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Lipid and stress dependence of amphotericin B ion selective channels in sterol-free membranes

Tracy Ruckwardt, Angela Scott, Jessica Scott, Peter Mikulecky, Scott C. Hartsel *

Department of Chemistry, Phillips 461, University of Wisconsin-Eau Claire, Eau Claire, WI 54702-4004, USA

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Abstract

The idea that amphotericin B (AmB) may not require sterols to form ion selective channels has recently been criticized on the grounds that egg phospholipids commonly used in experiments may contain small amounts of sterol which associate with AmB to form AmB/sterol pore channel structures. It was recently shown in this laboratory that modest osmotic stress can enhance the formation of AmB channels in sterol-free egg phosphatidylcholine (eggPC) membranes. We have tested AmB's ability to form ion channels/defects in synthetic palmitoyl oleoyl (POPC), dieicosenyl (DEPC) and natural eggPC osmotically stressed large unilamellar vesicles (LUV) using pyranine fluorescence detected ion/H^+ exchange. These sterol-free POPC LUV exhibit greatly increased sensitivity to cation selective AmB channel formation when osmotically stressed; even more than eggPC. Under these stressed conditions, AmB activity was observed at [AmB]/POPC ratios as low as 3.5×10^{-4}, corresponding to about 34 AmB molecules/vesicle. DEPC vesicles were almost completely unresponsive, demonstrating a strong bilayer thickness dependence. These results prove conclusively that AmB can form sterol-free channels and do so within therapeutic concentration ranges (>0.5–10×10^{-6} M) in a stress-dependent manner. This phenomenon may allow us to use osmotic stress changes in simple model systems to spectroscopically isolate and characterize the thus-far elusive AmB channel forming aggregate. In addition, this stress dependence may be responsible for the potentiation of renal toxicity of AmB in the ascending branch of the loop of Henle which is under greatest osmotic stress. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Large unilamellar vesicle; Membrane-active antibiotic; Pyranine; Ion channel

1. Introduction

Amphotericin B (AmB) is a polyene macrolide antibiotic used to combat systemic fungal infections, yet its effectiveness as an antifungal agent is marred by a toxicity that can cause chills, fever, nausea, vomiting, and nephrotoxicity [1]. AmB's selectivity for fungi is thought to hinge on its ability to form ion channels selectively in ergosterol-rich fungal membranes. Its toxicity is due to the fact that it can also form ion permeable pores in cholesterol-containing membranes. Newer liposomal drug delivery mixtures have been developed and tested and seem to reduce the drug's toxicity by reducing the concentration of free AmB in solution to below a certain threshold for self-association (see [1] for a recent review).

Abbreviations: AmB, Amphotericin B; LUV, large unilamellar vesicle(s); SUV, small unilamellar vesicle(s); FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine; DEPC, 1,2-dieicosenyl-*sn*-glycero-3-phosphorylcholine; eggPC, egg phosphatidylcholine

* Corresponding author. Fax: +1 (715) 836-4979; E-mail: hartsesc@uwec.edu

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Many studies have shown that AmB channels, when formed from addition to a single side of the bilayer, are monovalent cation selective [2]. While certain distinct spectra arise from interaction of AmB with sterol containing membranes [3,4], it has been difficult to demonstrate that these spectral species are directly involved with ion channel structures. Recent work done in this laboratory has lead to the conclusion that sterols do promote, but are not necessary to produce highly cationic selective AmB channels [5]. Although evidence for channel formation without sterols is not new [4,6,7], this idea has generated some controversy. The most widely accepted and oldest AmB channel models have included sterols as 'staves' in a barrel-type structure [8,9] and a very thorough recent paper by Brutyan and McPhie [10] showed that similar single AmB channels may be formed in the presence of ergosterol or cholesterol by one-sided addition at very low antibiotic concentrations. However, the study showed that the cholesterol/AmB channels have a much shorter lifetime than ergosterol/AmB and considering the well-known higher binding affinity of AmB for ergosterol-containing membranes [11], this alone could account for the differential toxicity toward fungi. Furthermore, these authors state that studies purporting to show sterol-free AmB membrane activities might be due to contaminating sterols possibly present in natural, i.e. egg, phospholipids, or from using excessively high (beyond therapeutic levels, $> 10^{-4}$ M) AmB concentrations. Other studies have shown that there are similarities between the channels formed in cholesterol-containing and sterol-free membranes at clinically relevant concentrations (0.5–10 μM) [12–14]. This has led to the proposal that AmB solution self-association is necessary for activity against cholesterol-containing and sterol-free membranes but that ergosterol-containing membranes are also sensitive to monomeric AmB and may form stable channel forming aggregate structures with this sterol at much lower AmB concentrations [12]. This model is supported by the data of Cohen [14] and Lambing et al. [15] which proposes that AmB oligomers themselves may act as 'preformed' channels, independent of sterol, which may evolve with time into other types of channel structures, depending upon the lipid, temperature, and sterol composition.

