Cationic lipids destabilize lysosomal membrane in vitro

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Abstract Addition of cationic lipids to plasmid DNA considerably increases the efficiency of transfection. The mechanism has not yet been elucidated. A possibility is that these compounds destabilize biological membranes (plasma, endosomal, lysosomal), facilitating the transfer of nucleic molecules through these membranes. We have investigated the problem by determining if a cationic lipid *N*-(1-(2,3-dioleoxy)propyl)-*N*,*N*,*N*,-trimethylammonium methyl-sulfate (DOTAP, Boehringer, Mannheim, Germany) affects the integrity of rat liver lysosomal membrane. We have measured the latency of β-galactosidase, a lysosomal enzyme, and found that incubation of lysosomes with low concentrations of DOTAP causes a striking increase in free activity of the hydrolase and even a release of the enzyme into the medium. This indicates that lysosomal membrane is deeply destabilized by the lipid. The phenomenon depends on pH, it is less pronounced at pH 5 than at pH 7.4. Anionic compounds, particularly anionic amphipathic lipids, can to some extent prevent this phenomenon. It can be observed with various cationic lipids. A possible explanation is that cationic liposomes interact with anionic lipids of lysosomal membrane, allowing a fusion between the lipid bilayers which results in a destabilization of the organelle membrane.

Key words: Cationic lipid; Lysosomal membrane destabilization

1. Introduction

Cationic lipids are widely used as plasmid DNA vectors in transfection experiments. They form complexes with nucleic acid molecules that are endocytosed [1–3]. A major problem is how after internalization, DNA is released from the complex and escapes from the intracellular vacuoles where it has been trapped. Indeed, to be transcribed, DNA must reach the nucleus in a free form via the cytosol [2]. A possibility is that cationic lipids may be able to react with the organelle membrane in a manner that destabilizes the lipid bilayer allowing a release of the vacuole content into the cytosol.

In the work presented here, using the lysosomal membrane as a model, we tried to investigate if cationic lipids can affect the integrity of biological membranes. The rationale of such an approach is that: (1) lysosomal membrane is a good representative of an endomembrane with which cationic lipids would have to interact after endocytosis of the DNA-lipid complex; (2) destabilization of lysosomal membrane can be easily studied by measuring the latency of lysosomal enzymes. In fact lysosomes may be considered big 'natural liposomes' enclosing enzymes that have no access to external substrates if the organelle membrane is intact. The activity of these enzymes towards such substrates increases if the membrane is injured. Its measurement may therefore be used to check organelle membrane integrity [4]. Our results show that cationic lipids cause a loss of latency of β-galactosidase, an enzyme located in lysosomes, indicating that these compounds are able to destabilize the membrane of these organelles.

2. Materials and methods

Experiments were performed on male Wistar rats weighing about 250 g. Liver total mitochondrial fraction (ML fraction) was prepared according to de Duve et al. [5]. Purified preparation of lysosomes was obtained by the method of Wattiaux et al. [6]. To investigate the effect of cationic lipids on lysosomal membrane, organelles were incubated at 37°C in a medium containing 0.25 M sucrose, 50 mM buffer, generally HEPES pH 7.4, in the absence or presence of the compound to be tested. After 20 min, samples were withdrawn for assay of free and total activities of β-galactosidase. In some experiments, samples were centrifuged for 10 min at 55000 rpm in the TLA 120.1 rotor of a TLX Beckman ultracentrifuge and the supernatant used to determine unsedimentable activity of the enzyme.

For β-galactosidase measurement, the lysosome preparation was incubated at 37°C in a total volume of 0.2 ml containing 5 mM methylumbelliferyl-β-galactopyranoside, 50 mM NaCl, 50 mM acetate buffer pH 5, 0.25 M sucrose in the absence (free activity) or presence (unsedimentable and total activities) of 0.1% Triton X-100. The assay was terminated after 10 min by adding 1 ml of 50 mM glycine buffer pH 10.5 containing 0.05% Triton X-100. The liberated methylumbelliferone was determined by measuring the fluorescence (excitation at 350 nm, emission at 450 nm) in a SPF 500 Aminco spectrofluorimeter.

