Biochimica et Biophysica Acta, 1108 (1992) 49–58

BBAMEM 75678

A sequential mechanism for the formation of aqueous channels by amphotericin B in liposomes. The effect of sterols and phospholipid composition

B. Eleazar Cohen

Center of Cell Biology, Faculty of Sciences, Central University of Venezuela, Caracas (Venezuela)

(Received 25 November 1991)
(Revised manuscript received 17 March 1992)

Key words: Amphotericin B; Polyene antibiotic; Liposomes; Ergosterol; Cholesterol; Phosphatidylethanolamine; Dimyristoylphosphatidylcholine

The kinetics of formation of amphotericin B (AmB) aqueous pores in ergosterol-containing DMPC or egg-PC liposomes was investigated using a stopped-flow method. The formation of aqueous pores by AmB occurred very rapidly (in milliseconds to seconds depending of the AmB concentration), and it was always preceded by the formation of transient, non-aqueous pre-pore structures. As anticipated, these non-aqueous pre-pore structures made the liposomes more permeable to urea without at the same time leading to a decrease of the reflection coefficient of urea or to an enhancement of glucose permeability. However, when liposomes were composed of egg-PC and cholesterol, the formation of non-aqueous and aqueous channels by AmB occurred after a lag time of several minutes. Such a time lag for AmB action was not observed in cholesterol-containing DMPC liposomes, an indication that the phospholipid composition is an important parameter in the formation of non-aqueous channels by AmB. Both non-aqueous and aqueous channels were always formed at lower concentrations of AmB in liposomes containing ergosterol while higher concentrations were needed in cholesterol-containing liposomes. Measurements of the permeabilizing effect of AmB on liposomes prepared without sterols indicate that non-aqueous channels were formed in DMPC (but not in egg-PC) at polyene concentrations identical to that found for cholesterol-containing liposomes. No evidence of the formation of aqueous channels by AmB was found in pure DMPC liposomes. These data are consistent with the concept that AmB forms non-aqueous channels without the direct participation of sterol molecules. The initially formed non-aqueous channels subsequently interact with the sterols in the membrane to form aqueous channels, having an enlarged diameter. This sequential mechanism for the formation of AmB aqueous pores in liposomes provides a rationale for the understanding of the effect of both the phospholipid composition and type of sterol in the interaction of AmB with natural membranes and artificial bilayers.

Introduction

The polyene antibiotic amphotericin B (AmB) is an efficient killer of fungi and parasitic protozoa, but is also toxic to mammalian cells (reviewed in Refs. 1 and 2). It has been assumed that such a selective toxicity of AmB for fungi is the result of its capacity to bind more strongly to ergosterol, the principal fungal sterol than to cholesterol, which is the principal sterol of mammalian cells [3,4]. For both types of membranes, a common mechanism of action for AmB has been proposed to involve the formation of aqueous pores of about 4 Å in radius [5,6,7]. According to this theory, these pores cause the membrane to be non-selective leaky and cell death ensues.

However, the details of the relationship between the formation of aqueous channels by AmB and its lethal action on sensitive cells is not yet fully understood. For example, it has been reported that AmB can induce changes in ion permeability in *Candida albicans* without causing cell death [8]. Others have failed to detect a correlation between the sensitivity of fungi to polyene antibiotics and the corresponding sterol content [9,10]. In *Leishmania mexicana*, the sensitivity to AmB has actually been shown to be enhaced after reducing the ergosterol content of the plasma membrane upon their transformation by heat into amastigote-like forms [11]. The reason for a more rapid response to AmB by heat-transformed leishmanias has been suggested to be based on changes in the degree of saturation of the phospholipid chains, which could conceivably facilitate

Correspondence to: B.E. Cohen, Center of Cell Biology, Faculty of Sciences, Central University of Venezuela, P.O. Box 47860, Caracas 1041, Venezuela.

the formation of aqueous channels [11,12,13]. Other effects of AmB, such as the lipid peroxidation observed in various cell types, could also play a role in the mechanism of action of AmB [14].

Previous models for formation of aqueous channels by AmB in membranes have emphasized formation of an initial complex between the antibiotic and sterols [6,7]. According to this concept, the complex consists of 8 to 10 sterol/AmB dimers packed to form a cylindrical transmembrane structure, the interior of which is lined by hydroxyl groups from the AmB molecules. However, it has seemed very unlikely that the rate-limiting step for formation of such a multimeric aqueous channel could actually be the simultaneous association of these AmB/sterol complexes as a single event. Rather, it is possible that AmB aqueous pores may be formed in a sequential mechanism involving the initial formation of a 'complex' between AmB and lipid molecules. In earlier studies, we reported that AmB can form channels in ergosterol-containing liposome [15] and *Leishmania* membrane vesicles [16], which differ in internal diameter. The simplest hypothesis to consider is that phospholipids themselves common to all membranes, interact with AmB and that complexes with other membrane components could then follow.

To test this hypothesis, we have used a rapid stopped-flow method to study the effect of phospholipid composition and type of sterol in the time course for channel formation by AmB. For this purpose, we have prepared large unilamellar liposomes composed of either DMPC or egg-PC, in the presence or absence of either ergosterol and cholesterol. We report here that in the absence of sterols, only the non-aqueous pre-pore structures are formed. However, if ergosterol or cholesterol are also present, the pre-pore non-aqueous structures progress to form AmB aqueous pores. We conclude that AmB may interact with biological membranes by a sequential mechanism in which an initial interaction with phospholipids to form non-aqueous pre-pore structures is followed by interaction of the complex with sterols to form complete aqueous pores.

