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Biochimica et Biophysica Acta 1564 (2002) 429–434

BIOCHIMICA ET BIOPHYSICA ACTA
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Cholesterol markedly reduces ion permeability induced by membrane-bound amphotericin B

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Received 8 January 2002; received in revised form 28 May 2002; accepted 13 June 2002

Abstract

It is widely accepted that amphotericin B (AmB) together with sterol makes a mixed molecular assemblage in phospholipid membrane. By adding AmB to lipids prior to preparation of large unilamellar vesicles (LUV), we directly measured the effect of cholesterol on assemblage formation by AmB without a step of drug's binding to phospholipid bilayers. Potassium ion flux assays based on ^{31}P-nuclear magnetic resonance (NMR) clearly demonstrated that cholesterol markedly inhibits ion permeability induced by membrane-bound AmB. This could be accounted for by a membrane-thickening effect of cholesterol since AmB actions are known to be markedly affected by the thickness of membrane. Upon addition of AmB to an LUV suspension, the ion flux gradually increased with increasing molar ratios of cholesterol up to 20 mol%. These biphasic effects of cholesterol could be accounted for, at least in part, by the ordering effect of cholesterol.

Keywords: Amphotericin B; Cholesterol; Ion channel; ^{31}P-NMR

1. Introduction

Membrane-active polyene macrolides form a category of clinically important antifungal agents, which have a broad spectrum against fungi and other prokaryotic microbes. Among those, amphotericin B (AmB) has been the drug of choice for serious systemic infections for over 30 years [1,2].

It has been revealed that AmB forms ion-permeable channels across fungal plasma membrane and subsequently leads to cell death, where sterols are thought to play an important role. The selective toxicity of AmB is derived, at least in part, from its greater affinity to ergosterol over cholesterol. In 1970s, a well-known barrel–stave model was proposed for the AmB channel assemblage; a barrel–stave complex is thought to comprise about eight pairs of AmB and sterol, in which sterols are expected to act as glue for stabilizing a channel assemblage [3,4]. This idea has attracted scientists' attention and made great contributions in accelerating mode-of-action studies for membrane-bound peptides or natural products. Recently, AmB turned out to form channels without sterols under certain conditions such as higher concentrations of AmB, osmotic gradient, and gel phase membranes [5–14]. Therefore, the channel structures comprising oligomeric pairs of AmB/sterol have been a subject of controversy; particularly, cholesterol has never shown to have a direct interaction with AmB in lipid bilayers, which should be prerequisite for the barrel–stave model. For addressing these complicated problems, Bolard et al. [15] have proposed a comprehensive model for AmB's action; in ergosterol-containing plasmamembrane, AmB forms transmembrane ion channels with the sterol while in cholesterol-containing membrane AmB does not. Since an AmB molecule is not long enough to span across normal lipid bilayers, AmB in up-right orientation form should lack channel activity for cholesterol-containing plasmamembrane. Head-to-tail aggregates of AmB, which are easily formed in an aqueous phase, should be somewhat longer than AmB monomers, hence facilitating formation of transmembrane channels in cholesterol-containing or sterol-free membranes. However, the effect of cholesterol on channel formation by membrane-bound AmB is still ambiguous. To gain a better understanding of sterol roles, we attempted to

Abbreviations: AmB, amphotericin B; CH, cholesterol; LUV, large unilamellar vesicle; EPC, egg yolk phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DEPC, 1,2-dieicosenyl-*sn*-glycero-3-phosphocholine; EDTA, *N*,*N*,*N*′,*N*′-ethylenediaminetetraacetic-acid; FCCP, carbonyl cyanid-*p*-trifluoro-methoxyphenyl hydrazone; DMSO, dimethylsulfoxide; NMR, nuclear magnetic resonance; UV, ultraviolet–visible

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0005-2736/02/$ - see front matter
PII: S0005-2736(02)00491-1

separate the AmB action into two steps: the incorporation into membrane and the assemblage formation in membrane. If cholesterol merely accelerates the first step, the ion-permeability of AmB after binding to membrane should not be greatly affected by changing cholesterol content in membrane. Conversely, if cholesterol stabilizes the AmB channel assemblage in membrane, the AmB action should be amplified in a cholesterol-dependent manner. Since most of previous experiments were carried out with AmB that was added to aqueous phase containing liposomes or other membrane preparations (*added-via-aqua AmB*), the two different effects of cholesterol before and after binding to membrane could hardly be distinguished. To address these questions, we attempted to carry out ion permeability assays using liposomes that contained AmB at the beginning of membrane preparation (*mixed-with-lipid AmB*). In this paper, we report the inhibitory action of cholesterol to AmB-induced ion currents and discuss the cholesterol-dependent activity of membrane-bound AmB and of aqueous oligomers of AmB.

