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Membrane permeabilizing activity of amphotericin B is affected by chain length of phosphatidylcholine added as minor constituent

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Abstract

The effect of acyl-chain length of phospholipid on the membrane permeabilizing activity of amphotericin B (AmB) was examined using egg phosphatidylcholine (eggPC) liposomes containing 5% or 20% phosphatidylcholine with various lengths of fatty acyl chains from C_{10} to C_{18}; 1,2-dicapryloyl-*sn*-glycero-3-phosphocholine (DCPC), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC). The membrane activity of AmB was evaluated by two methods; the drug was added to a liposome suspension (added-via-aqua), or mixed with lipids prior to liposome preparation (mixed-with-lipid). In both cases, K^+ influx by AmB was measured as pH change inside liposomes by ^{31}P-NMR. The C_{10} and C_{12} acyl phospholipids markedly enhanced the activity of AmB, the C_{14} and C_{16} lipids virtually showed no effect, and the C_{18} lipid was inhibitory to the AmB's action. Clear distinction between the C_{12} and C_{14} lipids, which differ only in acyl chains by two carbons, implies that molecular interaction between phospholipid and AmB is partly due to the matching of their hydrophobic length.

Keywords: Amphotericin B; Phosphatidylcholine; Ion channel; Hydrophobic length; Chain length

1. Introduction

Membrane-active polyene macrolides form a category of clinically important agents, which show a broad antibiotic spectrum against fungi and other eucaryotic microbes [1–3]. Amphotericin B (AmB), which is one of the best known drugs among these antibiotics, reveals its selective toxicity by forming ion-permeable channels across fungal plasma membrane in a sterol-specific manner. Besides the sterol effects, the drug's activity is reported to be greatly influenced by thickness of membrane [4,5]; more recently, Ruckwardt et al. [6] reported that the activity of AmB is markedly attenuated in the membrane comprising C_{20}-acyl phospholipids. In these studies, membranes consisting of single PC constituent were usually used, so the physicochemical effects of acyl-chain length such as the membrane thickness versus length of AmB channels were more emphasized. However, growing evidence has been presented for the molecular interaction between membrane phospholipid and AmB [7–11].

In this report, we utilized eggPC liposomes containing 5% or 20% PC bearing the different length of saturated acyl chains, and measured the membrane permeabilizing activity of AmB by two different methods; in added-via-aqua experiments, AmB dissolved in dimethylsulfoxide (DMSO) was added to a suspension of liposomes as routinely used in previous studies, and in mixed-with-lipid experiments, AmB was mixed with phospholipids prior to liposome preparation, by which the AmB–PC interaction in membrane could be evaluated without the influence of the membrane binding process [12].

Abbreviations: AmB, amphotericin B; LUV, large unilamellar vesicle; eggPC, egg yolk phosphatidylcholine; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DCPC, 1,2-dicapryloyl-*sn*-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMSO, dimethylsulfoxide; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; EDTA, *N*,*N*,*N*′,*N*′-ethylenediaminetetraacetic acid; FCCP, carbonyl cyanid-*p*-trifluoro-methoxyphenyl hydrazone; NMR, nuclear magnetic resonance; UV, ultraviolet-visible

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2. Materials and methods

2.1. Materials

AmB, egg yolk phosphatidylcholine (eggPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dicapryloyl-*sn*-glycero-3-phosphocholine (DCPC) were from Avanti Polar Lipid Inc. (Alabaster, AL), and 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) were from ICN biomedicals Inc. (Aurora, OH). FCCP (H^+ carrier) were obtained from Nacalai Tesque (Kyoto, Japan). Polycarbonate filters were from Nuclepore (Pleasanton, CA).