In a previous study, we have show that sterol-free egg phosphatidylcholine (eggPC) LUV are largely unaffected by AmB under iso-osmotic conditions [5]. However, the imposition of even a small osmotic stress (< 100 ΔmOsm) catalyzed significant formation of ion selective AmB channels as detected by increases in fluorescently detected electrogenic K^+ currents. In the current study, we use synthetic chromatographically pure lipids to show that the channels formed are actually due only to AmB and not to sterol or other lipid contaminants. Furthermore, this activity occurs well within the therapeutic range of this drug. POPC is generally considered to be analogous to the 'average' egg phospholipid and so it was chosen as a synthetic analog of eggPC.

2. Materials and methods

2.1. Materials

All lipids were purchased from Avanti Polar Lipids, (Pelham, AL). Purified amphotericin B was a generous gifts from Bristol-Myers Squibb Pharmaceuticals (Princeton, NJ). FCCP (carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone) was obtained from Sigma (St. Louis, MO). Valinomycin, a K^+ ionophore, was purchased from CalBiochem (La Jolla, CA). Laser-grade pyranine (1,3,6-pryrenetrisulfonic acid) was purchased from Eastman-Kodak (Rochester, NY).

2.2. Preparation and assay of lipid vesicle samples

The lipids were then dispersed in a 15 mM K_2HPO_4, 2 mM pyranine buffer with either 100 mM K_2SO_4 or 200 mM KCl and varying amounts of sucrose, pH 7.20. The lipid dispersions were then freeze-thawed and extruded ten times through 2000 and 1000 Å Nucleopore filters using an extruder (Lipex, Vancouver, BC). The external pyranine solution was removed and exchanged with a 15 mM K_2HPO_4 buffer through gel filtration with Sephadex G-25, thus forming a salt and/or osmotic gradient across the membrane. FCCP (3 μM) was added to allow for free exchange of H^+ ions. The osmolality was varied with added sucrose and was determined using an automatic osmometer (μOsmette, Precision Systems, Natick, MA).

The initial osmotic difference between the vesicle lumen and external medium is expressed as ΔmOsm, where a positive sign indicates inside hyperosmotic.

2.3. Measurement of K^+, Cl^-, and net ion currents in LUV

Ion currents were measured with an On Line Instrument Systems (OLIS, Jefferson, GA) converted Durrum D-110 stopped-flow spectrophotometer in the fluorescence mode. Stopped-flow techniques for measuring ion currents using the pH sensitive dye pyranine as a reporter molecule have been developed over the past few years and the concentrations, instrumentation, data handling and interpretation and other experimental conditions were identical to those in Wolf and Hartsel [5] with the main difference being that phosphate buffers were used and salt gradients were created by column exchange rather than rapid dilution and the vesicles allowed to equilibrate without AmB for at least 15 min. For the current studies, AmB (1.3 mM stock in DMSO) was introduced to vesicles from a buffer identical to the column exchange buffer via stopped flow syringes to the final concentrations at ambient temperature (22 ± 2°C). The final total lipid concentration was 1.1 mg/ml. Our assay measures pH changes inside the LUV that result from the AmB induced K^+ currents when sulfate is the counter ion (sulfate is not permeant) and net current when Cl^- is the counterion since Cl^- may be permeant to a lesser degree depending upon the conditions [5]. With the incorporation of the protonophore FCCP into the LUV bilayers, H^+ can equilibrate rapidly across the membrane. Hence, the imposed salt gradients coupled with the ionophoric action of AmB cause a H^+ for K^+ (or Cl^-) exchange that is limited by the rate of AmB induced K^+ or Cl^- efflux. The pyranine molecules entrapped in the target LUV provide a sensitive fluorescence assay for detecting interior vesicular pH changes. The initial rate of pH change induced by AmB was used for comparisons of AmB's channel activity under different osmotic conditions and is expressed in ΔpH s^{-1}. The points represent the average initial slope of the ΔpH versus time of two to three trials. In each case, a baseline fluorescence change of LUV in the absence of AmBwas subtracted from the data to rule out non-specfic stress-induced defects as a source of the observed change in fluorescence. In the stress range and time frame studied, there was never any evidence of non-specific leakage of LUV contents.