2. Materials and methods

2.1. Materials

AmB, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Egg yolk phosphatidylcholine (EPC), cholesterol and carbonyl cyanid-*p*-trifluoro-methoxyphenyl hydrazone (FCCP) (H^+ carrier) were obtained from Nacalai Tesque (Kyoto, Japan). Polycarbonate filters were from Nuclepore (Pleasanton, CA).

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

Lipids (4 mM) and AmB (0.1 mM) were dissolved in chloroform and methanol, respectively, to prepare stock solutions. A series of liposomes varying their cholesterol content was prepared by adding aliquots of 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 completely remove the solvent. Lipid films were hydrated with phosphate buffer (0.4 M potassium phosphate and 1 mM *N*,*N*,*N*′,*N*′-ethylenediaminetetraaceticacid (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, the resultant LUVs were then diluted four times in 0.4 M potassium sulfate. The final concentration of lipids was 13 mM. To quantify the final concentration of AmB, UV spectra were recorded on a Shimadzu UV spectrometer (UV-2500PC). An LUV suspension (100 μl) was lyophilized and resuspended in 3 ml of $CHCl_3$/MeOH (5:3) with a bath type sonicator (Shimadzu SUS-100). Insoluble salts were removed by centrifugation. All UV measurements were taken in a quartz cell of 1.0 cm path length over the wavelength range 300–440 nm.

2.3. Potassium permeability measurement

Potassium influx in liposomes elicited by AmB were measured by a proton–cation exchange method based on ^{31}P-nuclear magnetic resonance (NMR) chemical shifts reported by Gary-Bobo et al. [16–18]. 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 dimethylsulfoxide (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 an NMR tube and then added with a 100 mM $MnCl_2$ solution (4.4 μl) to quench the ^{31}P signal due to phosphate outside of liposomes. ^{31}P-NMR was recorded at 202 MHz with ^{1}H decoupling on a JNM GSX-spectrometer (JEOL, Akishima, Japan).

3. Results

Cation currents across liposome membrane can be monitored by pH-dependent shift of the ^{31}P-NMR resonance of phosphate [19]. In this method, pH of a liposome lumen is changed from initial pH 5.5 to pH 7.5, since efflux of H^+ via FCCP at the expense of K^+ influx can be monitored as chemical shifts of a phosphate signal. A signal at δ 1.2 corresponds to $H_2PO_4^-$ in intact liposomes at pH 5.5 whereas that at δ 3.1 deriving from HPO_4^{2-} in permeabilized liposomes at pH 7.5.

Fig. 1 shows ^{31}P-NMR spectra for measuring K^+ flux induced by AmB with various concentration of cholesterol in PC. In these experiments, AmB was added prior to preparation of liposomes (mixed-with-lipid AmB). In Fig. 1a–d, a signal at δ 1.2 increased whereas that at δ 3.1 decreased as the content of cholesterol was increased (the largest ion flux was observed in sterol-free liposomes, Fig. 1a) which clearly showed that cholesterol inhibits the K^+ influx induced by mixed-with-lipid AmB. To rule out the possibility that a trace amount of cholesterol present in egg PC affected the ion flux, synthetic sterol-free PC (POPC) instead of EPC was used for liposome preparations. The essentially same results were obtained with POPC liposomes as depicted in Fig. 1e. In contrast, when AmB was added to liposome suspensions (added-via-aqua AmB), quite different results were obtained (Fig. 2); Fig. 3 demonstrates marked difference between these two methods at various PC–cholesterol ratios. Added-via-aqua AmB shows small K^+ flux in the absence of cholesterol and has the maximum flux at 20%