2.2. Preparation of large unilamellar vesicles (LUVs) with or without AmB

Phospholipids (4 mM) and AmB (0.1 mM) were dissolved in chloroform and methanol, respectively, to prepare stock solutions. A series of liposomes were prepared by adding aliquots of eggPC and each phospholipid in the stock solutions into round-bottom glass tubes. Then, an AmB methanol solution was added to the tubes for the mixed-with-lipid AmB experiments. The solvent was evaporated to form lipid films at the bottom of tubes. Tubes were then left under vacuum for 6 h to remove the solvent completely. Lipid films were hydrated with phosphate buffer (0.4 M potassium phosphate and 1 mM EDTA dissolved in 40% D_2O at pH 5.5) by sonication, vortex mixing, and subsequently three times frozen/thawed to yield large vesicles. After the sizing of the liposomes using Liposofast® by filtering 19 times through a polycarbonate filter of 200-nm pore size [13], the resultant LUVs were then diluted four times in 0.4 M potassium sulfate. The final concentration of lipids was determined to be 19 mM using an assay kit (Phospholipid Test Wako, Wako Pure Chemical Industries).

2.3. Measurement of membrane permeabilizing activity

Membrane permeabilizing activity of AmB was measured as K^+ influx in various PC-containing liposomes with a proton–cation exchange method based on ^{31}P-NMR chemical shifts as reported by Herve et al. [14]. Briefly, FCCP dissolved in ethanol (1 mM) was added to a liposome suspension (0.4% v/v), which was then adjusted to pH 7.5 with potassium hydroxide. Then AmB in a DMSO solution (10 mM) was added to the LUV prepared without AmB for the added-via-aqua experiments. After each incubation period, 550 μl of the suspension was transferred into a NMR tube and then added with a 100 mM $MnCl_2$ solution (4.4 μl) to quench the ^{31}P-NMR signal due to phosphate outside of liposomes. ^{31}P-NMR was recorded at room temperature in the resonance frequency of 202 MHz with ^{1}H decoupling on a JNM GSX-spectrometer (JEOL, Akishima, Japan).

2.4. UV spectroscopy

UV spectra were measured using a UV-2500 spectrophotometer (Shimadzu, Kyoto, Japan) with a 1.0-cm pathlength quartz cell at room temperature. All data were recorded over the wavelength range 300–440 nm. To remove the light scattering by LUV, all spectra were corrected by subtracting the spectra of AmB-free liposomes from those of AmB-containing liposomes. Samples were prepared in the same way as that for NMR measurements except buffer and sample concentrations. Since the scattering by 19 mM LUV in the phosphate buffer was strong over the wavelength range, we adopted 4.8 mM LUV in 0.9% sucrose buffer. AmB was added at the AmB/lipid ratio of 0.0003 in both added-via-aqua and mixed-with-lipid experiments.

3. Results

3.1. Added-via-aqua experiments

Fig. 1 showed the results of the added-via-aqua experiments at the AmB/lipid molar ratio of 0.0001. In the ^{31}P-NMR spectra for LUV comprising 20% PC tested and 80% eggPC, the marked potentiation of AmB-induced membrane permeabilization was observed for DCPC (C_{10}) and DLPC (C_{12}) (Fig. 1a and b), in which a mode of ion flux belongs to an "all-or-none" type as depicted by two peaks at 1.2 and 3.1 ppm; the signal at 1.2 ppm was derived from intact liposomes while that at 3.1 ppm was from fully permeabilized ones. These effects were also observed for liposomes containing 5% DCPC or 5% DLPC (Fig. 1a′ and b′). The broad peaks between 1.2 and 3.1 ppm as seen for Fig. 1c–f were due to liposomes in which influx of K^+ did not reach equilibrium. In contrast to DCPC and DLPC, addition of 20% DMPC (C_{14}) and 20% DPPC (C_{16}) to eggPC liposomes did not induce any significant changes (Fig. 1c and d). When 20% DSPC (C_{18}) was added (Fig. 1e), a broad peak due to gradually permeabilized liposomes was shifted up-field, indicating that AmB's membrane activity was partly inhibited by DSPC.

