA (total DNA) were purified from the cell lysates as described in Section 2. Total DNA (2 μg) were dotted onto a nitrocellulose membrane or loaded into a 1% agarose gel, which was run at 60 V for 2 h. Blots were hybridized to a ^{32}P-labeled probe overnight for detection of plasmid using a phosphorimager. The number of plasmid copies was quantitated according to a plasmid standard and the results normalized by dividing by the number of cells. Experiments were performed in triplicate.

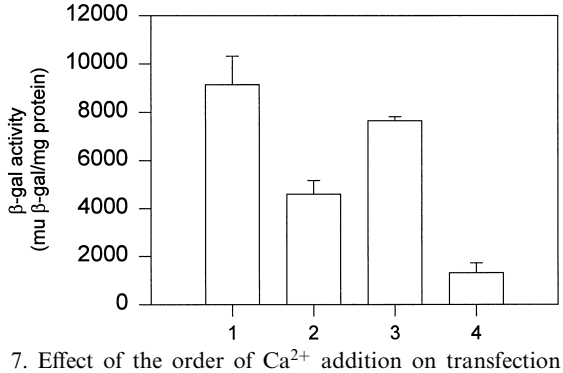

Fig. 7. Effect of the order of Ca^{2+} addition on transfection potency. Cells were transfected with DNA–cationic lipid complexes (0.25 μg DNA) that were mixed with 5 mM Ca^{2+} at different stages. 1, liposomes and plasmid DNA mixtures added to Ca^{2+} simultaneously; 2, Ca^{2+} mixed with cationic liposomes first; 3, Ca^{2+} mixed with plasmid DNA first; 4, Ca^{2+} added after formation of DNA–lipid complexes. Each experiment was performed in triplicate.

tems were compared after isolation of DNA from cells that had been transfected for 4 h, as described in Section 2. The results of the dot blot and Southern blot analyses are shown in Fig. 6. On average, each cell took up ~8000 copies of plasmid DNA when cells were transfected with complexes prepared in the absence of Ca^{2+}. This is consistent with previous work indicating that readily transfectable cells take up ~10^5 plasmids when transfected by complexes [7,37]. As shown in Fig. 6A, when cells were transfected with the complexes prepared in the presence of Ca^{2+} the number of plasmids taken up by each cell increased by more than 2.5-fold. A Southern analysis of the delivered DNA showed some degradation of the delivered plasmid for both the control and calcium-enhanced lipid–DNA complexes, as indicated in Fig. 6B by the smearing of the DNA bands and the appearance of additional DNA conformations as compared to the control plasmid. However, the results demonstrate that significantly more intact plasmid DNA is present in cells transfected with complexes prepared in the presence of Ca^{2+}.

3.7. The level of transfection enhancement is dependent on the time of Ca^{2+} addition

Experiments were performed to determine if the point at which Ca^{2+} is added during preparation of the complexes affects the level of transfection enhancement. Three situations were investigated, equivalent concentrations of Ca^{2+} (5 mM) were used for each. In the first situation, Ca^{2+} was incubated with either the DODAC/DOPE liposomes or the pCMVβ for 15 min prior to adding the other component, after which the mixtures were allowed to incubate for another 15 min before using for transfection. In the second case, liposomes and DNA were incubated for 30 min and Ca^{2+} was added just prior to applying the transfecting mixtures to cells. In the last case, liposomes and plasmid DNA were mixed together and immediately added to Ca^{2+} as described in Section 2, and the three components were incubated for 30 min before applying to cells.

As shown in Fig. 7, the greatest enhancement occurred when Ca^{2+}, plasmid DNA, and liposomes were mixed and incubated together. Incubation of plasmid DNA with Ca^{2+} before the addition of liposomes resulted in similar transfection levels. However, a more than 50% reduction in transfection potency was observed when Ca^{2+} was incubated first with the cationic liposomes and then with plasmid DNA. Most significantly, transfection levels were considerably reduced (~5-fold) when Ca^{2+} was added to pre-formed plasmid DNA–cationic lipid complexes immediately prior to the application to cells. These data suggest that the stimulatory effects of Ca^{2+} depend to some extent on an ability of the Ca^{2+} to interact directly with the plasmid DNA in the complex.

3.8. Ca^{2+}-enhanced transfection for complexes is observed in a variety of cell lines

In order to illustrate the generality of the transfection enhancing effect of Ca^{2+}, the influence of Ca^{2+} on complex-mediated transfection of a number of different cell lines was examined. Previous work has shown that the type of cell employed can cause large variations in cationic liposome-mediated transfection efficiencies [38]. This variation was also observed for the cell lines tested here. Transfection employing pCMVβ-DODAC/DOPE complexes prepared in the presence or absence of Ca^{2+} was performed on BHK, CV-1, 293, SKOV-3, and HS578T cells. In the absence of Ca^{2+}, BHK cells showed the highest transfection efficiencies among the cell lines examined (Fig. 8). Increased transfection was observed in the presence of Ca^{2+} for all cell