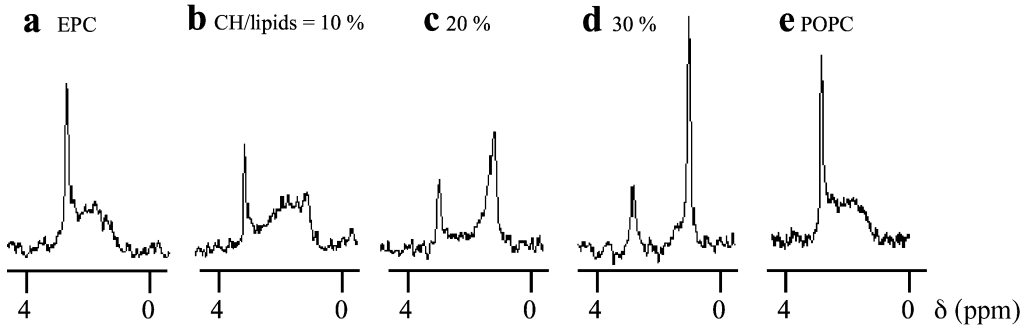

Fig. 1. ^{31}P-NMR signals of liposome-entrapped phosphate for various cholesterol (CH) contents in AmB-containing LUV (mixed-with-lipid). Signals due to external phosphate and phospholipid disappeared due to addition of Mn^{2+}. The liposome suspensions were incubated for 6 h at 25 °C under a pH gradient across the membrane: internal pH = 5.5, external pH = 7.5. The peak around δ 1.2 corresponds to $H_2PO_4^-$ at pH 5.5 of intact liposomes, and that around δ 3.1 corresponds to HPO_4^{2-} at pH 7.5 of the buffer.

cholesterol in PC. Above 20%, the activity was roughly equal between them (Fig. 3).

Since Moribe et al. [20] reported that AmB was partly excluded from liposomes during the preparation of liposomes, the amount of AmB retained in liposomes after repeated filtration for the LUV preparation in the presence of AmB (mixed-with-lipid) was determined by UV spectra. Under the concentrations of AmB in lipids used in this study (0.12–0.15% w/w), 92–100% of AmB was retained in the LUV liposomes.

4. Discussion

It is generally accepted that cholesterol enhances AmB-induced membrane permeabilization as implied by its potent nephrotoxicity. In the barrel–stave model, AmB and cholesterol form a cylindrical channel complex, where cholesterol is thought to participate in and stabilize the molecular assemblage. However, no direct evidence for this antibiotics/sterol complex has hitherto been obtained. Moreover, several groups have recently reported formation of AmB channels in the absence of sterol [5,9]. Based on ^{2}H-NMR experiments, Dufourc et al. [21,22] estimated the dissociation time constant of a possible AmB–cholesterol complex to be 10^{-5} s, which was orders of magnitude faster than an estimated open–close rate of the channels [23,24]. Cotero et al. [14] disclosed that functionally identical channels were formed regardless of cholesterol content in membrane, thereby denying the involvement of cholesterol in ion channel assemblages.

Although direct interaction between cholesterol and AmB is unlikely, it has been frequently reported that cholesterol has accelerating effects on AmB-induced membrane permeabilization. The findings reported herein demonstrate the biphasic effects of cholesterol (Fig. 3). With the added-via-aqua AmB, the ion flux gradually increased as cholesterol content was increased up to 20%. On the contrary, when AmB was mixed with lipids upon liposome preparation (the mixed-with-lipid AmB), ion flux decreased constantly with increasing concentrations of cholesterol in membrane. The former indicates that the incorporation of AmB into membranes is enhanced by cholesterol. The later reveals the inhibitory action of cholesterol to channel formation by membrane-bound AmB.

Polyene macrolides are known to possess greater affinity to highly ordered lipids in membranes [5,12,25,26]. Bolard

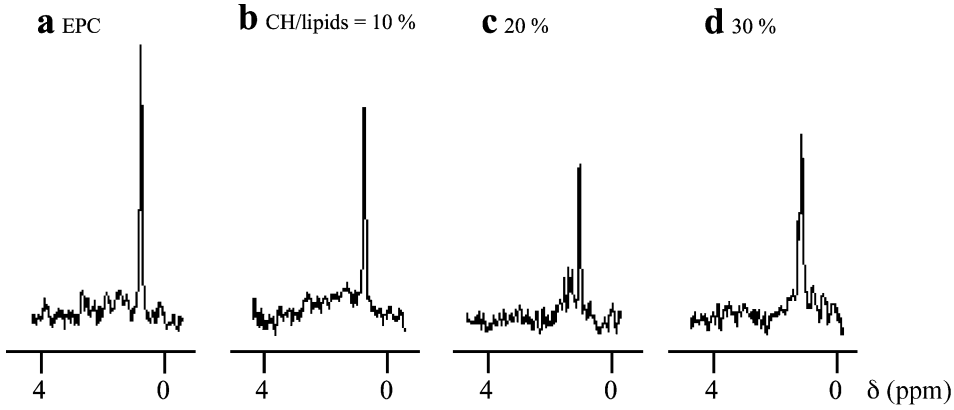

Fig. 2. ^{31}P-NMR signals of liposome-entrapped phosphate for various cholesterol (CH) contents in LUV (added-via-aqua). In these experiments, AmB was partly present as micelles outside the vesicles, which is the prominent difference from the LUV experiments in Fig. 1.