3.2. Mixed-with-lipid experiments

Fig. 2 showed the results of mixed-with-lipid experiments for LUV with the AmB/lipid molar ratio of 0.0001. In these experiments, 20% DCPC (C_{10}) and 20% DLPC (C_{12}) again enhanced the activity of AmB. The similar tendency to those in the added-via-aqua experiments is seen with respect to the chain lengths of PCs, including the inhibitory action by DSPC (C_{18}),

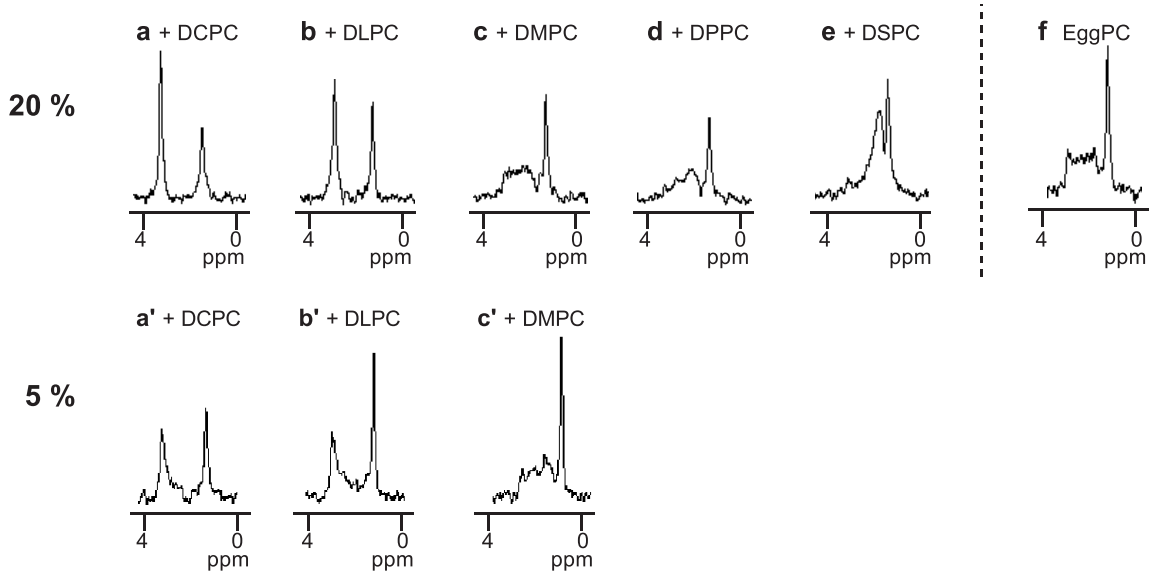

Fig. 1. ^{31}P-NMR signals of liposome-entrapped phosphate in LUV consisting of eggPC and saturated PC bearing C_{10}–C_{18} acyl chains (from a to e) in added-via-aqua experiments. Signals of external phosphate and phospholipids disappeared due to addition of Mn^{2+}. The liposomes were incubated for 6 h at 25 °C under a pH gradient across the membrane: internal pH = 5.5, external pH = 7.5. The concentrations of total lipids were 1.9 mM with the AmB/PC ratio of 0.0001. Liposomes for spectra a–e consist of 80% eggPC and 20% saturated PC as indicated. f is a spectrum of pure eggPC liposomes. Those for spectra a′–c′ contain 95% eggPC and 5% saturated PC.

although the mode of membrane permeabilization is clearly different. Gradual ion flux, rather than the all-or-none type, was observed in these experiments as one can see from the lack of the clear two peaks in the spectra. The same experiments were carried out with more concentrated AmB at the lipid/AmB molar ratio of 0.0003, and essentially similar results to those in Fig. 2 were obtained (data not shown). The leakage of ions in the absence of AmB was insignificant either in the added-via-aqua experiments or in the mixed-with-lipid experiments, and the extent of the leakage was not influenced by chain length of PCs tested.