Materials and Methods

Materials. Analytical quality reagents were used whenever possible. Amphotericin B (AmB), egg phosphatidylcholine (egg-PC) and dimyristoylphosphatidylcholine (DMPC) were purchased from Sigma. Ergosterol (Sigma) was purified as described previously [15]. In all experiments, AmB was dissolved in dimethylformamide (DMF) at a concentration of 1 mg/ml.

Preparation of liposomes. Large unilamellar liposomes were formed by the reverse-phase evaporation method as described by Szoka and Papahadjopoulos [17]. For the preparation of LUV, 2 ml of an aqueous phase containing 60 mOsm potassium phosphate buffer (pH 7.0) were mixed with 6 ml diethyl ether containing 20 μmoles of a lipid mixture (egg-PC + 16 mol% ergosterol). For the corresponding preparation of liposomes composed of DMPC with or without cholesterol or ergosterol, the ether phase was substituted by 10 ml of a mixture of chloroform/diethyl ether (1:1, v/v). In both cases, the resulting two-phase system was sonicated in a cylindrical bath sonicator, until the mixture become a one-phase dispersion. Liposomes were formed after the organic solvent was removed under reduced pressure in a rotary evaporator. This preparation of liposomes was filtered through a polycarbonate membrane (Nuclepore, pore size 0.4 μm) without loss of lipid. The ergosterol content of such liposomes was found to be identical to the lipid composition in the original solution, indicating that the incorporation of sterol was maximal. Liposomes were found by negative-staining electron microscopy to be homogeneous in size (average diameter 0.1 $\pm$ 0.04 μm), being predominantly unilamellar. Oligolamellar vesicles were also occassionally observed.

Osmotic measurements. Volume changes of liposomes occurring after an exposure to an osmotic gradient were followed by measuring the 90° light scattering intensity at 450 nm, in a Durrum stopped-flow spectrophotometer (D-110). As shown previously [18], the light scattering increase observed at 90° to the incident beam corresponds to the shrinking of the liposomes due to water efflux. As predicted by the Boyle-Van't Hoff equation, a linear relationship was found between the reciprocal of the total light scattering changes (at equilibrium) and the reciprocal of impermeable solute concentration in the external solution. Thus the 90° light scattering changes could be directly related to changes in liposome volume.

The effect of AmB on the permeability coefficients for different solutes was measured by the 'maximum slope' method developed by Hill and Cohen [19]. The rate of swelling and shrinkage of liposomes as well as the extent of the minimum volume (V_{min}) depend on the value of the solute's reflection coefficient. On the other hand, the maximum slope of the volume changes after attaining the minimum volume is proportional to the solute permeability coefficient, provided that the so-called 'drag term' in the equation, which describes the solute flow [19], is small and can be neglected.

Before mixing in the Stop-flow apparatus, the liposomes were diluted to a lipid concentration of 1 mM concentration and incubated with different concentrations of AmB. For mixing in the stop-flow, it was found most convenient to use a 4:1 ratio drive syringe, which permits one volume of vesicles contained in syringe I to be mixed with four volumes of an hyperosmotic solute solution (600 mosM) contained in syringe II. In all cases, AmB was dissolved in DMF and the stock solu-

tion added (in μl) so that the organic solvent final concentration was less than 0.5% by volume. Control experiments indicated that at this concentration DMF itself had no effect on light scattering changes.All measurements were done with water continuously circulating through the drive syringes and mixing cuvette at a constant temperature.

In order to measure the effect of AmB on the total extents of the volume changes of liposomes suspended in hyperosmotic solutions, the light scattering changes were stored in a computer until no changes were observed. The corresponding rate constants k (in s^{-1}) for solute permeation were calculated by dividing the maximum rate of swelling after V_{min} by the total extent of the light scattering changes. For a first-order process it can be demostrated that $k = P \cdot A/V$, where P is the solute permeability, A is the total area of liposomes and V is the liposome volume. We have taken k values as proportional to the solute permeability coefficient since the A/V ratio has nearly the same value in all the liposomes prepared by the present method.

Results

Formation of non-aqueous channels and aqueous channels by AmB across ergosterol-containing DMPC liposomes

We have previously reported that in the time course of the permeabilization by AmB of eggPC/ergosterol liposomes [15] two types of channels appear to be formed depending of the AmB concentration and the time elapsed after mixing. Fig. 1 shows the effect of increasing concentrations of AmB on the biphasic light scattering of DMPC/ergosterol liposomes after rapid mixing with a hyperosmotic urea solution. Up to an AmB concentration of 0.8 μM, modest but significative increments in the total volume changes were observed (Fig. 1, top). However, beyond 1.2 μM AmB, the extents of shrinkage decreased abruptly (Fig. 1, bottom). This finding demostrates that the reflection coefficient for urea has decreased (see Methods). This observation is consistent with previous data indicating that addition of AmB to planar lipid bilayers leads to a decrease of the reflection coeffcient of urea from unity in untreated membranes to a value near 0.6 [5,6].

