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Simultaneous Measurements of K^+ and Calcein Release from Liposomes and the Determination of Pore Size Formed in a Membrane

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The changes induced by biologically active substances in the permeability to K^+ and calcein of liposomes composed of egg phosphatidylcholine and cholesterol were measured simultaneously in order to rapidly screen the sizes of pores formed in a membrane, using different sized markers. The substances examined in the present study were classified into three types based on differences in the rates at which K^+ and calcein were released. The first type released only K^+, and included gramicidin A. The second type predominantly released K^+, preceding the release of calcein, and included amphotericin B and nystatin. The third type, including antimicrobial peptides, such as gramicidin S, alamethicin, and melittin, and several membrane-active drugs, like celecoxib (non-steroidal anti-inflammatory drug), 1-dodecylazacycloheptan-2-one (named azone; skin permeation enhancer), and chlorpromazine (tranquilizer), caused the release of K^+ and calcein simultaneously. Thus, the sizes of pores formed in a liposomal membrane increased in the following order: types one, two, and three. We determined the size more precisely by conducting an osmotic protection experiment, measuring the release of calcein in the presence of osmotic protectants of different sizes. The radii of pores formed by the second type, amphotericin B and nystatin, were 0.36 – 0.46 nm, while the radii of pores formed by the third type were much larger, 0.63 – 0.67 nm or more. The permeability changes induced by substances of the third type are discussed in connection with a transient pore formed in a lipid packing mismatch taking place during the phase transition of dipalmitoylphosphatidylcholine liposomes.

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Introduction

Assays of the permeability of liposomes are fundamental for investigations of the interactions between biologically active substances and membranes.[1,2] Rapid changes in the permeability of liposomal membranes can be brought about by interactions with drugs, calcium-mediated fusion, or antibody/complement-mediated lysis of antigen-bearing liposomes. Among various markers so far developed, calcein has most widely been used for such measurements.[1,3-7] Calcein fluoresces very weakly at high concentrations because of self-quenching, but its fluorescence increases at lower concentrations as quenching is reduced. Thus, the calcein entrapped inside liposomes at high concentrations is weakly fluorescent, while the calcein that has leaked out of the liposomes is highly fluorescent. Calcein-loaded liposomes have been widely used for permeability assays, because liposomal leakage can be measured *in situ* without any separation of liposomes in an assay medium.[1,3-7]

In the present study, we were particularly interested in the combination of calcein and K^+ for permeability assays and attempted to monitor simultaneously the release of calcein and K^+ from liposomes to obtain information about the sizes of pores formed in membranes. Because K^+ leakage is the first event in the permeabilization of a membrane, measurements of differences in the rates of release of K^+ and calcein, which is larger than K^+, can provide significant information about the sizes of pores made by membrane-active substances. We tested various membrane-active substances; the channel-forming peptide gramicidin A,[8,9] polyene antibiotics such as amphotericin B, nystatin, and filipin,[8,10,11] antimicrobial peptides such as gramicidin S, alamethicin, and melittin,[8,9,12-14] and membrane-disrupting drugs such as celecoxib (non-steroidal anti-inflammatory drug),[6] 1-dodecylazacycloheptan-2-one (named azone; skin permeation enhancer),[15] and chlorpromazine (tranquilizer).[16,17] Although simultaneous monitoring of the different rates of K^+ and calcein release from liposomes provided significant information about the sizes of pores generated by these substances, we further determined the sizes more precisely by conducting an osmotic protection experiment.[3,18] This experiment is based on the fact that, if the solute added to the outer medium does not pass through pores formed in the liposomal membrane, the release of calcein will not be induced because the osmotic pressure of the inner calcein is balanced with that of the solute. Thus, the sizes of pores in the membrane can be evaluated by examining whether the solutes can protect against the release of calcein. We used a series of sugars as

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solutes and estimated the sizes of pores formed by the various membrane-disrupting substances described above. Furthermore, we determined the size of a pore formed transiently during the phase transition of dipalmitoylphosphatidylcholine (DPPC) liposomes to obtain further insight into the mechanism of increasing permeability induced by membrane-active substances.