3.3. UV spectroscopy

The UV spectra of AmB in LUV containing 20% PC with various acyl-chain lengths were shown in Fig. 3. A dotted curve in Fig. 3A denotes the UV spectrum of an aqueous suspension of AmB (1.4 μM in sucrose buffer) without liposomes, which gives rise to the absorption maxima characteristic of AmB at 409, 385 and 364 nm. In the spectra of AmB in eggPC LUV and those containing 20% PC bearing C_{10}, C_{12}, C_{14}, C_{16} or C_{18} acyl group, the absorptions of these triplet peaks were red-shifted to 413, 387 and 368 nm with somewhat stronger absorbance (Fig. 3B). Their λ_{max} were the same as those in eggPC-only LUV (Fig. 3A) while their ε values were markedly varied with their acyl-chain lengths. This propensity was observed both in the added-via-aqua and mixed-with-lipid experiments (Fig. 3B and C). The spectra of AmB for DMPC, DPPC, and eggPC-only LUVs in Fig. 3C gave rise to the weak triplet peaks but a prominent band at 330–340 nm. In comparison, the spectra of AmB for 20% DCPC (C_{10}), DLPC (C_{12}) and DSPC (C_{18}) showed the larger

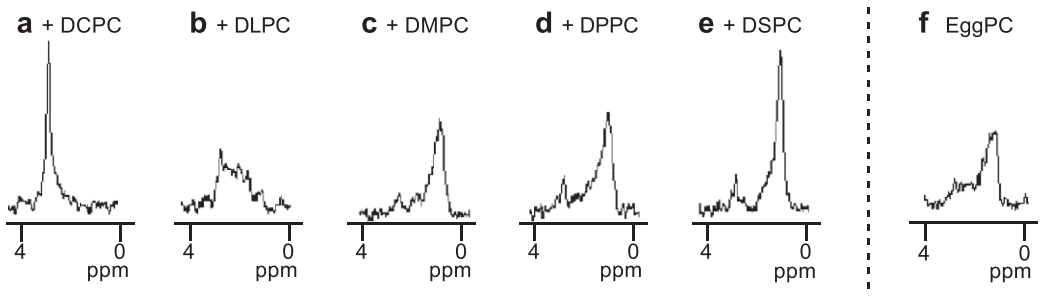

Fig. 2. ^{31}P-NMR signals of liposome-entrapped phosphate in LUV consisting of eggPC and saturated PC bearing C_{10}–C_{18} acyl chains (from a to e) in mixed-with-lipid experiments. The concentrations of total lipids were 1.9 mM with the AmB/PC ratio of 0.0001. Liposomes for spectra a–e consist of 80% eggPC and 20% saturated PC as indicated. f is a spectrum of pure eggPC liposomes.