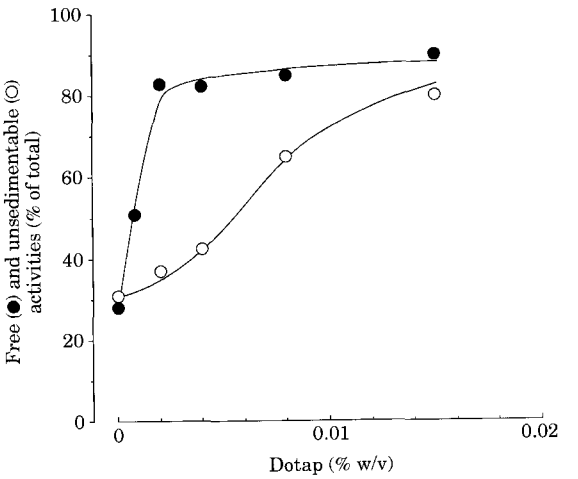

Fig. 1. Effect of DOTAP on free and unsedimentable β-galactosidase activities. Purified lysosomes were incubated at 37°C for 20 min in 0.25 M sucrose, 50 mM HEPES buffer, pH 7.4 in the presence of various concentrations of DOTAP. After that, free, unsedimentable and total β-galactosidase activities were measured. Ordinate: free (●) or unsedimentable (○) activity as a percentage of total activity.

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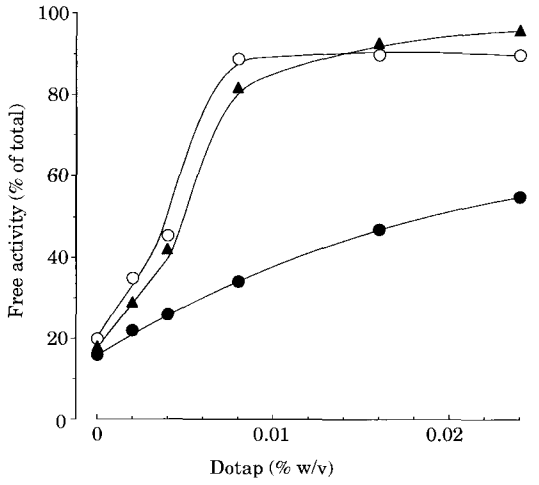

Fig. 2. Effect of pH on activation of lysosomes by DOTAP. The ML fraction was incubated at 37°C for 20 min in 0.25 M sucrose, 50 mM acetate buffer pH 5 (●) or pH 6 (▲) or 50 mM HEPES buffer pH 7.4 (○). After that, free and total β-galactosidase activities were measured. Ordinate: free activity as a percentage of total activity.

3. Results

Most of the experiments were performed with *N*-(1-(2,3-dioleoxy)propyl)-*N*,*N*,*N*,-trimethylammonium methyl-sulfate (DOTAP, Boehringer, Mannheim, Germany), an artificial, cationic lipid frequently used for transfection. To investigate the effect of DOTAP on lysosomal membrane, first we incubated purified lysosomes in the presence of increasing concentrations of DOTAP and measured free, unsedimentable and total activities of β-galactosidase, a lysosomal enzyme. As reported in Fig. 1, hydrolase free activity is strongly increased by DOTAP. Paradoxically, the release of β-galactosidase in the medium does not parallel the unmasking of the hydrolase as seen with other lysosome disrupting agents: detergents, hypotonicity, permeant molecules. Higher concentrations of DOTAP are required to observe a significant release of β-galactosidase in unsedimentable form. This suggests that interaction of DOTAP with lysosomal membrane may have two effects on membrane integrity. At a low concentration, cationic lipid destabilizes the membrane and increases its permeability, allowing the low molecular weight external substrate to have access to hydrolases but not permitting hydrolases to leave the organelles. At higher lipid concentrations, a disruption of the membrane takes place causing the enzymes to escape from the lysosomes. It is to be noted that the total activity of β-galactosidase is not modified by DOTAP. Similar results were obtained when a total mitochondrial fraction [5], containing up to 70% of liver lysosomes but obviously less purified, was used. Subsequently, experiments were performed with total mitochondrial fractions, owing to the ease of preparation in comparison to purified lysosomes.

