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SHORT-CHAIN PHOSPHOLIPIDS ENHANCE AMPHIPATHIC PEPTIDE-MEDIATED GENE TRANSFER

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Addition of short-chain phospholipids to the gramicidin S-DNA-dioleoyl phosphatidylethanolamine complex enhanced up to 6-fold β-galactosidase expression in several cell-lines *in vitro*. Among the compounds tested, the most potent in enhancing transfection were the dicapryl- and the dicapryloyl phosphatidylcholine. In contrast, no significant enhancement of transfection was seen when short-chain phospholipids were mixed with cationic lipids. Short-chain phospholipid and gramicidin S may act simultaneously on the cell membrane to enhance gene transfer, yet resulting in no toxicity.

The design of artificial, non-viral systems to deliver genes into mammalian cells has become a research area of considerable interest (1). Many different techniques have been developed, including cationic lipids (2, 3), cationic peptide / DNA complexes (4), polymers (5, 6) and receptor-mediated delivery (7). However, despite a few examples of *in vivo* gene expression after non-viral DNA delivery (8), there remains a need for improved gene delivery efficiency using these reagents.

The amphipathic, cyclic peptide gramicidin S has been shown to facilitate plasmid DNA entry into mammalian cells *in vitro* (4). The transfecting complex was formed by mixing plasmid DNA with gramicidin S at a 1:1 +/- charge ratio and then adding the hexagonal phase-competent lipid dioleoyl phosphatidylethanolamine (DOPE) in a 5 fold molar excess to the peptide. The resulting transfecting particles mediated high levels of transfection of many adherent cell-lines. To further improve the transfection efficiency of the gramicidin S-DNA-DOPE complex, the addition of other effectors was investigated. Among possible effectors, we studied the effect of short-chain phospholipids (SCPL) and lysophospholipids (LPL). These phospholipids have a polar head group which exceeds in cross-section the hydrophobic tail (cone shape) and hence display membrane perturbing and hemolytic activity (9, 10). We found

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Abbreviations: DOPE, dioleoyl phosphatidylethanolamine; DOTAP, dioleoyloxy trimethylammonium propane; DOTMA, dioleoyloxypropyl trimethylammonium chloride; FCS, fetal calf serum; LPL, lysophospholipid; PBS, phosphate buffer saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SCPL, short-chain phospholipid.

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that incorporation of SCPL into the gramicidin S-DNA-DOPE complex enhances up to 6 fold β-galactosidase activity after DNA transfection of different cell types, without any toxicity.

MATERIAL AND METHODS

Preparation of the transfecting complex pCH110 (Promega) and pCMVβgal (Clontech) plasmids containing the β-galactosidase reporter gene under the control of the SV40 and the CMV promoter, respectively, were grown using standard techniques and purified by column chromatography (Qiagen, Germany). Lipids were obtained from Avanti Polar Lipids Inc. (Alabaster, USA). Dioleoyl phosphatidylethanolamine (DOPE) dispersions with or without SCPL and LPL were prepared by drying the lipids from chloroform:methanol stock solutions and then rehydrating the film with 30 mM Tris Cl pH 8.5. Final lipid concentration was 1 mM. Gramicidin S was obtained from Sigma and the gramicidin S-DNA-DOPE complexes, with or without SCPL or LPL, were prepared as previously described (4). Dioleoyloxy trimethylammonium propane (DOTAP, Avanti Polar Lipids Inc.) liposomes and dioleoyloxy trimethylammonium chloride (DOTMA):DOPE liposomes, with or without SCPL, were prepared by drying the lipids under vacuum and then rehydrating the lipid film with distilled water at a 1 mM final concentration. The vesicles were sonicated during 10 min. DOTAP and DOTMA:DOPE liposomes, with or without SCPL, were subsequently used at a 2:1 +/- charge ratio with plasmid DNA.

Transfection experiments Cells, COS-1 (monkey kidney fibroblasts), HepG2 (human hepatocytes), CV-1 (monkey kidney fibroblasts), 3T3 (mouse embryo fibroblasts) were plated at a density of about $2x10^4$ cells per well in 96-well plates and grown 24 h in 10% fetal calf serum (FCS) containing medium. Transfection took place in FCS free medium and 5 h later media was removed and replaced by 10% FCS containing medium. 0.2 or 1 μg of plasmid DNA was applied per well. After 48 h, β-galactosidase activity was measured using the O-nitrophenyl galactopyranoside assay (11). Day-to-day β-galactosidase activity values usually varied by about 2 fold depending upon cell density and condition of the cells.