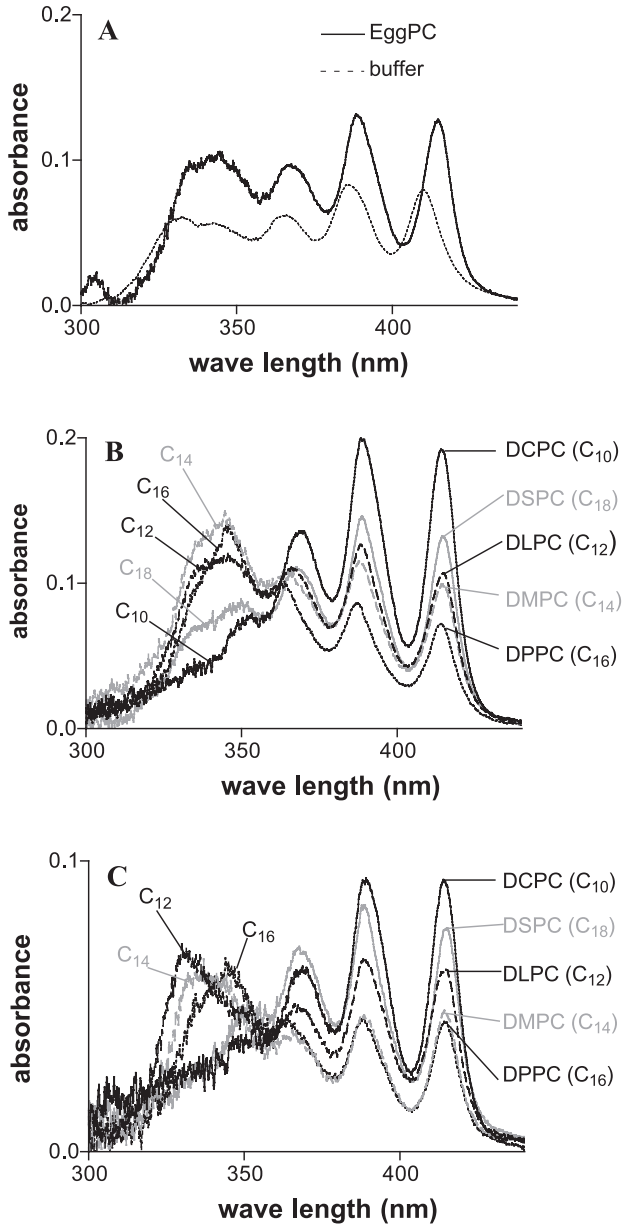

Fig. 3. UV spectra of AmB in LUVs consisting of eggPC and saturated PC bearing C_{10}–C_{18} acyl chains. (A) AmB suspension in the sucrose buffer, and eggPC-only LUVs in the added-via-aqua experiment. (B) LUVs containing 20% PCs with various acyl-chain lengths in the added-via-aqua experiments. (C) LUVs containing 20% PCs with various acyl-chain lengths in the mixed-with-lipid experiments. The UV spectrum of AmB for eggPC-only LUVs by this method is almost superimposable on that of DMPC-containing LUV, and omitted for clarity. The concentrations of total lipids were 4.8 mM with the AmB/lipid ratio of 0.0003.

triplet peaks, and those for DCPC and DSPC lack the distinctive UV maxima at 330–340 nm (Fig. 3C).

4. Discussion

This study disclosed the marked stimulations of AmB's action by DCPC and DLPC even as a minor constituent in liposomes. Moreover, their ion flux belongs to a typical all-or-none type in the added-via-aqua experiments. The large K^+ influx appears to be due to a stable channel complex, which continuously elicits an ion flow (equal to the H^+ counter-flow in these experiments) to reach equilibrium, leaving only fully permeabilized liposomes and intact ones. Such a stable assemblage has been thought to occur only in the presence of membrane sterols [15]. The present study demonstrated that the AmB channels could be stabilized with short-chain PC. It is noteworthy that DMPC, which is practically devoid of any effects even at 20%, possesses only two carbons longer chains than DLPC, which induces all-or-none flux even at 5% addition. These marked differences with a minute structural change imply the presence of a certain interaction between fatty acyl chains and AmB upon channel formation, since it is not likely that 5% addition of a similar PC in eggPC liposomes greatly alters the membrane thickness, which has been thought as the major membrane property controlling the AmB's activity.

The hydrophobic lengths of DCPC and DLPC in the fluid-phase membrane are shorter than that of POPC, a major constituent of eggPC (Table 1) [16–20]. The length of the hydrophobic polyene part of AmB was 22 Å [5], longer than that of DCPC or DLPC but shorter than POPC (Table 1). According to "mattress model" proposed by Mouritsen and Bloom [21], membrane lipids surrounding AmB shrink to fit to the AmB assemblage (Fig. 4A). In contrast, DCPC and DLPC need not reduce their lengths to allow the AmB complex to span across the membrane (Fig. 4B). The higher affinity between these saturated PCs and AmB may stabilize the ion channels with only 5% content of the lipid. These lipid–AmB interactions are thought to be effected by AmB's recruiting matched PC from the membrane since these PCs in the fluid phase are miscible [22].

The inhibition of the AmB's action by DSPC was observed for the both experiments (Figs. 1 and 2). In the added-via-aqua method, the broad signal in Fig. 1e was observed near a peak at 1.2 ppm, which indicated that a gradual type of K^+ influx in liposomes by AmB was significantly attenuated. In the mixed-with-lipid method in Fig. 2, DSPC further exhibited the inhibition of the AmB-induced flux as seen for the larger peak at 1.2 ppm. The membrane-permeabilizing activity of AmB in these experi-

Table 1
Lengths of hydrophobic region of bilayer membranes consisting of POPC and saturated phosphatidylcholines

PC	Phase at 25 °C	Length of hydrophobic region (Å)	
		Fluid phase (f)	Gel phase (g)
POPC (C_{16}, $C_{18:1}$)	f	25.8[a]	–
DCPC ($2 \times C_{10}$)	f	15.5[b]	–
DLPC ($2 \times C_{12}$)	f	19.5[b]	27[c]
DMPC ($2 \times C_{14}$)	f	23[b]	31.5[d, e]
DPPC ($2 \times C_{16}$)	g	26[b]	36[d]
DSPC ($2 \times C_{18}$)	g	29.5[b]	40.5[c]

[a–e]: Data are cited from Refs. [16–20].