The light scattering curves presented in Fig. 1 also show that at AmB concentrations below the 'critical' concentration value of 1.2 μM (Fig. 1), we could detect an increase in the rate of swelling of liposomes after they had attained their minimum volumes. When the corresponding changes of the rate constants for urea permeation (Δk(urea)) were calculated (see Methods) and plotted against the external AmB concentration (Fig. 2, empty circles) a linear relationship was obtained up to an AmB concentration of 1.6 μM. Beyond

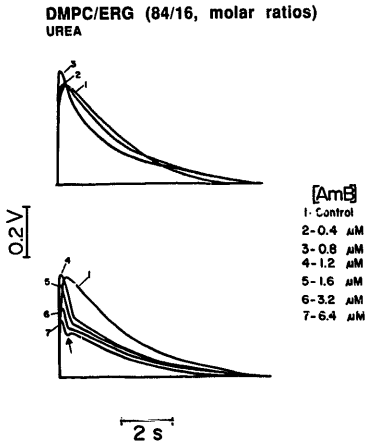

Fig. 1. Stopped-flow traces of volume changes in urea-suspended liposomes treated with AmB. Ordinate: Light scattering intensity changes at 90° (in mV). Liposomes were prepared from DMPC and ergosterol (84:16, molar ratios) in a 60 mosM buffer phosphate solution (pH 7.0) They were then rapidly mixed (1:4, volume ratios) with a 600 mosM urea solution containing increasing AmB concentrations. The final AmB concentrations were as follows. 1, control; 2, 0.4 μM; 3, 0.8 μM; 4, 1.2 μM; 5, 1.6 μM; 6, 3.2 μM; 7, 6.4 μM. The curved traces show the first sweep of the oscilloscope and the horizontal lines indicate the final light scattering equilibrium values. Final lipid concentration 0.2 mM; Temp. 30°C.

this concentration, the measured Δk were smaller than those expected from a linear dose-response.

It follows that the channels that are formed by AmB at low AmB concentrations (< 1.2 μM) allow a significant increment of the urea permeability across DMPC/ergosterol liposomes (Fig. 2, empty circles) without at the same time exerting any reduction in the reflection coefficient of urea (Fig. 1, top). Such structures will be referred to now as non-aqueous AmB channels.

Formation of AmB aqueous channels – from non-aqueous channels

The results shown in Fig. 1 (bottom) also indicate that a few seconds after attaining V_{min}, the swelling portion of the curves corresponding to AmB concentrations higher than 0.8 μM, exhibit a characteristic break or discontinuity. It can be observed in Fig. 1 that such breaks occurred at progressively shorter times after mixing, as the AmB concentration was increased.

When an AmB concentration of 6.4 μM was reached, we clearly observed an actual minimum in the time course of the swelling (arrow in Fig. 1).

The possibility arises that the presence of such kinks during the time course of the swelling could be directly related to rearrangements of lipids and AmB leading to the formation of AmB aqueous channels. If this were so, one might expect that any kinks due to rearrangements of the AmB-phospholipid complex might be eliminated by lengthy exposures of liposomes to AmB prior to mixing. In order to investigate this possibility, liposomes were therefore pre-incubated for 15 min with different AmB concentrations prior to mixing with the hyperosmotic urea solution (Fig. 3). It can be seen in Fig. 3 that no 'kinks' were observed when liposomes were pre-incubated with either 2 μM AmB (Fig. 3A, curve 1) or 4 μM AmB (Fig. 3B, curve 1) and then mixed in the stop-flow apparatus with an urea solution containing no AmB. This lack of kinks can be taken as an indication that the formation of the aqueous pores occurred during the pre-incubation period. This conclusion is reinforced by the observation that the extents of shrinkage of such incubated liposomes (curves 1 in Figs. 3A and 3B, respectively) were smaller than those exhibited by control liposomes treated with the same final AmB concentration (curves 3 in Figs. 3A and 3B).

As a further test of the hypothesis, we pre-incubated liposomes in AmB and then allowed the osmotic gradient to occur in different final concentrations of AmB. As shown in both Figs. 3A and 3B (curves 2), kinks were again observed only if the additional amount of the antibiotic that was added to the hyperosmotic aqueous solution was greater than the 'critical' concentration of 0.8 μM. It follows that the presence of kinks in the swelling portion of DMPC/ergosterol liposomes exposed to AmB is connected with the formation of aqueous pores by the antibiotic, and that kinks are a consequence of interactions between AmB and the membrane occurring over a time scale much greater than seconds.

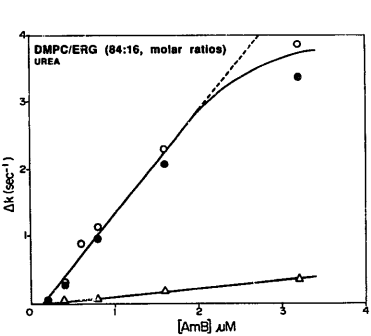

Fig. 2. The effect of AmB on the urea permeability of liposomes. Ordinate: changes of the rate constant for urea permeation (Δk in s^{-1}). Abscissa: final AmB concentration (in μM). ○, Liposomes were mixed with a hyperosmotic urea solution containing increasing AmB concentrations. ●, Liposomes were pre-incubated for 15 min with increasing AmB concentrations and then mixed with a hyperosmotic urea solution containing AmB at different concentrations. △, Liposomes were pre-incubated for 15 min with increasing AmB concentrations and then mixed with a hyperosmotic urea solution. The rest of experimental details as in Fig. 1.