Experimental

Reagents

The reagents were obtained from the following sources: calcein and sodium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate (NaTFPB) were from Dojindo Laboratories (Kumamoto, Japan); egg phosphatidylcholine (PC) was from Lipid Products (Red Hill, Surry, UK); cholesterol, DPPC, amphotericin B, nystatin, filipin (as filipin complex, approximately 75% by UV spectral analysis), gramicidin S, alamethicin, melittin (from bee venom, approximately 93% by high-performance liquid chromatography), chlorpromazine hydrochloride, and valinomycin were from Sigma (St. Louis, MO, USA); gramicidin A was from Calbiochem (La Jolla, CA, USA); celecoxib was from LKT Laboratories (St. Paul, MN, USA); azone was from Koei Chemical (Osaka, Japan); bis(2-ethylhexyl) sebacate was from Fluka (Buchas, Switzerland); poly(vinyl chloride) (PVC; degree of polymerization, 1020) was from Nacalai Tesque (Kyoto, Japan); D-mannitol, sucrose, and raffinose were from Wako (Osaka, Japan); and maltotriose, maltotetraose, and maltopentaose were from Hayashibara (Okayama, Japan). All other materials were of analytical reagent grade.

Electrode system

A K^+-selective electrode was constructed using a PVC-based membrane, as reported previously.[19,20] The PVC membrane had the following composition: 1 mg of valinomycin, 10 mol% of NaTFPB relative to valinomycin, 60 µl (55 mg) of bis(2-ethylhexyl) sebacate, and PVC (30 mg). The materials were dissolved in tetrahydrofuran (about 1 ml) and poured into a flat Petri dish (28 mm in diameter). The solvent was then evaporated off at room temperature. The resulting membrane was excised and attached to a PVC tube (4 mm o.d., 3 mm i.d.) with a tetrahydrofuran adhesive. The sensor membrane was conditioned overnight in a solution of 10 mM KCl. The electrochemical cell arrangement was Ag, AgCl/internal solution/ sensor membrane/sample solution/1 M NH_4NO_3 (salt bridge)/ 10 mM KCl/Ag, AgCl. The internal solution was the same as that used to condition the membrane. Potential measurements were made with a voltmeter produced by a field-effect transistor operational amplifier (LF356; National Semiconductor, Sunnyvale, CA, USA; input resistance $>10^{12}$ Ω) connected to a recorder. The potential values were converted to the percent release of K^+ using a previously reported equation.[21]

Preparation of liposomes

Liposomes were prepared using the reversed-phase evaporation method,[2,3,6,21] as follows. Aliquots of lipid stock solutions containing egg PC (10 µmol, 7.7 mg) and cholesterol (7.5 µmol, 2.9 mg) dissolved in chloroform/methanol (1:2, v/v) were placed in a centrifuge 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 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–KOH (pH 7.4). The mixture was ultrasonicated (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*g* for 15 min) and washed once with 150 mM NaCl and 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4) to remove any untrapped calcein potassium salt. The final pellet of liposomes was suspended in 5 ml of the above-mentioned 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

