Application of Calcein-Loaded Liposomes for the Determination of Membrane Channel Size

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Received May 6, 1999; accepted June 3, 1999

We found that calcein-loaded liposomes can be used to evaluate the sizes of channels in membranes by measuring changes in calcein release when the molecular size of the solute added to the outer suspension medium is changed. If the solute added to the outer medium can enter the inner aqueous phase through the channel, the osmotic pressure of the inner phase increases, causing bursting of the liposomes and the release of calcein. Thus, the size of the channel formed in the liposomal membrane can be determined by examining whether the solute added to the outer medium induces calcein release. We used a series of sugars as solutes and estimated the channel size formed by polyene antibiotics. The results agreed well with those conducted in a similar manner using erythrocytes, demonstrating that this method should be useful for examining channel sizes in membranes.

Key words liposome membrane permeability assay; calcein; channel size determination; fluorescent probe; osmotic protection experiment

Calcein-loaded liposomes have been widely used to investigate the permeability properties of liposomal membranes.^{1–4} Calcein fluoresces very weakly at high concentrations because of self-quenching, but its fluorescence increases at lower concentrations as quenching is reduced. Thus, calcein entrapped inside liposomes at high concentrations is weakly fluorescent, but calcein that leaks out of the liposomes is highly fluorescent. Calcein-loaded liposomes have been widely used for liposome membrane permeability assays, because liposomal leakage can be measured *in situ* without any separation of liposomes in an assay medium.^{1–4}

We are particularly interested in finding new applications for calcein-loaded liposomes and have attempted to determine channel sizes formed in liposomal membranes. This experiment is similar to an osmotic protection experiment using erythrocytes to measure the release of hemoglobin (so-called hemolysis) in the presence of a protectant.^{5–8} In the erythrocyte experiment, if the solute added to the outer medium does not pass through a channel formed in the erythrocyte membrane, hemolysis will not be induced because the osmotic pressure of the intracellular hemoglobin is balanced with that of the solute. Thus, the size of the channels in the membrane can be evaluated by examining whether the substances added can protect against hemolysis. We applied this principle to measure liposome permeability using calcein.

MATERIALS AND METHODS

Reagents The sources of the reagents are as follows: calcein from Dojindo Laboratories (Kumamoto, Japan); egg phosphatidylcholine (PC) from the Green Cross Corporation (Osaka, Japan); cholesterol, amphotericin B, filipin (as filipin complex, approximately 75% by UV spectral analysis), nystatin and Triton X-100 from Sigma (St. Louis, MO, USA); D-mannitol, sucrose and raffinose from Wako (Osaka, Japan). Other chemicals used were of analytical-reagent grade.

Preparation of Liposomes Liposomes were prepared using the reversed-phase evaporation method^{9,10} as follows. Aliquots of lipid stock solutions containing egg PC (10 μ mol, 7.7 mg) and cholesterol (7.5 μ mol, 2.9 mg) dissolved in chloroform/methanol (1:2, v/v) were placed in a cen-

trifuge test tube (10 ml; Nichiden-Rika, Kobe, Japan). The solvent was evaporated using a centrifugal evaporator (RD400; Yamato, Tokyo, Japan), and the residual lipid was dried under a vacuum for several hours. The lipid was then dissolved in 1.5 ml of diethyl ether, followed by the addition of 1 ml of an aqueous solution of 100 mM calcein–NaOH (pH 7.4). The mixture was sonicated (5201; Ohtake Works, Tokyo, Japan) at 50 W for 1 min at 0 °C to obtain a homogeneous emulsion. Immediately, it was transferred to a round-bottom flask (20 ml), and the diethyl ether solvent was removed using a conventional rotary evaporator under reduced pressure (using an aspirator) at 25 °C. After the diethyl ether was completely removed by passing nitrogen gas through the mixture, a homogeneous suspension of liposomes was formed. The liposomes were centrifuged (105000 $\times g$, 20 min) and washed twice with 150 mM NaCl and 5 mM 4-(2-hydroxyethyl)-2-piperazineethanesulfonic acid (HEPES)–NaOH (pH 7.4) to remove the untrapped calcein. The final pellet of liposomes was suspended in 5 ml of the above washing solution. The osmotic pressure of the inner and outer aqueous solutions was measured with an OS osmometer (Fiske, Needham, MA, USA); both were approximately 300 mOsm.