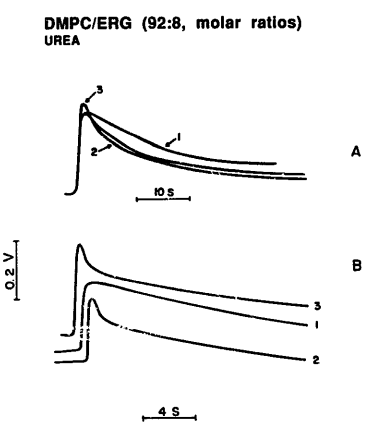

Fig. 3. Stopped-flow traces of the volume changes of urea-suspended liposomes treated with AmB under different conditions. Ordinate: Light scattering intensity changes at 90° (in mV). (A) Curve 1: Liposomes were pre-incubated with 2 μM AmB and then mixed with a hyperosmotic urea solution containing no AmB (final AmB concentration 0.4 μM) Curve 2: Liposomes were pre-incubated with 2 μM AmB and then mixed with a hyperosmotic urea solution containing 0.5 μM AmB (final AmB concentration 0.8 μM). Curve 3: Liposomes were mixed with a hyperosmotic urea solution containing 0. 5 μM AmB (final AmB concentration 0.4 μM). (b) Curve 1: Liposomes were pre-incubated with 4 μM AmB and then mixed with a hyperosmotic urea solution containing no AmB (final AmB concentration 0.8 μM). Curve 2: Liposomes were pre-incubated with 4 μM AmB and then mixed with a hyperosmotic urea solution containing 1.0 μM AmB (final AmB concentration 1.6 μM). Curve 3: Liposomes were mixed with a hyperosmotic urea solution containing 1.0 μM AmB (final AmB concentration 0.8 μM). Liposomes were prepared from DMPC and ergosterol (92:8, molar ratios) in a 60 mosM buffer phosphate solution (pH 7.0) as described in Methods. Aliquots of liposomes were pre-incubated for 15 min with different AmB concentrations. The rest of experimental details as in Fig. 1.

The corresponding changes of the rate constant for urea permeation (Δk(urea)) across liposomes exposed to AmB with or without pre-incubation with AmB are plotted in Fig. 2. It can be seen in Fig. 2 that Δk(urea) for pre-incubated liposomes (triangles) are much smaller that those measured for non-incubated liposomes (empty circles) exposed to the same final AmB concentration. Clearly, the osmotic method to measure solute permeabilities grossly underestimates the true permeability coefficient of urea when measurements are carried out after the formation of the aqueous pores.

However, when the AmB-incubated liposomes were exposed to an urea solution also containing AmB (Fig. 2, filled circles) the magnitude of the Δk(urea) exhibited by these liposomes were essentially identical to those measured for non-incubated liposomes, under equivalent concentrations. This result is not surprising since in all cases (see Methods) the rate of urea permeabilization was calculated by taking the maximum slope (after the minimum volume) from the portion of the swelling curves preceding the formation of kinks.

The permeability of the AmB aqueous channels to glucose

The aqueous pores that are formed by AmB in planar lipid bilayers, liposomes or natural membrane vesicles are known to exhibit an small but significative permeability to glucose [4,5,15,16]. This behaviour was also observed when ergosterol-containing DMPC liposomes were mixed with a hyperosmotic glucose solution containing AmB at increasing concentrations (Fig. 4).

It can be seen in Fig. 4 that no changes of glucose permeability were induced by AmB until the 'critical' concentration of 0.8 μM AmB was added to the system. Beyond this concentration, a linear enhancement of glucose permeability was measured with raising AmB concentrations.

As far as the magnitude of the measured enhancement of glucose permeability is concerned, it is important to note that k(glucose) at 0.8 μM AmB (Fig. 4) is about 500-times smaller that k(urea) at the same AmB concentration and temperature (Table I).

The formation of non-aqueous channels by AmB across liposomes prepared without sterols

We then proceded to test the hypothesis that sterols, per se, had influenced in the eventual development of aqueous pores. As shown in Fig. 5, AmB exerted a bimodal effect on the permeability of urea across liposomes prepared with DMPC but without any sterol present. Thus, at low AmB concentrations (< 0.8 μM) the k(urea) decreased with respect to the untreated (control) liposomes but beyond such a concentration, a modest but significative enhancement of k(urea) was observed. The total volume changes at V_{min} of such sterol-free liposomes suspended in urea do not differ from the control values, even at polyene concentrations as high as 10 μM.

The behaviour of DMPC liposomes that were incubated with AmB for 15 min previously to its mixing with the urea solution was also investigated. Under this condition, an enhancement of urea permeability was also measured but such changes were not accompanied by variations in the extents at V_{min} (not shown). It

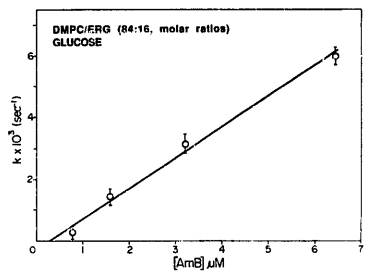

Fig. 4. The effect of AmB on the glucose permeability of liposomes. Ordinate: the rate constant for glucose permeation (k in s^{-1}). Abissa: the final AmB concentration (in μM). Liposomes were prepared from DMPC and ergosterol (84:16, molar ratios) as described in Methods. They were rapidly mixed with a 600 mosM glucose solution containing AmB at increasing concentrations.