The permeability was assayed at 25°C as follows. To monitor the efflux of calcein and K^+ simultaneously, the K^+ and reference electrodes were immersed in a conventional quartz cell with a light path length of 1 cm for measuring the fluorescence and set to a cuvette holder of an Ocean Optics USB2000 miniature fiber-optic spectrometer (Dunedin, FL, USA). Fiber-optic cables were connected to conduct excitation and fluorescence, and Ocean Optics OOIBase32 software was used to process the data. Figure 1 shows a device for simultaneous measurements using potentiometry and fluorometry. An aliquot of liposomal suspension (30 µl) was diluted with 1.97 ml of the following assay solution: (a) 150 mM NaCl and 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4); (b) 180 mM mannitol, 50 mM NaCl, and 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4); (c) 170 mM sugar containing either sucrose, raffinose, maltotriose, maltotetraose or maltopentaose, 50 mM NaCl, and 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4). The concentration of mannitol was relatively high to make the osmotic pressure of all the solutions approximately 300 mOsm. The total volume of the liposomal suspension in the measuring cell was 2 ml, and the final phospholipid concentration was 30 µM. The suspension was constantly stirred with a stirrer bar. Subsequently, a small aliquot of a membrane-active substance was added, and incubation continued for several more minutes. Amphotericin B, nystatin, filipin, and celecoxib were dissolved in dimethyl sulfoxide; gramicidin A, gramicidin S, alamethicin, and azone were dissolved in ethanol; and melittin and chlorpromazine hydrochloride were dissolved in distilled water. The final concentrations of dimethyl sulfoxide or ethanol in assay media were less than 1% v/v. The release of calcein out of the liposomes was determined by measuring the total fluorescent intensity through a high-pass OF2-OG515 filter (pass > 515 nm; Ocean Optics) and using an USB-LS-450 LED light source module (Ocean Optics) having a maximum wavelength of 470 nm. The total amounts of calcein and K^+ in each liposome were determined after disrupting the liposomes by adding melittin (final concentration: 20 µM).[6,21] The following molecular radii of sugars were used:[22,23] mannitol, 0.36 nm; sucrose, 0.46 nm; raffinose, 0.57 nm. The apparent radii (R) of maltotriose, maltotetraose, and maltopentaose were calculated from the molecular weights (M) of the sugars using the following equation:[24]

$$R = R_{\text{raffinose}} \times \left(\frac{M}{M_{\text{raffinose}}} \right)^{1/3},$$

$R_{\text{raffinose}} = 0.57$ nm.[22,23]

The molecular radius was thus 0.57 nm for maltotriose, 0.63 nm for maltotetraose, and 0.67 nm for maltopentaose.

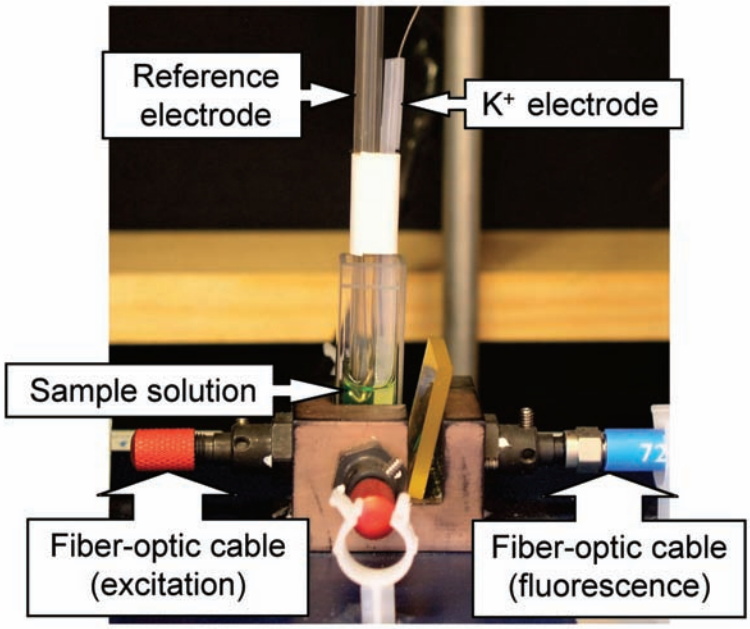

Fig. 1 Device for the simultaneous measurements of K^+ and calcein release from liposomes.