Toxicity assay The toxicity of the transfecting complex with or without SCPL was evaluated on COS-1 cells using the same protocol as for the transfection. The toxicity was measured using a dye reduction assay (12), as previously described (5).

Physico-chemical characterization The size and the zeta potential of transfecting complexes with or without SCPL were measured using a Zetasizer 4 (Malvern Instruments) with 30 mM Tris Cl buffer pH 8.5 as the dilution buffer.

RESULTS

Dicapryl PC (C10 PC) was mixed with DOPE in proportions ranging from 5 to 75 mol% before preparation of complexes with pCH110 plasmid and gramicidin S. The transfecting complex was then applied to COS-1 cells and β-galactosidase activity and toxicity were measured 48 h later (figure 1). Dicapryl PC (C10 PC) enhanced 4 to 5 fold gramicidin S-mediated transfection when incorporated at a fraction of 5 or 10 mol% (i.e., a final concentration of 1 or 2 μM in the transfecting well). However, at concentrations above 25 mol% some toxicity appeared and transfection efficiency started to decrease. At a

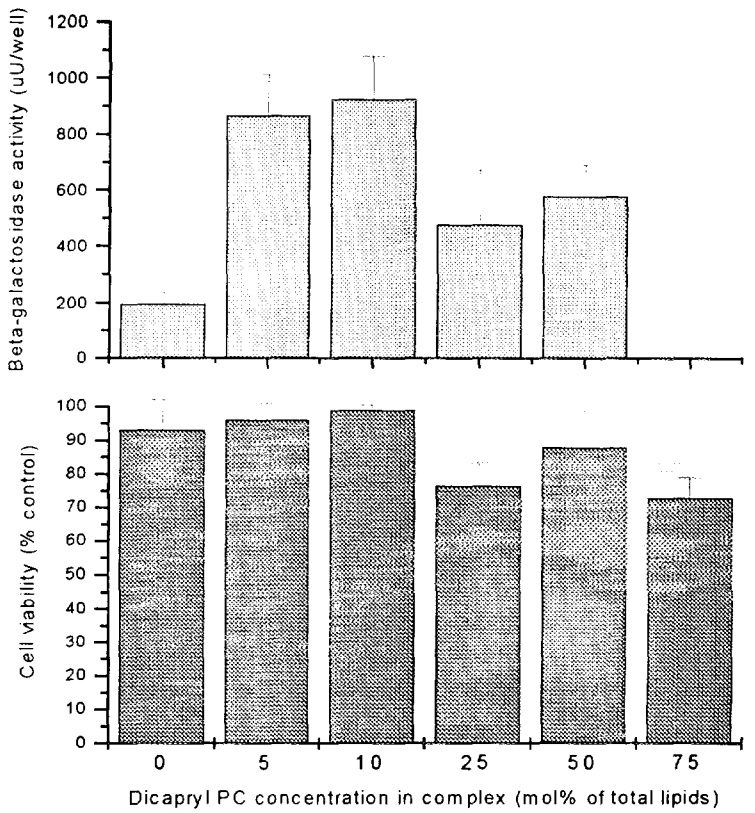

Fig. 1. Transfection efficiency (upper panel) and toxicity (lower panel) of gramicidin S-DNA-DOPE complex containing various amounts of dicapryl PC (expressed as mol% of total lipids). COS-1 cells were transfected with 0.2 μg of pCH110 plasmid per well and β-galactosidase activity and toxicity were measured 48 h posttransfection. Results are the mean±SD of 4 or 8 transfecting wells

concentration of 75 mol% of dicapryl PC (C10 PC), transfection was completely abolished, although toxicity was similar to that observed at 25 mol%. Beside dicapryl PC (C10 PC), dicapryloyl PC (C8 PC) and to a lower extent diundecanoyl PC (C11 PC) also improved transfection (figure 2). Dilauroyl PC (C12 PC) and dicapryloyl PE (C8 PE) did not significantly enhance β-galactosidase activity. When plasmid DNA was mixed only with DOPE:dicapriloyl PC 3:1 (molar ratio) no transfection was observed, indicating that SCPL by themselves are unable to deliver DNA into cells. Furthermore, the addition of DOPE:dicapryloyl PC 3:1 (molar ratio) to the cells 5 min. prior to applying the gramicidin S-DNA-DOPE complex did not result in any significant enhancement of the transfection efficiency (data not shown). These data suggest that both gramicidin S and dicapryloyl PC (C8 PC) must act simultaneously on cells to mediate an optimal transfection.