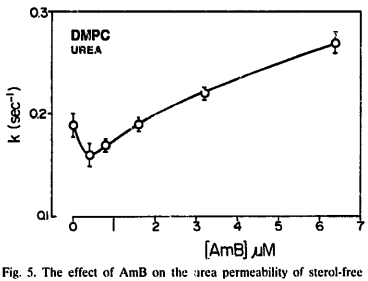

Fig. 5. The effect of AmB on the urea permeability of sterol-free liposomes. Ordinate: the rate constant for urea permeation (k in s^{-1}). Abissa: the final AmB concentration (in μM). Liposomes were prepared from DMPC in a 60 mosM buffer phosphate solution (pH = 7.0) They were rapidly mixed with a 600 mosM urea solution containing AmB at increasing concentrations. Final lipid concentration 0.4 mM; Temp. 30°C.

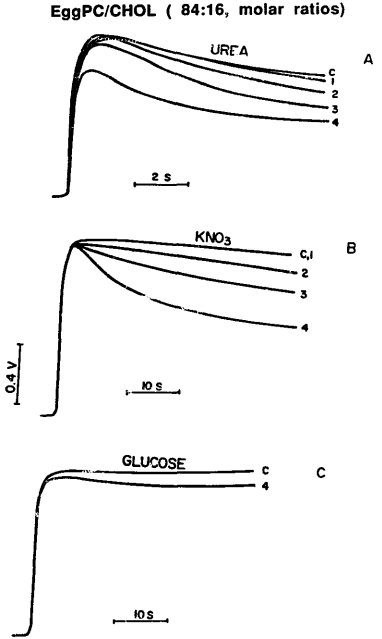

Fig. 6. Stopped-flow traces of volume changes of cholesterol-containing liposomes suspended in hyperosmotic solutions of different solutes. Ordinate: Light scattering intensity changes at 90° (in mV). (A) Urea; (B) KNO_3; (C) glucose. Liposomes were prepared from egg-PC and cholesterol (84:16, molar ratios) in a 60 mosM phosphate buffer (pH 7.0). Aliquots of liposomes (1 mM) were pre-incubated for 15 min with increasing concentration of AmB. They were rapidly mixed with a hyperosmotic 600 mosM solution of the tested solute. The final AmB concentrations (after 1:4 volume mixing) were as follows: C, control; 1, 1.0 μM; 2, 3.0 μM; 3, 5.0 μM; 4, 10.0 μM. Final lipid concentration 0.2 mM; Temp. 20°C.

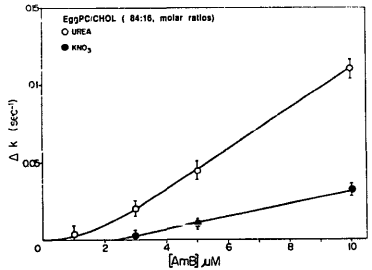

Fig. 7. The effect of AmB on the permeability of cholesterol-containing liposomes to urea and potassium nitrate. Ordinate: changes of the rate constant for solute permeation (Δk in s^{-1}). Abscissa: final AmB concentration (in μM). ○, Urea; ●, KNO_3. The rest of experimental details as in Fig. 6.

follows that in pure DMPC liposomes, the permeability to urea that is induced by AmB occurred only by way of the formation of non-aqueous channels.

As anticipated, when liposomes were prepared from egg-PC in the absence of sterols, no enhancement of urea permeability nor any changes of the total volume change at V_{min} were measured up to AmB concentrations as high 10 μM, independently of its being pre-incubated with the antibiotic.

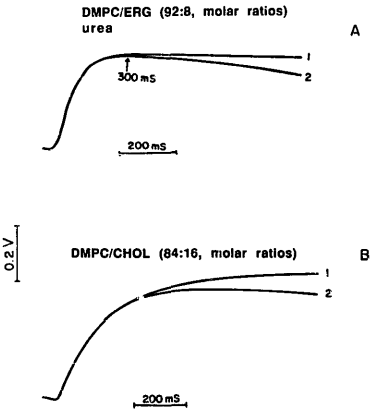

Fig. 8. Stopped-flow traces of AmB-induced volume changes in ergosterol and cholesterol-containing liposomes. Ordinate: Light scattering intensity changes it 90° (in mV). (A) Ergosterol-containing DMPC liposomes (92:8, molar ratios). Curve 1: Liposomes were mixed with a 600 mosM hyperosmotic urea solution. Curve 2: Liposomes were mixed with a 600 mosM hyperosmotic urea solution containing AmB. Final AmB concentration 0.8 μM. (B) Cholesterol-containing liposomes (84:16, molar ratios). Curve 1: Liposomes were mixed with a 600 mosM hyperosmotic urea solution. Curve 2: Liposomes were mixed with a 600 mosM hyperosmotic urea solution containing AmB. Final AmB concentration 6.4 μM. The rest of experimental details as in Fig. 3.