The effect of DOTAP on lysosomal membrane may involve ionic or (and) hydrophobic interactions. The importance of ionic interactions is illustrated by the following results. As shown in Fig. 2, the lysosomolytic effect of DOTAP is attenuated when the pH is low enough, the increase of β-galactosidase free activity induced by the lipid is similar at pH 7.4 and at pH 6 but is significantly inhibited at pH 5. Polyanionic compounds, e.g. dextran sulfate, heparin, hyaluronic acid, ATP, prevent the unmasking of β-galactosidase by DOTAP. Of particular interest are the results obtained with amphipathic lipids (Fig. 3). Anionic compounds are able to prevent the effect of DOTAP on lysosomes while zwitterionic and non-ionic molecules are inefficient. Plasmid DNA behaves like other polyanionic compounds and counteracts the lysosomolytic effect of DOTAP. Inhibition depends on the concentration ratio DOTAP/DNA (Fig. 4). Interestingly, at con-

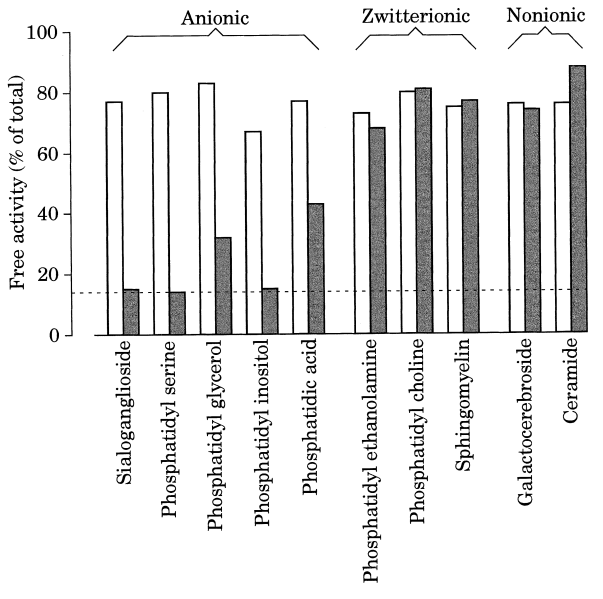

Fig. 3. Effect of amphipathic lipids on activation of lysosomes by DOTAP. The ML fraction was incubated at 37°C for 20 min in 0.25 M sucrose, 50 mM HEPES buffer pH 7.4, 0.008% (w/v) DOTAP in the absence (open bar) or presence (closed bar) of 0.008% (w/v) amphipathic lipid. After that, free and total β-galactosidase activities were measured. Ordinate: free activity as a percentage of total activity. The dotted line indicates the mean free activity of β-galactosidase at the end of the 20 min incubation period in the absence of DOTAP.

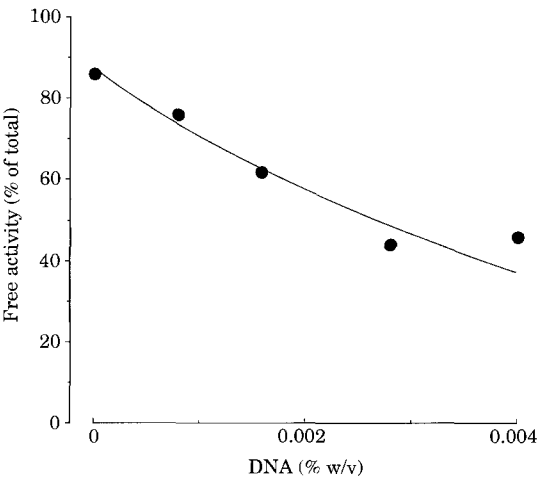

Fig. 4. Effect of plasmid DNA on activation of lysosomes by DOTAP. The ML fraction was incubated at 37°C for 20 min in 0.25 M sucrose, 50 mM HEPES buffer pH 7.4, 0.008% (w/v) DOTAP in the presence of various amounts of plasmid DNA (pcDNA/Amp). After that, free and total β-galactosidase activities were measured. Ordinate: free activity as a percentage of total activity.

Table 1
Effect of various cationic lipid preparations on free activity of β-galactosidase

Compound	β-Galactosidase free activity (% of total)
None	15.2
TR1	86.1
TR2	52.5
TR3	25.0
TR4	62.1
TR5	74.5
TR6	81.3
TR7	27.6
TR8	74.1
Lipofectamine	93.4
DMRIE	67.8
Lipofectin	80.7
Cellfectin	67.3
DOSPER	78.7
DOTAP	78.9
Triton X-100	22.6

The ML fraction was incubated at 37°C for 20 min in 0.25 M sucrose, 50 mM HEPES buffer pH 7.4 and 0.004% (w/v) cationic lipid. After that, free and total β-galactosidase activities were measured. TR1 to TR8 are eight different liposome preparations (Perfect Transfection Kit, Invitrogen, Abingdon, UK) whose nature is not given by the vendor. Lipofectamine, Lipofectin, DMRIE and Cellfectin are from Gibco Merelbeke, Belgium, DOSPER and DOTAP from Boehringer, Mannheim, Germany. For the sake of comparison, the effect of Triton X-100, at the same concentration, is given.

centration ratios (five or more) which are usual in transfection experiments, a large proportion of DOTAP lysosomolytic capacity is still present.