In the case of LPL, only lysolauroyl PC (C12 lysoPC) slightly enhanced 2 to 3 fold gramicidin S-mediated transfection (figure 3). Surprisingly, the other LPL tested not only failed to improve β-galactosidase activity but even abolished transfection at low concentration.

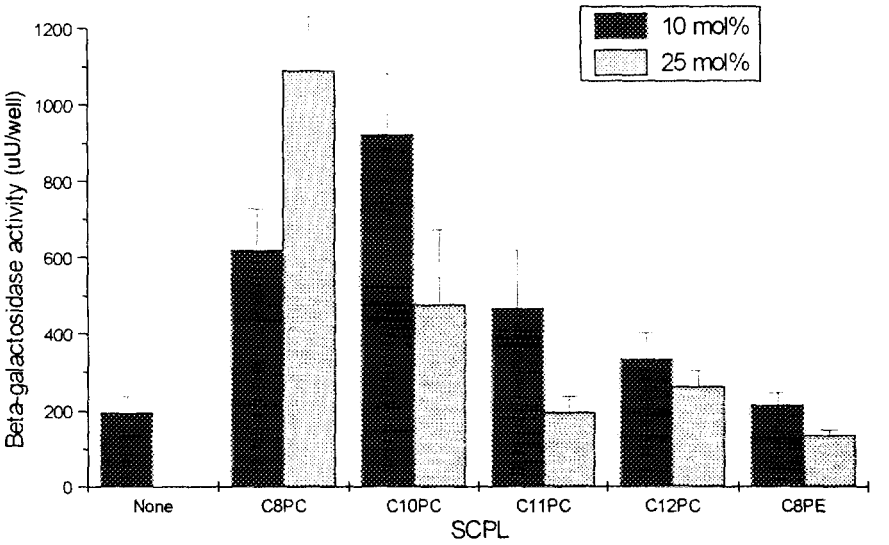

Fig.2. Transfection efficiency of gramicidin S-DNA-DOPE complex containing 10 mol% or 25 mol% (referred to total lipids) of various SCPL. COS-1 cells were transfected with 0.2 μg of pCH110 plasmid per well and β-galactosidase activity was measured 48 h posttransfection. Results are the mean± SD of 4 to 12 transfecting wells.

The transfection enhancing activity of dicapryloyl PC (C8 PC) was also investigated in other cell-lines, using the pCMVβgal as the expression vector (figure 4). In all the cell-lines tested, the incorporation of 25 mol% of dicapryloyl PC (C8 PC) into the transfecting particles improved gene expression. However, this enhancement was modest in CV-1 and 3T3 cells.

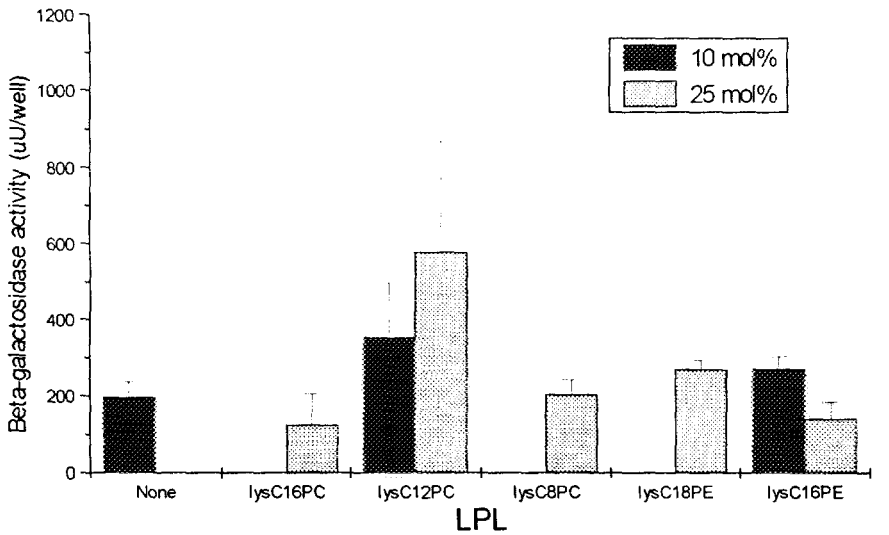

Fig.3. Transfection efficiency of gramicidin S-DNA-DOPE complex containing 10 mol% or 25 mol% (referred to total lipids) of various LPL. COS-1 cells were transfected with 0.2 μg of pCH110 plasmid per well and β-galactosidase activity was measured 48 h posttransfection. Results are the mean± SD of 4 or 8 transfecting wells.