Determination of amount of calcein released from DPPC liposomes

Multilamellar liposomes composed of DPPC were prepared, using procedures similar to those described previously.[2] The dried thin film of DPPC (20 μmol, 14.7 mg) was swollen in 2 ml of 100 mM calcein–KOH (pH 7.4) at 55°C, centrifuged (22000*g* for 10 min), and washed once to remove untrapped marker. The final lipid pellet was suspended in 2 ml of 150 mM NaCl and 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4), and an aliquot (100 μl) of this liposomal suspension was diluted with 200 μl of required assay mixture to produce a final suspension of either (a) 180 mM mannitol, 50 mM NaCl, and 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4) or (b) 170 mM sugar containing sucrose, raffinose, maltotriose, maltotetraose or maltopentaose, 50 mM NaCl, and 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4), as in the case of an assay examining the membrane-active substances described above. Each liposomal suspension was incubated at 35°C or 45°C for 10 min, and then centrifuged at 22000*g* for 10 min. However, in some cases, especially a suspension containing maltotetraose and maltopentaose, liposomes did not sediment and floated on the solution, because of the high density of the solution containing these sugars. We carefully pipetted the transparent part of the solution (25 μl), diluted it with 975 μl of 150 mM NaCl and 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4), and determined the release of calcein from the liposomes by measuring the fluorescence intensity at 510 nm (excitation at 490 nm) on a Hitachi F-4500 fluorescence spectrophotometer. The fluorescence intensity corresponding to 100% release was determined by treating the liposomes ultrasonically at 60°C for 1 min using a Sibata SU-3T ultrasonic cleaner (Tokyo, Japan).

Results and Discussion

Simultaneous measurements of K^+ and calcein release

In order to directly confirm whether the combination of the K^+ electrode and fluorometry using calcein can give useful information about liposomal membrane permeability measurements, we monitored the time response of K^+ and calcein release simultaneously in a solution containing 150 mM NaCl and 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4). We tested three typical substances: gramicidin A, amphotericin B, and gramicidin S. Gramicidin A is known to make channels specific for monovalent cations,[8,9] and amphotericin B forms a complex with cholesterol, making larger size pores in the membrane than gramicidin A.[8,10,11] Gramicidin S perturbs lipid packing, resulting in membrane permeabilization,[25] whose pore size is reported to be larger than that of amphotericin B.[18] Such differences in the sizes of pores were expected to significantly influence the time response of K^+ and calcein release. In the present experiments, we added the membrane-active substances stepwise to obtain dose-response characteristics from one experiment and, finally, melittin (20 μM) was added to determine the total amounts (100% level) of markers trapped in liposomes, as shown in Fig. 2. Equivalent amounts obtained after the addition of melittin (20 μM) and a treatment with chloroform/methanol (1:2, v/v) demonstrated that melittin caused the complete release of both markers. Gramicidin A induced only the release of K^+, demonstrating that this peptide inhibited the passage of a large molecule, calcein, forming an ion-channel passing K^+. Amphotericin B caused the release of K^+, preceding the release of calcein, while gramicidin S gave similar profiles of K^+ and calcein release, which was consistent with a previous result showing that gramicidin S formed a larger size of pore than amphotericin B.[18] Thus, the relative sizes of the pores formed in liposomal membranes can be easily compared from these experiments. It should be pointed out that the dose-response behavior of membrane-active substances can also be estimated approximately in the present assay. Figure 3 shows dose-response curves of amphotericin B and gramicidin S based on the response profiles shown in Fig. 2.