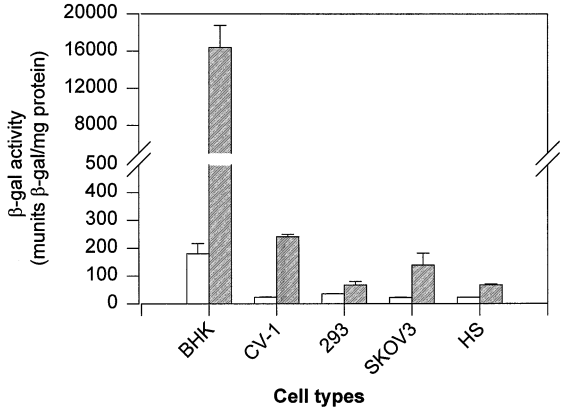

Fig. 8. Effect of Ca^{2+} on transfection for different cell lines. Cells were transfected with 0.25 μg pCMVβ complexed to DODAC/DOPE in the presence (gray bars) or absence (open bars) of Ca^{2+}(5 mM). Cells were exposed to the complexes for 4 h and then the transfection mixtures were replaced with the appropriate culture media for a further 20 h before assaying for β-gal expression. Experiments were performed in triplicate.

lines, although different cell lines demonstrated different degrees of enhancement (from 3- to 40-fold).

3.9. *Ca^{2+} enhances the transfection potency of complexes containing a variety of cationic lipids*

The stimulatory effect of Ca^{2+} on the transfection properties of plasmid DNA–cationic lipid complexes containing a variety of cationic lipids were examined. Prior to application to cells, plasmid DNA was complexed to liposomes containing either 50 mol% of DODAC, DOTMA, DSDAC or DDAB and 50 mol% DOPE. Enhanced β-gal expression was observed for all the cationic liposome formulations examined in the presence of Ca^{2+}, with DNA–lipid complexes containing DOTMA showing the highest transfection efficiency, followed by DODAC, DSDAC, and DDAB (Fig. 9A).

It was of interest to include DODAC/DOPC liposomes in order to see if Ca^{2+} could enhance transfection for a 'non-fusogenic' formulation. It has been demonstrated that only the fusogenic lipid DOPE is able to show helper activity in facilitating cationic liposome-mediated gene transfer [10,34,39]. Results presented in Fig. 9A showed that cells transfected with the DODAC/DOPC formulation exhibited very low levels of β-gal activity. Transfection efficiencies increased more than 5-fold when Ca^{2+} was included, although the enhanced transfection was still significantly lower than for the DOPE-containing formulation in the absence of Ca^{2+}. The ability of Ca^{2+} to enhance transfection for the DODAC/DOPC system was further investigated for increasing concentrations of Ca^{2+} in complexes prepared at a 1.5 charge ratio. As shown in Fig. 9B, significant enhancements in transfection were observed in the presence of 6–25 mM Ca^{2+}, where increases in β-gal activities from 0 to 600 mU per mg of total cellular protein were observed. As was observed for the DODAC/DOPE system, DODAC/DOPC transfection diminished when 50 mM or higher Ca^{2+} concentrations were used.

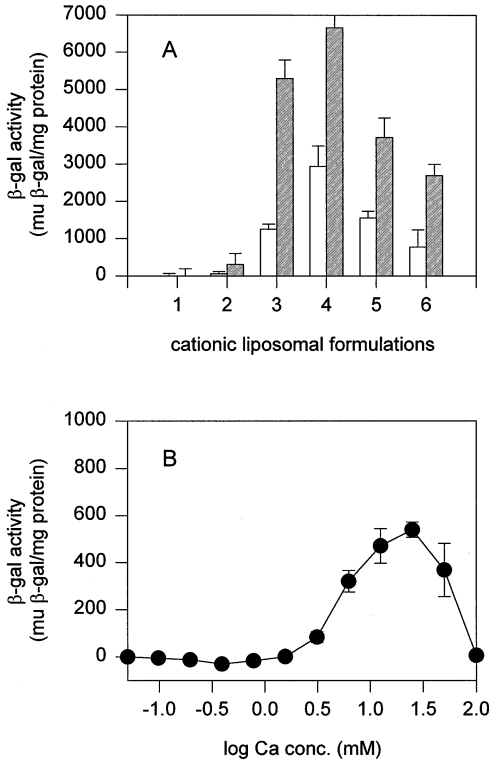

Fig. 9. Effect of Ca^{2+} on transfection properties of different liposomal formulations. (A) Transfection properties of complexes containing DODAC, DOTMA, DSDAC, or DDAB prepared at a 1:1 molar ratio with DOPE, as well as a 'non-fusogenic' formulation (DODAC/DOPC), in the presence (gray bars) or absence (open bars) of 10 mM Ca^{2+}. DNA was mixed with: 1, Ca^{2+} only; 2, DODAC/DOPC; 3, DODAC/DOPE; 4, DOTMA/DOPE; 5, DSDAC/DOPE; or 6, DDAB/DOPE. (B) Effect of increasing Ca^{2+} concentration on transfection properties of 'non-fusogenic' DNA–lipid complexes. DNA was complexed to DODAC/DOPC and added to 0–100 mM Ca^{2+}. All liposome formulations were complexed to 0.25 μg pCMVβ at 1.5 charge ratio. Experiments were performed in triplicate.