Assay Procedure A liposome permeability assay was carried out at 30 °C as follows. An aliquot of liposome suspension (0.3 ml) was diluted with 19.7 ml of the following assay solution to make a 30 μ M phospholipid suspension: (a) 150 mM NaCl and 5 mM HEPES–NaOH (pH 7.4); (b) 300 mM sugar containing one of D-mannitol, sucrose or raffinose and 5 mM HEPES–NaOH (pH 7.4). A 500 μ l volume of this liposome suspension was pipetted in a test tube, and a small aliquot of a polyene antibiotic dissolved in dimethyl sulfoxide was added and incubated for 10 min at 30 °C. The dimethyl sulfoxide concentration was less than 1% v/v. The release of calcein out of the liposomes was determined by measuring the fluorescence intensity at 520 nm (excitation at 490 nm) on a Hitachi MPF-4 fluorospectrophotometer. The fluorescence intensity corresponding to 100% release was determined by adding a 200 mM Triton X-100 solution (5 μ l) to 500 μ l of the sample. The following molecular diameters of substances were used^{11,12}: mannitol, 0.7 nm; sucrose, 0.9 nm; raffinose, 1.1 nm.

Erythrocytes Human erythrocytes were washed twice with buffer (150 mM NaCl and 5 mM HEPES-NaOH (pH 7.4)) and suspended in this buffer at a concentration of 5% hematocrit. Then, an aliquot of erythrocyte suspension (8 ml) was diluted with 72 ml of an appropriate assay solution to make the following erythrocyte suspension at 0.5% hematocrit: (a) 150 mM NaCl and 5 mM HEPES-NaOH (pH 7.4); (b) 135 mM NaCl, 5 mM HEPES-NaOH (pH 7.4) and 30 mM sugar containing one of D-mannitol, sucrose or raffinose. A 30 mM concentration of sugar was chosen to balance the erythrocytes with the concentration of inner hemoglobin.⁶⁻⁸⁾ The total amount of phospholipid concentration contained in the erythrocyte membrane at 0.5% hematocrit was 30 μ M in egg PC equivalent,¹³⁾ which was the same phospholipid concentration as the liposomes. A 2 ml volume of this erythrocyte suspension was pipetted in a test tube and incubated with a polyene antibiotic for 10 min at 30 °C. Cells were centrifuged at 1200 $\times g$ for 5 min at 4 °C, and hemolysis was determined by measuring the absorbance at 540 nm. The total amount of hemoglobin was estimated by adding Triton X-100 solution, similarly to the liposome experiments.

RESULTS AND DISCUSSION

An osmotic protection experiment using erythrocytes is widely used to measure the size of channels formed in erythrocyte membranes.⁵⁻⁸⁾ We applied this technique to calcein-loaded liposomes and tried to determine the channel size formed by amphotericin B, which has been shown to form channels in cholesterol-containing liposomes.^{5,9,10,14,15)}

Fig. 1. Dose-Response Curves of Calcein Release in 150 mM NaCl (a), 300 mM Mannitol (b), 300 mM Sucrose (c) and 300 mM Raffinose (d) Buffered with 5 mM HEPES-NaOH (pH 7.4)

Fig. 2. Dose-Response Curves of Hemolysis in 150 mM NaCl (a) and 135 mM NaCl Containing One of 30 mM Mannitol (b), 30 mM Sucrose (c) or 30 mM Raffinose (d) Buffered with 5 mM HEPES-NaOH (pH 7.4)

Fig. 3. Relationship between Calcein Release and Hemolysis Induced by Polyene Antibiotics and the Molecular Diameter of Sugars Amphotericin B (a), nystatin (b) and filipin (c). Solid line for calcein release and dashed line for hemolysis.