Determination of the sizes of pores

We then used the calcein-loaded liposomes to determine the sizes of pores formed in the liposomal membrane. This experiment is similar to an osmotic protection experiment using

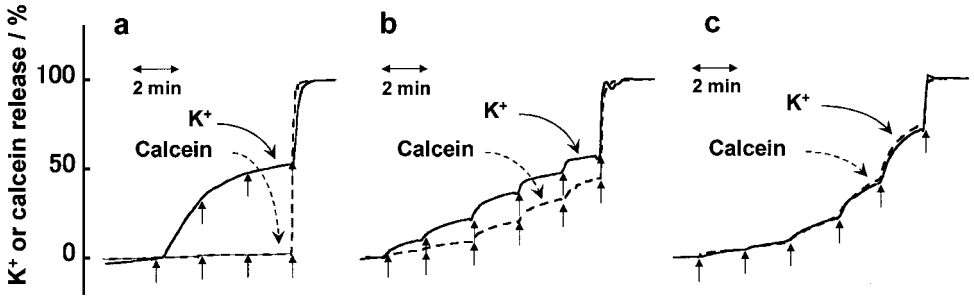

Fig. 2 K^+ and calcein release from liposomes composed of egg PC and cholesterol induced by gramicidin A (a), amphotericin B (b), and gramicidin S (c). Liposomes were suspended in a solution containing 150 mM NaCl and 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4). The solid and dashed lines indicate traces for K^+ and calcein release, respectively. The biologically active substances were added stepwise, and the arrows indicate when. In each chart, the final arrow shows when melittin was added at a final concentration of 20 μM to induce changes in the permeability of liposomes completely. The first, second, and third arrows of (a) indicate the times of the addition of gramicidin A at final concentrations of 0.005, 0.01, and 0.02 μM, respectively. The first, second, third, fourth, and fifth arrows of (b) indicate the times of the addition of amphotericin B at final concentrations of 1, 2, 5, 10, and 20 μg ml^{-1}, respectively, while those of (c) indicate the times of the addition of gramicidin S at final concentrations of 0.1, 0.2, 0.5, 1, and 2 μM, respectively.

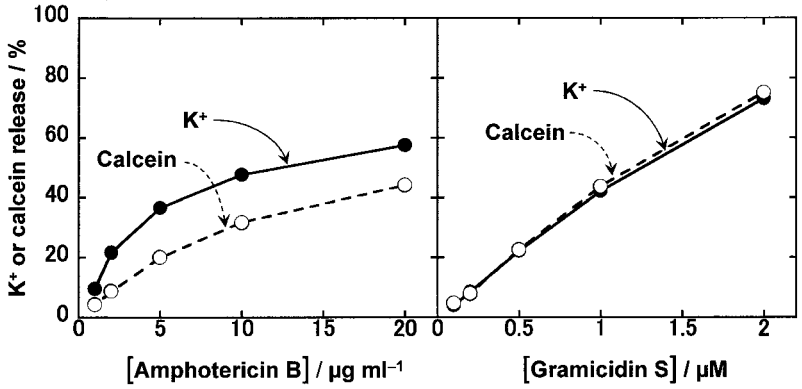

Fig. 3 Dose-response curves of K^+ and calcein release induced by amphotericin B and gramicidin S.

erythrocytes to measure the release of hemoglobin (so-called hemolysis) in the presence of a protectant.[3,18] In the erythrocyte's experiment, if the solute added to the outer medium does not pass through a channel formed in the erythrocyte's membrane, hemolysis will not be induced because the osmotic pressure of the intracellular hemoglobin is balanced by that of the solute. Thus, the size of the pore in the membrane can be evaluated by examining whether the substance added can protect against hemolysis. We applied this principle to the present liposomes and tried to determine the sizes of pores formed in liposomal membranes by amphotericin B and gramicidin S. We performed this experiment at the concentrations giving 50% calcein release, estimated from the dose-response curves shown in Fig. 3, which for amphotericin B and gramicidin S were around 20 μg ml^{-1} and 1 μM, respectively. We incubated liposomes with these substances for 5 min, because the release occurred rapidly, as shown in Fig. 2. Figure 4 shows the release of calcein induced by amphotericin B and gramicidin S as a function of the molecular radii of sugars. Amphotericin completely suppressed the release of calcein in media containing sucrose or raffinose, indicating that amphotericin B made a larger pore than mannitol (0.36 nm) and a smaller one than sucrose (0.46 nm). This result was consistent with the size of pores formed in the membranes of erythrocytes reported previously.[3,26] The protection of calcein release by gramicidin S was observed only when maltopentaose was added to the assay medium, indicating that the radius of pores was between that of maltotetraose (0.63 nm) and maltopentaose (0.67 nm). Such large pores were also observed in erythrocyte membranes.[18] These results demonstrate that the calcein-loaded liposomes can be used to estimate the size of pores formed in liposomes. It should be pointed out that the use of the present osmotic protection method is restricted to measurements of pores smaller than the molecular size of calcein (near to maltopentaose in this study). When pores exceeding the size of calcein are formed in membranes, calcein release takes place irrespective of osmotic lysis.