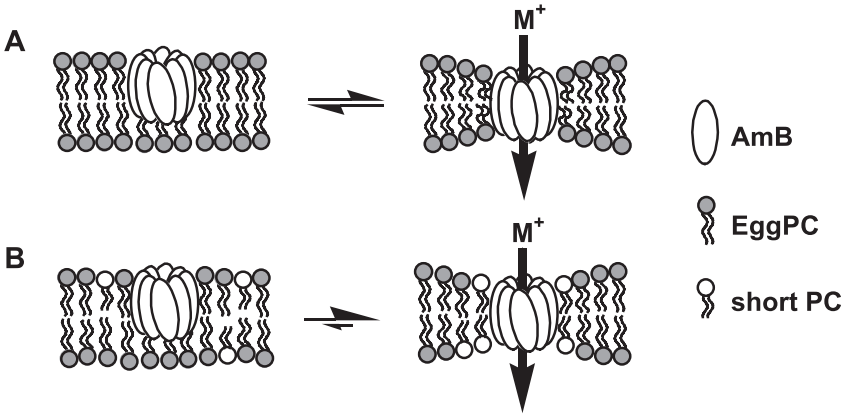

Fig. 4. Schematic model for open/close flickers of AmB channel.

ments was plotted against the number of carbon atoms in an acyl group in Fig. 5. The plot clearly demonstrates the inhibitory action of C_{18} as well as the stimulatory effect of short-chain acyl groups C_{10} and C_{12}. When the ^{31}P-NMR experiments were carried out with 5% DSPC in eggPC, no significant inhibition was observed (data not shown). DSPC should be in the gel phase and undergo phase separation from eggPC at room temperature [22]. Compared with the stimulatory effects by DCPC and DLPC, the inhibition by DSPC requires higher concentrations. This may imply that microdomains formed by DSPC efficiently extract AmB from the surrounding eggPC domains. Although DPPC is also known to form the gel phase, no significant inhibitory effects were observed (Fig. 5). The minute changes in the membrane thickness may influence the AmB's activity, but no experimental evidence is available to account for the difference between DPPS and DSPC.

In the mixed-with-lipid experiments, the all-or-none type of K^+ flux was not observed for PCs tested. This may be explained by the following speculations: AmB is known to form an aggregate in aqueous media. Upon addition through

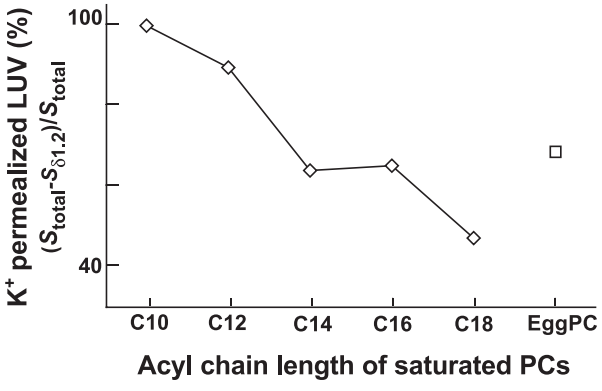

Fig. 5. Relation between K^+ influx activity of AmB and acyl-chain length of saturated PC in mixed-with-lipid experiments (20% PC tested and 80% eggPC, AmB/lipid = 0.0001). The activity of AmB is represented by the percentage of permeabilized liposomes (LUV) including those in the process of gradual influx: S_{total}, integration of the area between 1.2 to 3.1 ppm; $S_{\delta 1.2}$, integration of the peak at 1.2ppm.