4. Discussion

The studies reported here show that the presence of Ca^{2+} during formation of DNA–cationic lipid complexes can result in significant improvements in transfection properties in vitro. Three areas warrant further discussion. First, it is of interest to compare the improvements in transfection efficiency achieved in the presence of Ca^{2+} with that achieved by other protocols. A second area concerns the interactions of Ca^{2+} with the components of the complexes. Finally, we discuss possible mechanisms whereby Ca^{2+} results in enhanced transfection potency of plasmid DNA–cationic lipid complexes.

Compared to viral vectors, plasmid DNA–cationic liposome complexes are relatively inefficient gene transfer agents [7]. Transfection efficiency may be limited by the ability of complexes to enter the cell, release of plasmid from the endosomal compartment, the stability of plasmid in the cytoplasm, and entry of plasmid into the nucleus. A variety of agents have been proposed to enhance the transfection potency of complexes by removing one or more of these limitations. Lysosomotropic agents, such as chloroquine have been suggested to promote escape of endocytosed materials from the endosome [10,15]. However, although some increase in transfection has been detected ($\sim$4-fold), the effect of chloroquine has been found to vary for different types of complexes to the extent that it could inhibit transfection [15]. DNA-binding molecules, such as polylysine have been proposed to inhibit breakdown of intracellular DNA and thus increase transfection, resulting in a 3- to 9-fold increase in transfection efficiency [19,40,41]. The results presented here demonstrate that the presence of Ca^{2+} can enhance the transfection potency of complexes by factors ranging from 3- to 20-fold depending on Ca^{2+} concentrations employed. Optimal Ca^{2+} concentrations to stimulate DNA–cationic lipid complexes transfection ranged between 5 and 25 mM. This is considerably higher than the Ca^{2+} concentrations found in culture media such as DMEM, which contains $\sim$1.3 mM Ca^{2+}. The generality of this stimulatory effect was confirmed by transfecting a number of different cell lines as well as employing various cationic liposomal systems, which further established Ca^{2+} as a potent stimulatory agent for enhancing cationic liposome-mediated cell transfection. In this regard, a recent report has shown that Ca^{2+} ions can enhance nuclear protein-mediated transfection, but not cationic liposome-mediated transfection [31]. This discrepancy likely arises from preparation of the complexes in the absence of Ca^{2+}, with subsequent addition of Ca^{2+} immediately prior to application to cells. The results presented here show that the transfection potency is significantly reduced under such situations.

It is likely, but not yet proven, that the ability of Ca^{2+} to enhance the transfection properties of complexes involves a direct association with the plasmid DNA in the complex. Two lines of evidence support this conclusion. First, the observation that efficient transfection in the presence of calcium is observed at lower cationic lipid-to-plasmid DNA charge ratios than in the absence of calcium suggests that calcium may substitute to some extent for cationic lipid in the complex. Second, enhanced transfection potencies were observed if complexes were incubated with Ca^{2+} for 30 min immediately after complex formation, but transfection levels were much reduced if Ca^{2+} was added immediately before application to cells. It has been shown that plasmid in plasmid DNA–cationic lipid complexes is at least partially protected from the external aqueous environment [42], which would preclude rapid interaction with external Ca^{2+}.

The mechanism whereby Ca^{2+} enhances the transfection potency of complexes appears to be directly related to an ability to increase uptake of complexes into cells, resulting in increased levels of intact intracellular plasmid DNA. The mechanism whereby Ca^{2+} could stimulate uptake is not yet clear, but likely arises from increased intracellular levels of Ca^{2+}. It has been shown that increased intracellular Ca^{2+} levels could lead to increases in the rates of endocytosis [27,28] and thus could promote the uptake of complexes. The dependence of transfection enhancement on the time and order of Ca^{2+} addition suggests that complex-associated Ca^{2+}, as opposed to free Ca^{2+}, plays a primary role in stimulating uptake. It is possible that the complex-associated Ca^{2+} can gain access to the intracellular compartment more readily, or that the complex-associated Ca^{2+} simply represents the largest pool of Ca^{2+} in the endosome following uptake of the complex.

In summary, we have shown that Ca^{2+} can act as

a potent stimulatory agent to enhance transfection properties of cationic DNA–lipid complexes. The protocol is simple and appears to be of utility in a variety of cell lines and for a variety of cationic lipids. The mechanism whereby Ca^{2+} stimulates transfection appears to involve enhanced uptake of plasmid DNA. It is anticipated that this protocol will find general applicability as a means to enhance cationic liposome mediated transfection.

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