Application of the method using various membrane-active substances

The same experiments were performed using various membrane-active substances: polyene antibiotics (nystatin and filipin), antimicrobial peptides (alamethicin and melittin), a non-steroidal anti-inflammatory drug (celecoxib), a skin permeation enhancer (azone), and a tranquilizer (chlorpromazine). Table 1 summarizes ED_{50} values

Table 1 ED_{50} values (concentrations giving 50% K^+ or calcein release) of membrane-active substances and the sizes of pores formed in liposomes composed of egg PC/cholesterol

Type[a]	Substance	ED_{50}/μM or μg ml^{-1} [b]		Pore size/nm[c]
		K^+	Calcein	
1	Gramicidin A	0.01	> 0.2	~0.25
2	Amphotericin B	10	20	0.36 – 0.46
	Nystatin	20	50	0.36 – 0.46
3	Filipin	10	10	> 0.67
	Gramicidin S	1	1	0.63 – 0.67
	Alamethicin	10	10	> 0.67
	Melittin	0.01	0.01	0.63 – 0.67
	Celecoxib	50	50	> 0.67
	Azone	500	500	> 0.67
	Chlorpromazine	100	100	> 0.67

a. The substances are classified into three types according to differences in ED_{50} values of K^+ and calcein release as described in the text.
b. ED_{50} values were expressed in μM, except for those of polyene antibiotics (amphotericin B, nystatin, and filipin) which were expressed in μg ml^{-1}.
c. Pore sizes formed in liposomes were expressed as radii. These were determined at ED_{50} values for calcein release, except for that of gramicidin A which did not induce any calcein release up to a concentration of 0.2 μM. The pore size of gramicidin A was estimated as around the hydrated ion radius of K^+ (0.25 nm).[27]

(concentrations giving 50% K^+ or calcein release) for these substances and the sizes of pores formed in liposomes, along with those for gramicidin A, amphotericin B, and gramicidin S discussed above. As was expected, substances giving the same ED_{50} values for K^+ and calcein release (defined as type 3 in Table 1) made larger pores in the membrane. The pore sizes observed with polyene antibiotics were similar to those for erythrocytes reported previously;[3,26] that is, amphotericin B and nystatin, so-called "large polyenes",[26] formed smaller pores (type 2 in Table 1), while filipin, a so-called "small polyene",[26] made larger pores. Amphiphilic peptides, such as gramicidin S, alamethicin, and melittin, formed large pores in liposomal membranes. Although alamethicin is known to form relatively small voltage-gated ion-channels in planar lipid membranes,[8,9,14] it formed large pores in the present liposomal membrane. Melittin is known to form large pores in erythrocyte membranes,[18] consistent with this experiment. Other membrane-active drugs, azone and chlorpromazine, afforded large pores in liposomal membranes, which again supported the results of the osmotic protection experiment using human erythrocytes.[15,17]