an aqueous phase, aggregates of AmB bind to membrane [23] and distribute unevenly among liposomes: some of those containing more AmBs are fully permeabilized while the others with fewer AmBs are not. Another possible account is that the channel assemblages formed in water by the added-via-aqua method are different from those in lipids by the mixed-with-lipid method; e.g. double-sided channels, which may occur in the latter method, have smaller ion conduction [4,24], resulting in a gradual type of ion flux. The double-sided channels may play a certain roll in the mixed-with-lipid experiments but the single channels, as described above, are presumably responsible for the ion flux because the hydrophobic length of the double-sided channel, approximately 40 Å, which is much longer than the thickness of eggPC bilayers, should reduce their stability in the membranes.

The UV spectra in Fig. 3 allow us to discuss more in details the interactions between AmB/AmB and AmB/lipids. The red shifts induced by AmB's binding to membrane are ascribable to a lipophilic environment surrounding the heptaene chromophore [25,26]. The triplet peaks of AmB in all the LUV preparations were red-shifted from that in the aqueous suspension, hence implying that under these conditions (1.4 μM AmB at the AmB/lipid ratio of 0.0003), AmB mostly binds to membrane. The UV bands near 340 nm as well as the attenuated triplet peaks at longer wavelength are presumably derived from the close interaction between the chromophores, which may not be due to channel assemblages comprising around eight molecules of AmB but due to large clusters involving much more AmB molecules [26,27]; the heptaene chromophores of AmB in the aggregate are thought to reside in close vicinity (< 6 Å), which leads to appearance of the 340-nm band and the reduction of the triplet peaks, while in the channel assemblages the inter-chromophore distance can be estimated as more than 8 Å based on molecular dynamics simulations [28], resulting in larger absorptions for the triplet peaks than those in the aggregates. This is further supported by the observations that the ε value at 413 nm increases in parallel with the stimulatory effects by PCs on the AmB's

actions (Figs. 1 and 3), except for the DSPC-containing LUV.

The higher triplet peaks are observed for the shortest C_{10}-acyl DCPC and the longest C_{18}-acyl DSPC, whereas their absorptions near 340 nm are obscure (Fig. 3B). These spectral features suggest that addition of DCPC or DSPC hampers formation of AmB aggregates in membrane and stabilizes channel assemblages, which may possess the similar structures judging from their resemblance in UV spectra. In the DCPC-containing LUV, thickness of the membrane is 15.5 Å for DCPC bilayer and 20.7 Å for DCPC/eggPC bilayer (see Table 1), both of which are somewhat shorter than the length of the hydrophobic part of AmB (22 Å). These thinner bilayers may stabilize AmB's channel assemblage and facilitate the channel to span across the membrane, thereby leading to large ion flux (Fig. 4). Similar phenomena have been reported for membrane-active peptides; their α-helix binds more stably to the membrane bearing an equal or shorter hydrophobic length than that of the peptide [29].

In contrast to DCPC, the DSPC-containing membrane, in which AmB also shows no clear UV band at 340 nm, inhibits the AmB's action. The hydrophobic length of DSPC in a single layer is 20.3 Å in the gel phase (Table 1). The microdomain of DSPC at room temperature is thought to have enough thickness to accommodate AmB or its assemblages within a single layer. The hydrophobic matching between the DSPC single layer and the heptaene side of AmB may hold AmB in the microdomain and prevent formation of a large aggregate, resulting in a loss of the UV peak near 340 nm. However, the AmB assemblage falls short of reaching the other side of the DSPC-containing membrane, which results in the weak membrane activity.

To our knowledge, the present study first reveals that a small difference in the length of acyl chains of PC, even as a minor constituent, greatly influences the membrane-permeabilizing activity of AmB. The UV spectra further demonstrate that AmB–AmB interaction is heavily dependent on the chain lengths of PC. The results imply that the AmB–PC interaction in membrane, presumably based on hydrophobic matching between AmB and acyl chains of PC, may play an important role in the biological or pharmacological actions of AmB. Further studies on the mode of interactions between AmB and membrane lipids are currently under way.

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