Formation of non-aqueous and aqueous channels by AmB in cholesterol-containing liposomes

When egg-PC liposomes were prepared with cholesterol and then mixed directly with the AmB containing urea solution, no enhancement of the urea permeability nor any changes of the total volume changes at V_{min} were observed (data not shown). However, if liposomes were pre-incubated for 15 min with AmB concentrations higher than 0.8 μM (Fig. 6A), the rate of swelling after V_{min} increased. The calculated changes of the rate constant for urea permeabilization (Δk(urea)) across egg-PC/cholesterol liposomes are plotted in Fig. 7 as a function of the final AmB concentration.

It can be seen in Fig. 6A that such an enhancement of urea permeability induced by AmB across egg-PC/cholesterol liposomes was not accompanied by any changes in the magnitude of the total extents at V_{min} for AmB concentrations lower than 10 μM (curves 1 to 3 in Fig. 6A). This observation indicates that at such a concentration range, AmB seems only to generate the formation of non-aqueous channels. At 10 μM AmB, the total extent abruptly decreased (Fig. 6A, curve 4) indicating the formation of aqueous pores. In fact, at this concentration, a modest but significative increment of glucose permeability could be measured (Fig. 6C).

Similar incubation conditions to those employed for urea and glucose were used to investigate the effect of AmB on the permeability of egg-PC/cholesterol liposomes to potassium nitrate (Fig. 6B). It can be observed in Fig. 7 that the KNO_3 rate constant was not affected significantly by AmB until a concentration of about 3 μM AmB was added to the system.

Comparison between the rate of formation of non-aqueous channels by AmB across ergosterol and cholesterol-containing liposomes

The fact that urea permeabilization across egg-PC/cholesterol liposomes is not accompanied by any variations in the total extents of V_{min} or in the glucose permeability indicates that AmB concentrations lower than 10 μM seem to generate the formation of non-aqueous channels. However, the fact that we could observe such non-aqueous channels only after the incubation of egg-PC/cholesterol liposomes for several minutes with AmB contrasts with the observed formation of the same type of channels just after rapidly mixing of AmB with DMPC/ergosterol (Fig. 1) or egg-PC/ergosterol liposomes (Cohen, 1986) [15]. It was thus possible that the choice of phospholipid could influence the nature of the interaction betwen AmB and the membrane. To test this hypothesis, we compared the rates of formation of AmB non-aqueous channel in DMPC/cholesterol liposomes with DMPC, ergosterol liposomes (Fig. 8). As shown in Fig. 8, the time course of the permeabilization that is induced by AmB across DMPC/cholesterol liposomes is essentially similar to that shown by DMPC/ergosterol liposomes.

TABLE I

The effect of AmB on the urea permeability of liposomes

$k = P \cdot V / A$ (P = solute permeability coefficient; V = volume of liposomes; A = area of liposomes. All k values quoted are an average of five determinations (± S.D.).

Composition (molar ratios)	T (°C)	[AmB] (μM)	k (s^{-1})	Δk [a] (s^{-1})	Q_{10} [b]
(1) DMPC liposomes					
(a) no-sterol	30	0	0.19 ± 0.03		
	30	6.4	0.27 ± 0.01	0.08	
(b) DMPC/CHOL	30	0	0.05 ± 0.002		
(84:16)	30	6.4	0.14 ± 0.01	0.09	
DMPC/CHOL	40	0	0.21 ± 0.02		
(84:16)	40	6.4	0.25 ± 0.02	0.04	0.44
(c) DMPC/ERG	20	0	0.04 ± 0.005		
(92:8)	20	0.8	0.19 ± 0.01	0.15	
DMPC/ERG	30	0	0.14 ± 0.01		
(92:8)	30	0.8	0.86 ± 0.06	0.72	4.8
(2) Egg-PC liposomes					
(a) eggPC/ERG	20	0	0.17 ± 0.01		
(84:16)	20	0.8	0.23 ± 0.01	0.06	
eggPC/ERG	30	0	0.33 ± 0.04		
(84:16)	30	0.8	0.40 ± 0.07	0.07	1.2
(b) eggPC/CHOL	20	0	0.06 ± 0.003		
(84:16)	20	6.4 [c]	0.12 ± 0.005	0.06	

[a] $\Delta k = k(\text{AmB}) - k(0)$.

[b] $Q_{10} = k(T + 10)/k(T)$.

[c] Liposomes were incubated for 15 min with AmB before mixing.

The effect of temperature on AmB-induced urea permeability across ergosterol- and cholesterol-containing liposomes

The effect of temperature on the AmB-induced permeability of urea across ergosterol- and cholesterol-containing liposomes was investigated by measuring permeabilities at two temperatures (Table I, column 2). From the k(urea) values the corresponding Q_{10} values were calculated (Table I, column 6). It can be observed that the Q_{10} value is large and positive for ergosterol-containing DMPC liposomes but negative for cholesterol-containing DMPC liposomes. It can also be observed in Table I that the permeability to urea which is induced by AmB across egg-PC/ergosterol liposomes was not greatly affected by temperature.

Discussion

The present kinetic studies of the differential permeabilization of liposomes to small non-electrolytes indicates clearly that addition of AmB to liposomes leads to the formation of non-aqueous and aqueous channels, differing in internal diameter. The formation of non-aqueous channels was characterized by the ability of AmB to cause an increase in urea permeability without inducing either a decrease of the reflection coefficient for this solute or an increase of glucose permeability. Indeed, this last set of permeability properties is characteristic of the formation of aqueous pores by AmB in planar lipid bilayers [5,6].