Multiple cationic liposomes are now used in transfection experiments. We have tested 14 of them with respect to their ability to lyse lysosomes: all are able to destabilize lysosomal membrane, however, some lipids are more lysosomolytic than others (Table 1). At the same concentration, most of these substances are even more efficient than Triton X-100, a detergent widely used as a membranolytic agent.

4. Discussion

Our results clearly indicate that in vitro, cationic lipids are able to destabilize lysosomal membrane, allowing the lysosome content to be released outside the organelle.

Apparently, the lysosomolytic effect of cationic lipids depends on interaction between the cationic lipids and anionic molecules present in the membrane. It is inhibited by lowering the pH, which decreases the anionic charge of the cytoplasmic face of the lysosome membrane. It is to be noted that the charge of DOTAP is independent of pH, hence the pH effect arises from changes affecting the organelle membrane. Moreover, it is prevented when the positive charges are neutralized by polyanionic compounds, in particular acidic phospholipids and acidic gangliosides. Interaction between lysosomal membrane and cationic lipids is probably favored by the fact that the cytoplasmic-facing monolayer of lysosomal membrane is enriched in acidic phospholipids. A plausible hypothesis is that such an interaction mediates a fusion between the lysosomal membrane and cationic liposomes as it takes place between negatively charged phosphatidylserine liposomes and cationic liposomes [7]. Intermixing of lysosomal membrane anionic lipids with cationic lipids could form neutral ion pairs, as described by Xu and Szoka [8], which destabilize the membrane, increasing its permeability and accessibility of external substrates to lysosome hydrolases, and even leading to a leakage of these enzymes into the medium. Obviously, if cationic lipids are endowed with similar lysosomolytic properties when they are located inside the organelles, as is the case in vivo, they could facilitate the release of plasmid DNA from lysosomes into the cytosol. This supposes the presence of anionic lipids in the inner monolayer of the lysosome membrane. Such a possibility is not excluded since acidic gangliosides are present in the inner monolayer of the organelle membrane. Moreover, a flip-flop of anionic lipids could take place resulting from an initial destabilization of the membrane caused by cationic lipids, leading to a progressive loss of asymmetry in lipid composition of the bilayer, as suggested by the recent observations of Xu and Szoka [8]. A drawback could be that the pH of lysosomes is acidic and, as we have shown, a lowering of the pH inhibits the lysosomolytic effect of DOTAP. However, destabilization of the lysosomal membrane by the cationic lipid is still significant even at pH 5, the physiological pH of lysosomes. Finally, we have found that DNA, a polyanion, opposes the DOTAP effect on lysosomes. However, the concentration ratio DNA/DOTAP that is required to observe a significant inhibition is higher than that usually present in complexes used for transfection. Therefore, in such complexes, DOTAP remains endowed with lysosomolytic properties.

After endocytosis, DNA-lipid complexes initially probably remain for a long time in an endosomal or prelysosomal compartment before reaching lysosomes [9]. Therefore, it is possible that the escape of DNA into cytosol mainly takes place in that compartment. We think that if, as we have described, cationic lipids are lysosomolytic they are also endosomolytic, owing to the kinship that exists between the membranes of the two organelles, and that, therefore, cationic lipids can contribute to the release of DNA from the endosome; endosomal membrane destabilization by cationic lipids could even be more efficient because the endosome pH is higher than the lysosome pH.

From a practical point of view, the lysosomolytic power of a cationic lipid could be a characteristic that would be of interest to check when designing new cationic lipids, as it is probably linked to the capacity of these compounds to induce the release of plasmids from lysosomes or endosomes after their uptake by the cell and thus to their efficiency for transfection. On the other hand, it could be worthwhile to investigate factors that stimulate lysosomolysis caused by cationic lipids. In this respect, it is possible that the increase in transfection efficiency caused by lysosomotropic compounds such as chloroquine [10] results in part from the beneficial effect of endosome and lysosome alkalinization induced by the weak base on the lysosomolytic capacity of cationic lipids complexed with plasmid DNA.

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