The size of transient pores formed during the phase transition of liposomes

Then, we were interested in the mode of action of substances forming large pores. These substances are known to perturb lipid packing markedly,[6,10-15,28-31] causing a mismatch in stable lipid packing, leading to most probably transient pores in the membrane.[14] Such a mismatch in lipid packing is also observed at the interface of the fluid and ordered domain during the phase transition of phospholipids.[32,33] Thus, we determined the size of transient pores formed during the phase transition of DPPC liposomes, in order to obtain further insight into the mechanism of increases in the permeability of liposomal membrane induced by the substances defined as type 3. We measured the release of calcein in the presence of osmotic protectants below and

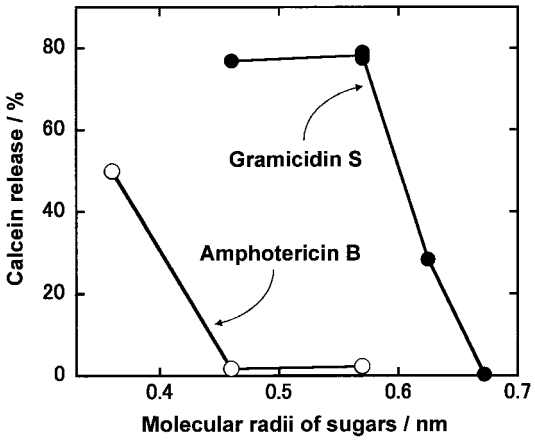

Fig. 4 Calcein release induced by amphotericin B and gramicidin S as a function of the molecular radii of sugars. The following radii were used: mannitol, 0.36 nm; sucrose, 0.46 nm; raffinose, 0.57 nm; maltotriose, 0.57 nm; maltotetraose, 0.63 nm; and maltopentaose, 0.67 nm.

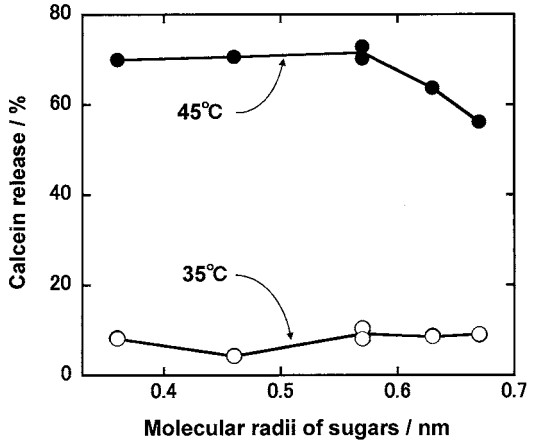

Fig. 5 Calcein release from DPPC liposomes induced with increases in temperatures (35 or 45°C) as a function of the molecular radii of sugars. The two temperatures corresponded to below and above the phase-transition temperature of DPPC liposomes (41.5°C). The molecular radii of sugars are shown in Fig. 4.

above the phase-transition temperature (41.5°C) of DPPC liposomes. In this experiment, the temperature was raised from room temperature (about 25°C) to 35 or 45°C, and thus the liposomes passed the phase transition, causing a packing mismatch in the case of 45°C. As shown in Fig. 5, the release of calcein at 35°C below the phase-transition temperature was not significant, while the case at 45°C above the phase-transition temperature was remarkable. Moreover, the release of calcein was observed even in the presence of maltopentaose, indicating that the transient pores produced during the phase transition had a radius above 0.67 nm. Such a large size was consistent with a previous result showing that the pores formed at the phase transition permitted the passage of inulin (molecular radius, 0.70 nm[23]).[34] These results support the view that the changes in permeability induced by substances of type 3 (giving large pores at ED_{50} values for calcein release) are attributable to their ability to perturb lipid packing, making an unstable domain, like a packing mismatch.

Conclusions

Simultaneous measurements of K^+ and calcein release from liposomes enabled us to predict the sizes of pores formed in membranes. These sizes could more precisely be determined by measuring the amount of calcein released in the presence of protectants of different sizes. The present method was used to analyze the sizes of pores formed by various membrane-active substances, and also those made during the phase transition of DPPC liposomes.

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