The formation of aqueous channels by AmB in liposomes may occur by a sequential mechanism in which an initial interaction with phospholipids to form non-aqueous pre-pore structures is followed by the interaction of the complex with sterols to form aqueous pores. This hypothesis is supported by the finding that the Δk(urea) values corresponding to non-aqueous channels formed at 30°C by adding AmB to liposomes composed of either pure DMPC, DMPC/cholesterol or egg-PC/ergosterol are identical (Table I, column 5), regardless of the presence or not of sterol and the AmB concentration. The operational meaning of these set of data is obviously that non-aqueous channels are formed by the interaction of AmB molecules with phospholipids, without the participation of sterol molecules.

However, the present data also indicate that non-aqueous channels are formed at lower AmB concentration in ergosterol-containing liposomes than in those containing cholesterol, regardless of the phospholipid composition of the liposomes (Table I). From Table I (column 5) we note similar Δk values (at 30°C) for either ergosterol-containing egg-PC liposomes treated with 0.8 μM AmB or cholesterol-containing egg-PC or DMPC liposomes treated with 6.4 μM AmB. These results strongly suggest that an specific interaction between AmB and an ergosterol-containing membrane matrix is essential, both for an increased initial process of insertion of AmB molecules into a target membrane as well as for the rapid formation of aqueous channels from non-aqueous pre-pore structures.

In ergosterol-containing liposomes composed of either egg-PC [15] or DMPC (Fig. 1) the formation of non-aqueous channels have been found to be a transient phenomena leading in a time scale of ms to s (depending on the AmB concentration) to the formation of aqueous pores. By contrast, when liposomes were prepared from egg-PC and cholesterol, pre-incubation of liposomes with AmB for several minutes is always needed for the formation of non-aqueous channels or aqueous pores. The occurrence of a time lag of several minutes for the process of channel formation by AmB has also been reported in planar lipid bilayers [20] and erythrocytes [21]. However, such a time lag for AmB channel formation does not occur when cholesterol-containing liposomes are prepared with DMPC instead of egg-PC (Fig. 8). It therefore appears that this phenomena can be related to the phospholipid composition of the liposomes. In this respect, it is important to note that the binding of AmB to egg-PC liposomes (with or without constituent sterols) reached saturation at rather low AmB/lipid ratios, a result not observed in DMPC liposomes [22].

The present data also indicate (Table I, column 5) that the increment in the rate constant for urea permeation across ergosterol-containing DMPC liposomes treated with 0.8 μM AmB is about one order of magnitud higher ($\Delta k = 0.72\ s^{-1}$) than for ergosterol-containing egg-PC liposomes treated with the same concentration of AmB ($\Delta k = 0.07\ s^{-1}$) at the same temperature (30°C). Such a differential enhancement by AmB of the process of urea permeation across DMPC/ERG liposomes as compared with egg-PC/ERG liposomes can be explained on the basis that DMPC liposomes have a thinner lipid bilayer than egg-PC liposomes. In fact, it is known that AmB channel formation is very sensitive to the fatty acyl chain length which determines the thickness of the bilayer in which the polyene is inserted [23,24]. The formation of an increased number of channels by AmB in DMPC/ergosterol liposomes may be also facilitated by the existence of an stronger rate of adsorption of monomeric AmB molecules into saturated liposomes as compared to unsaturated egg-PC liposomes [22,25].

Mechanism for the formation of aqueous pores by AmB across liposomes

In a previous attempt to elucidate the initial events that leads to the formation of aqueous pores by different polyene antibiotics, it was found that AmB as well as nystatin and candicidin were able to induce a significative enhancement of water permeability across er-

gosterol-containing liposomes [18] and *Leishmania* membrane vesicles [26] at much shorter times than those required for the onset of a significative salt permeability. It was also shown in that work that most of the AmB-induced enhancement of water permeability occurred below 0.5 μM AmB; beyond this concentration, no further increment of water permeability across such vesicles could be demostrated in spite that the corresponding salt permeability increased linearly from this concentration up to AmB concentrations as high as 3.2 μM [26].

On the basis of the present data, such an initial enhancement of water permeability can be ascribed to the primary interaction of AmB with membrane ergosterol leading to the formation of aqueous pores by the following sequential mechanism:

(a) At AmB concentrations lower than 0.5 μM, AmB interacts specifically with membrane ergosterol, leading to a transient enhancement of water permeability. Since AmB self-association is known to occur at 0.5 μM [27], it is proposed that AmB monomers are solely responsible of such an initial interaction with membrane ergosterol.

(b) The establishment of an AmB–ergosterol interaction also may leads to a local membrane rearragement that allows the insertion of AmB oligomers (dimers or tetramers) into the membrane to form non-aqueous channels.

(c) After a certain time, that may depend on various parameters such as the AmB/lipid concentration ratio and the membrane lipid composition, the non-aqueous channels interact with the entire lenght of the ergosterol molecules present in the membrane to form aqueous pores with an enlarged diameter.