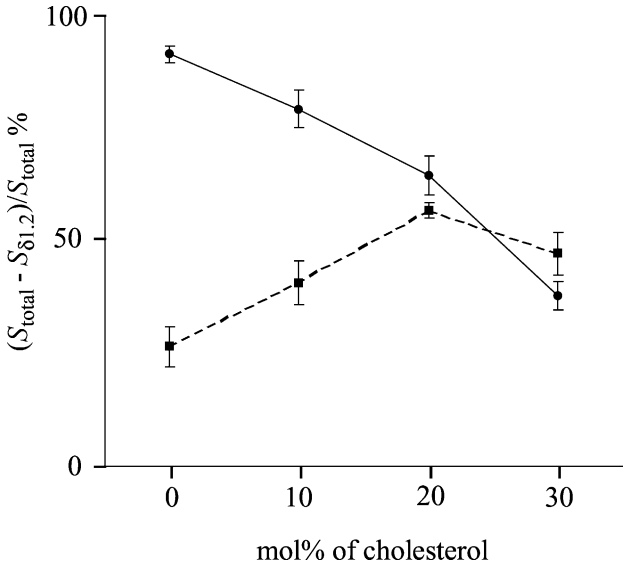

Fig. 3. AmB-induced ion permeability dependent on cholesterol concentrations. $S_{\delta\ 1.2}$ is the area of a ^{31}P-NMR peak at 1.2 ppm, which was calculated from curve-fitting for a Lorentzian line-shape. For mixed-with-lipid AmB, S_{total} is the sum of peak areas from 1.2 to 3.1 ppm. For added-via-aqua AmB, S_{total} was obtained from the area of peak at 1.2 ppm of intact LUV (negative control). Thus, $S_{total-1.2}/S_{total}$ corresponds to a portion of liposomes permeabilized by AmB. Mixed-with-lipid AmB, solid line with (●); added-via-aqua AmB, broken line with (■). Error bars were obtained from three independent experiments.

et al. [27] demonstrated that AmB partitions more abundantly to rigid and/or ordered gel. In our experimental conditions, lipid bilayers can be regarded as a liquid crystalline phase, where cholesterol increases the ordering of phospholipids. For the added-via-aqua AmB, its preferential incorporation into rigid membrane results in the concerted augment of ion flux with increasing cholesterol content (Figs. 2 and 3). On the other hand, with the mixed-with-lipid AmB, the ion flux is markedly decreased with increasing cholesterol content (Figs. 1 and 3). To our knowledge, this may be the first experimental evidence that cholesterol markedly inhibits formation of ion channels in membrane of liquid crystalline phase. In the experiments with the mixed-with-lipid AmB, a step of AmB incorporation to membrane was skipped, and formation of channel assemblages in membrane was selectively observed. We speculate that cholesterol reduces the stability of the molecular assemblages and this inhibitory action is caused not by AmB–sterol assembling but by sterol–phospholipid interaction. Ruckwardt et al. [13] demonstrated that AmB strictly recognizes bilayer thickness. Under osmotic stress, AmB induced large ion flux in POPC, whereas showing only marginal flux in 1,2-dieicosenyl-*sn*-glycero-3-phosphocholine (DEPC). The difference in bilayer thickness between POPC and DEPC is about 3 Å [13]. Nezil and Bloom [28] reported that cholesterol (33 mol%) increased the thickness of POPC bilayers by about 4 Å, which corresponds to the difference in the bilayer thickness between POPC and DEPC. Thus, the thickened bilayers may be one of plausible accounts for the inhibition of AmB action by cholesterol (Fig. 4).