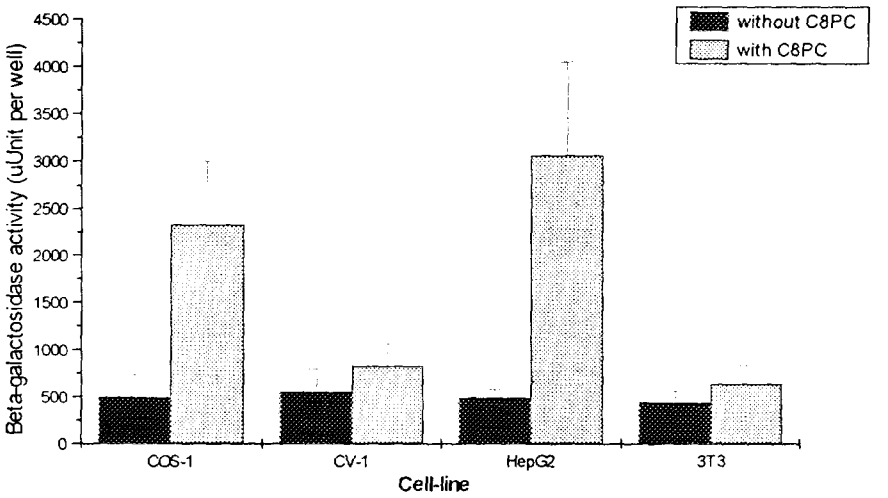

Fig.4. Transfection efficiency of gramicidin S-DNA-DOPE complex containing or not 25 mol% (referred to total lipids) of dicapryloyl PC in various cell-lines. Cells were transfected with 1 µg of pCMVβgal plasmid per well and β-galactosidase activity was measured 48 h post transfection. Results are the mean± SD of 4 transfecting wells.

In order to see if the incorporation of 25 mol% of dicapryloyl PC (C8 PC) into the gramicidin S-DNA-DOPE complex would modify the physicochemical characteristics of the transfecting complex, the size and the zeta potential of the complex with or without dicapryloyl PC (C8 PC) were measured. There was no difference between both complexes in terms of size (between 200 and 300 nm) and zeta potential (between -0.2 and -1 mV) (data not shown).

Finally, the effect of SCPL on cationic lipid-mediated transfection was investigated. Dicapryloyl PC (C8 PC) was incorporated into DOTAP or DOTMA:DOPE liposomes at molar ratios ranging from 5 to 75% and β-galactosidase activity was measured 48 h after the transfection of COS-1 cells. No significant enhancement (less than 1.5 fold) of transfection was observed upon the addition of dicapryloyl PC (C8 PC) (data not shown).

DISCUSSION

Addition of SCPL to the gramicidin S-DNA-DOPE complex enhanced the transfection rate of various cell-lines. The most potent compounds in enhancing transfection were the dicapryl PC (C10 PC) and the dicapryloyl PC (C8 PC). Only SCPL which are known to be hemolytic (9) were able to improve gene transfer.

Although the exact mechanism whereby SCPL enhance gramicidin S-mediated gene delivery is still unclear, SCPL may enhance the partition of gramicidin S into the membrane (13) by destabilizing the lipid bilayer (14). We believe that SCPL do not permeabilize the whole cell membrane but that they are rather delivered locally in the plasma membrane of the cell where they play a synergistic role with the gramicidin S. This hypothesis is based upon the facts that

first, SCPL enhance transfection at a concentration well below their hemolytic concentration (about 10^{-4}M) and second that the addition of SCPL prior to the gramicidin S-based complex did not result in any enhancement of gene expression.

In addition, it is unlikely that the transfection enhancement observed was due to a major structural modification of the complex upon the addition of SCPL. Indeed, improved gene delivery was seen with SCPL concentration as low as 5 mol% and the size as well as the zeta potential of the transfecting particles were not modified when dicapryloyl PC (C8 PC) was incorporated at a 25 mol% ratio into the complex. However, the complete inhibition of transfection at 75 mol% of dicapryl PC (C10 PC) in the complex cannot be explained by a toxic effect (figure 1) and could therefore be due to a weaker binding of the lipids onto the peptide in the presence of high amounts of SCPL.