The amphotericin B was allowed to react with liposomes for 10 min, because this reaction time was adequate to clearly distinguish differences in the release of markers having different sizes induced by amphotericin B,⁹⁾ and also to measure calcein release for the present purpose. Figure 1 shows the dose-response curves of calcein release upon the addition of amphotericin B in various outer suspension media. When liposomes were suspended in a solution containing 150 mM NaCl and 5 mM HEPES-NaOH (pH 7.4), the release of calcein began to occur at around 2–5 μ g/ml (line a). A similar dose-response curve was observed in a medium containing 300 mM mannitol and 5 mM HEPES-NaOH (pH 7.4) (line b), while the release of calcein was suppressed completely in media containing sucrose (line c) or raffinose (line d). These results indicate that the channel formed by amphotericin B is bigger than mannitol (0.7 nm) and smaller than sucrose (0.9 nm). For comparison, we measured the size of the channel formed by amphotericin B in erythrocyte membranes by osmotic protection assays. Figure 2 shows the dose-response curves of hemolysis upon the addition of amphotericin B in different suspension media. Amphotericin B induced hemolysis in a medium containing mannitol (line b) whose curve almost overlapped with that of a solution containing 150 mM NaCl and 5 mM HEPES-NaOH (pH 7.4) (line a), while hemolysis was suppressed completely in media containing sucrose (line c) or raffinose (line d), indicating that amphotericin B made channels bigger than mannitol (0.7 nm) and smaller than sucrose (0.9 nm). These results agree with the calcein-loaded liposome experiments, and suggest that calcein-loaded liposomes can be used for evaluating the channel size formed in membranes.

Table 1. ED₅₀ Values (Concentrations Giving 50% Calcein Release or Hemolysis; $\mu\text{g/ml}$ Unit) of Polyene Antibiotics on Calcein-Loaded Liposomes and Human Erythrocytes

	Liposomes	Erythrocytes
Amphotericin B	5—10	2
Nystatin	20—50	20—50
Filipin	5—10	2—5

Measured in a buffer solution containing 150 mM NaCl and 5 mM HEPES-NaOH (pH 7.4).

We further estimated the size of channels formed in liposomal membranes and erythrocytes induced by the polyene antibiotics nystatin and filipin. Figure 3 shows the percentage of calcein release and hemolysis at 100 $\mu\text{g/ml}$ antibiotics in the presence of sugars. This concentration is higher than the ED₅₀ value (concentration giving 50% release of calcein or hemolysis) for each antibiotic in physiological saline, as shown in Table 1. For comparison, the results from the amphotericin B experiments are also shown. For all the polyene antibiotics examined, there was a good correlation between the results from the artificial liposomal membrane and biological erythrocyte membrane experiments. Nystatin formed channels bigger than mannitol (0.7 nm) and smaller than sucrose (0.9 nm), while filipin formed channels larger than raffinose (1.1 nm) in both liposomes and erythrocytes. These results demonstrate that both the calcein release from liposomes and hemolysis in erythrocytes are induced by the same mechanism, and that calcein-loaded liposomes can be used to estimate channel size in liposomes. It should be pointed out, however, that the channel size exceeding the molecular size of calcein (molecular weight of 618.5 as a proton-dissociated form) cannot be determined by the present method.

While the experimental data presented here are limited, the findings demonstrate the analytical usefulness of calcein-

loaded liposomes for channel size determination. Unlike erythrocytes, the lipid compositions of liposomes can be easily modified and, therefore, this method can determine pore size in membranes with different lipid compositions. The present method is a new technique for liposome membrane permeability assays.

Acknowledgement We wish to thank Ms. Naomi Hanada, who carried out some of the preliminary experiments that were extended in this study.

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