Previous studies including those of molecular dynamics calculations have indicated that AmB molecule takes relatively rigid conformation [29], in which its heptane backbone takes an extended zigzag geometry and this leads to stretching the polyhydroxyl chain on the other side. AmB has an amphoteric charged pair at one side of the molecular, which should always stay in the bilayer–water interface. Upon forming an ion channel, the tail side of AmB should penetrate into the hydrophobic region of membrane. In this situation, the bilayer thickness should be roughly equal to the double of AmB molecular axis for a double-layered AmB channel to span across membrane (Fig. 4). This could

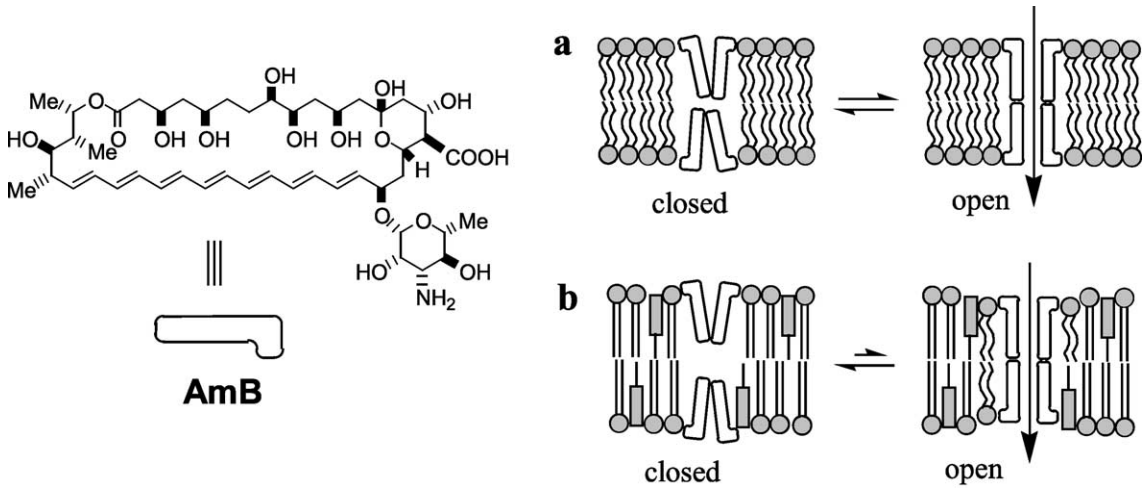

Fig. 4. Hypothetical AmB channel in pure EPC and cholesterol-containing membranes. In sterol free EPC, the bilayer thickness is small (about 36 Å) enough for AmB to form a K^+ permeable channel (a). In cholesterol-containing EPC, the bilayer is slightly thicker than that of the sterol-free membrane by a few angstrom (b). An AmB channel in cholesterol-containing membrane is destabilized and ion current becomes smaller than that in sterol-free membrane. Cholesterol is depicted as a gray rectangle.

be one of possible explanations for the biphasic action of cholesterol (Fig. 3); over 30%, the membrane thickness exceeds the length of tail-to-tail dimers, and this inhibition surpasses the stimulatory effect on AmB's binding to membrane by cholesterol.

The difference between ergosterol and cholesterol in the binding affinity to AmB is generally regarded as the molecular basis of AmB's sterol selectivity [30]. Nevertheless, indirect effects, such as membrane ordering, surface polarity, and bilayer thickness, may also play an important role in the AmB channel formation in sterol-containing membrane. It was demonstrated by solid state NMR that the ordering effect of ergosterol on PC bilayers is quite different from that of cholesterol [31,32]. The surface polarity and bilayer thickness of ergosterol-containing membrane should be different from those of cholesterol-containing membrane because these properties are strongly affected by the membrane order [33,34]. These indirect effects may be partly responsible for acceleration of AmB action in ergosterol membrane.

In conclusion, we have demonstrated that cholesterol, once bound to membrane, markedly inhibits AmB-induced membrane permeabilization. This finding adds a new interpretation to the molecular mechanism of cholesterol on AmB channel formation. The indirect effect of the bilayer thickening and ordering may be the predominant action of cholesterol for the AmB channel formation rather than direct interaction between the sterol and AmB. Detailed studies of AmB–sterol–phospholipid reciprocal action, particularly for ergosterol, are currently underway.

Acknowledgements

We are grateful to Dr. Nobuaki Matsumori and Prof. Tohru Oishi in our laboratory, and to Prof. Kazuo Tachibana, The University of Tokyo, for discussion and suggestions. This study was supported by a Grant-In-Aid for Scientific Research on Priority Area (A) from the Ministry of Education, Sciences, Sports, Culture, and Technology, Japan; by a grant from the CREST, Japan Science and Technology Corporation; and by the Yamada Science Foundation.

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