Addition of LPL into the gramicidin S-DNA-DOPE complex did not result in a clear enhancement of transfection as seen with the SCPL (figure 3). Transfection was even sometimes completely abolished, depending of the added compound and its concentration, albeit no obvious toxicity was observed. Although they are potent hemolytic agents (9, 15), LPL, unlike SCPL, can also inhibit late fusion events (16). Several experimental data, such as the absence of chloroquine-mediated enhancement of the transfection, suggest that DNA delivery by the gramicidin S-DNA-DOPE complex involves entry of the DNA through the plasma membrane of the cell (4). In this regard, DOPE plays a crucial role by promoting uptake and probably membrane fusion. It is therefore likely that the addition of LPL somehow limits DOPE-mediated fusion and subsequently, decreases transfection. The slight transfection improvement observed with the lauroyl lysoPC (C12 lysoPC) can be the result of the two opposite effects, membrane disruption and fusion inhibition.

Cationic lipid-mediated transfection could not be significantly augmented by the addition of dicapryloyl PC (C8 PC) to DOTAP or DOTMA:DOPE liposomes. This observation can be related to the different mechanism of action of the cationic lipids and the gramicidin S-based system. Cationic lipids, unlike the gramicidin S-DNA-DOPE complex, deliver plasmid DNA after endocytosis has occurred (4, 17, 18). SCPL therefore appear only efficient when incorporated in a transfection system which delivers DNA primarily through the plasma membrane of the cell.

In conclusion, SCPL and especially dicapryl (C10 PC) and dicapryloyl PC (C8 PC) enhance gramicidin S-mediated transfection *in vitro*. This effect is related to the membrane perturbing

activity of the SCPL. This observation could open new strategies to improve gene delivery using non-viral systems, especially those requiring direct fusion with the plasma membrane of the cell.

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REFERENCES

1. Behr, J. P. (1993) *Acc. Chem. Res.* **26**, 274-278
2. Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielsen M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7413-7417
3. Behr, J. P. (1994) *Bioconj. Chem.* **5**, 382-389
4. Legendre, J. Y., and Szoka, F. C. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 893-897
5. Haensler, J., and Szoka, F. C. (1993) *Bioconjugate Chem.* **4**, 372-379
6. Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., and Behr, J. P. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 7297-7301
7. Wagner, E., Curiel, D., and Cotten, M. (1994) *Adv. Drug Del. Rev.* **14**, 113-135
8. Caplen, N. J., Alton, E., Middleton, P. G., Dorin, J. R., Stevenson, B. J., Gao, X., Durham, S. R., Jeffery, P. K., Hodson, M. E., Coutelle, C., Huang, L., Porteous, D. J., Williamson, R., and Geddes, D. M. (1995) *Nature Med.* **1**, 39-45
9. Reman, F. C., Demel, R. A., De Gier, J., Van Deenen, L. L. M., Eibl, H., and Westphal, O. (1969) *Chem. Phys. Lipids* **3**, 221-233
10. Chernomordik, L. V., Kozlov, M. M., Melikyan, G. B., Abidor, I. G., Markin, V. S., and Chizmadhev Y. A. (1985) *Biochim. Biophys. Acta* **812**, 643-655
11. Felgner, J. H., Kumar, R., Sridhar, C. N., Wheeler, C. J., Tsai, Y. J., Border, R., Ramsey, P., Martin, M., and Felgner, P. L. (1994) *J. Biol. Chem.* **269**, 2550-2561
12. Mosmann, T. R. (1983) *J. Immunol. Methods* **65**, 55-63
13. Wu, E., Jacobson, K., Szoka, F. C., and Portis, A. Jr. (1978) *Biochemistry* **17**, 5543-5550
14. Tanaka, Y., Mashino, K., Inoue, K., and Nojima, S. (1983) *J. Biochem.* **94**, 833-840
15. Matsuzaki, K., Handa, T., Miyajima, K., Mikura, Y., Shimizu, H., and Toguchi H. (1988) *Chem. Pharm. Bull.* **36**, 4253-4260
16. Chernomordik, L. V., Vogel, S. S., Sokoloff, A., Onaran, H. O., Leikana, E. A., and Zimmerberg, J. (1993) *FEBS lett.* **318**, 71-76
17. Legendre, J. Y., and Szoka, F. C. (1992) *Pharm. Res.* **9**, 1235-1242
18. Wrobel, I., and Collins, D. (1995) *Biochim. Biophys. Acta* **1235**, 296-30