Other authors have proposed the need of special conditions in the membrane phase in order to facilitate the penetration of polyene molecules i.e. an ordered membrane phase [13,14]. In the present mechanism, it is proposed that the initial interaction of AmB with ergosterol-containing membranes allows for the proper insertion and rearragement of AmB small oligomers, at relatively low AmB concentrations [28]. In this respect, the energy required for such an initial interaction of AmB with membrane ergosterol may account for the positive temperature dependence measured for the process of solute permeation across ergosterol-containing membranes (Table I, column 6). Such a positive temperature dependence was not observed for the corresponding urea permeation across cholesterol-containing liposomes(Table I, column 6). This finding is in agreement with the observation that in cholesterol-containing membranes, the interaction between AmB and the sterol is much weaker than in ergosterol-containing membranes [3,4,22]. It follows that in cholesterol-containing liposomes the participation of the sterol is restricted to the formation of the aqueous pores, with no role in the process of formation of the non-aqueous structures. As a result, non-aqueous AmB channels are formed in cholesterol-containing liposomes at concentrations wich are similar to those found in sterol-free liposomes(Table I).

In the absence of ergosterol, an ordering effect appears to be exerted by the AmB molecules themselves upon its interaction with the membrane/water interface. Support for this proposition can be found in the measured reduction of the basal permeability of the DMPC liposomes to urea at low AmB concentrations (< 0.8 μM)) (Fig. 5). This finding is also consistent with the observation that ergosterol-containing liposomes suspended in a hyperosmotic urea solution, exhibited a modest but significant enhancement of the total volume changes at V_{min}, with increasing AmB concentrations (Fig. 1, top).

Finally, the present sequential mechanism for the formation of aqueous pores by AmB provides a rationale for the observation that both the phospholipid composition and the type of sterol are involved in determining the rate of formation of such structures. The separate modulation of phospholipid composition and sterol content in different cell types may explains some of the complexities of AmB biological action.

Acknowledgements

This work was supported in part by CDCH-UCV Grant No. 03.10.2044.89 and Commission of European Communities, Grant No. C/1-0517-F (ED). I am grateful to Drs. H.V. Pollard, J. Bolard and J.A. Urbina for carefully reading the manuscript before publication.

References

1 Gale, E.F. (1984) in Macrolide Antibiotics. Chemistry, Biology and Practice (Omura, S., ed.), pp. 425–455, Academic Press, New York.
2 Bolard, J. (1986) Biochim. Biophys. Acta 64, 257–304.
3 Norman, A.W., Demel, R.A., De Kruijff, B., Geurts van Kessel, W.S.H. and Van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 290, 1–14.
4 De Kruijff, B., Gerritsen, W.J., Oerlemans, A., Demel, R.A. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 339, 30–43.
5 Finkelstein, A. and Holz, R. (1973) in Membranes, Vol. 2. Lipid bilayers and Antibiotics (Eisenman, G., ed. pp. 377–408, Marcel Decker, New York.
6 Andreoli, T.E. (1974) Ann. NY Acad. Sci. 235, 448–468.
7 De Kruijff, B. and Demel, R.A. (1974) Biochim. Biophys. Acta 339, 57–70.
8 Chen, C.C., Chou, D.L. and Feingold, D.S. (1970) Antimicrob. Ag. Chemother. 13, 914–917.
9 Danilenko, I.L. and Stepanyuk, V.V. (1982) Biochim. Biophys. Acta 691, 201–210.
10 Jirku, V., Cejkova, A. and Paca, J. (1981) Experientia 37, 39–40.
11 Ramos, H., Milhaud, J., Cohen, B.E. and Bolard, J. (1990) Antimicrob. Ag. Chemother. 34, 1584–1589.
12 HsuChen, C.C. and Feingold, D.S. (1973) Biochem. Biophys. Res. Commun. 31, 972–978.

13 Archer, D.W. (1976) Biochim. Biophys. Acta 436, 68–76.
14 Brajtburg, J., Powderly, W.G., Kobayashi, G.S. and Medoff, G. (1990) Antimicrob. Ag. Chemother. 34, 381–384.
15 Cohen, B.E. (1986) Biochim. Biophys. Acta 857, 117–122.
16 Cohen, B.E. and Gamargo, M. (1987) Drugs. Exptl Clin. Res. 13, 539–546.
17 Szoka, F. and Papahadjopoulos, D. (1979) Proc. Natl. Acad. Sci. USA 75, 4194–4198.
18 Cohen, B.E. (1983) in Liposome Letters (Bangham, A.D., ed.), pp. 127–135.
19 Hill, M.W. and Cohen, B.E. (1972) Biochim. Biophys. Acta 290, 403–407.
20 Cass, A., Finkelstein, A. and Krespi, V. (1970) J. Gen. Physiol. 56, 100–125.
21 Wietzerbin, J., Szponarki, W. and Gary-BoBo, C.M. (1990) Biochim. Biophys. Acta 1026, 93–98.
22 Milhaud, J., Hartman, M.A. and Bolard, J. (1989) Biochimie 71, 49–56.
23 Van Hoogevest, P. and De Kruijff, B. (1978) Biochim. Biophys. Acta 511, 397–407.
24 Kleinberg, M.E. and Finkelstein, A. (1984) J. Membr. Biol. 80, 257–269.
25 Chen, W.C. and Bittman, R. (1977) Biochemistry, 16, 4145–4149.
26 Cohen, B.E., Ramos, H., Gamargo, M. and Urbina, J. (1986) Biochim. Biophys. Acta 860, 57–65.
27 Mazerski, J., Bolard, J. and Borowski, E. (1982) Biochim. Biophys. Acta 719, 11–17.
28 Urbina, J.A., Cohen, B.E., Perozo, E. and Cornivelli, L. (1987) Biochim. Biophys. Acta 897, 467–473.