3. Results

The pyranine fluorescence detected ion current method employed here has proven useful in detecting AmB-induced ion currents under inside hyperosmotic salt gradient and iso-osmotic conditions [5]. Fig. 1 shows the result of the exposure of synthetic sterol-free POPC vesicles to AmB under varying osmotic stresses ranging from 0 to +880 ΔmOsm. It is apparent that there is both a ΔmOsm and concentration dependence to electrogenic ion currents induced by AmB. POPC also showed a small but significant channel formation under iso-osmotic conditions. Additional concentration dependence studies with POPC LUV at +221 ΔmOsm showed that small, but significant, K^+ currents were already apparent at 0.5 μM, but not 0.1 μM AmB (data not shown). Fig. 2 shows a set of typical POPC experimental data for a +550 ΔmOsm gradient with KCl

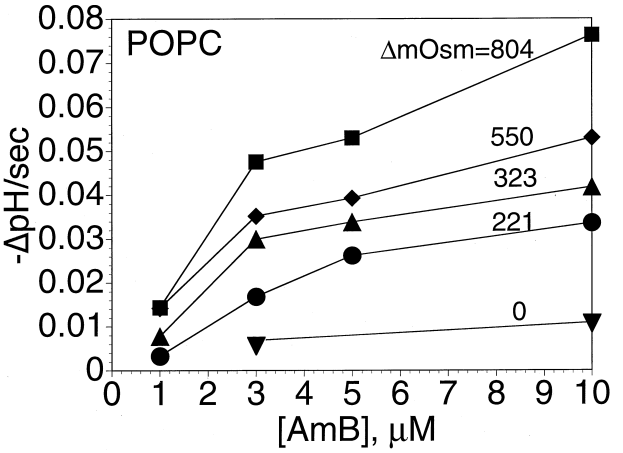

Fig. 1. Comparison of the initial pH change induced by 1.0, 3.0, 5.0 and 10 μM AmB added to 1.1 mg/ml sterol-free POPC vesicles with varying osmotic gradients (inside hyper-). The interior has 100 mM K_2SO_4, 15 mM K_2HPO_4, pH 7.20 and sucrose inside to produce an osmotic and K^+ gradient against the 15 mM K_2HPO_4, pH 7.20 external buffer. The ΔmOsm was measured experimentally. The rate of pH change, and hence K^+ currents, increases as a function of increasing [AmB] and ΔmOsm.

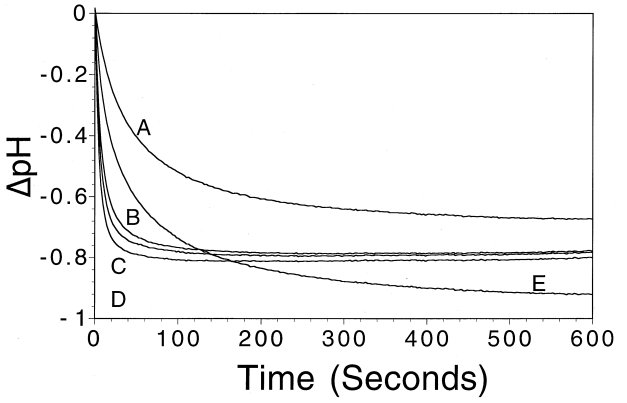

Fig. 2. Time course plot of the change in pH resulting from electrogenic K^+ ion current from sterol-free POPC vesicles with a KCl gradient. Leakage induced by (A) 1.0 μM AmB, (B) 3.0 μM AmB, (C) 5.0 μM AmB, (D) 10 μM AmB and 0.2 μM valinomycin (E) as a completely K^+ selective control. Vesicles contain 200 mM KCl, 15 mM K_2HPO_4 and sucrose inside to produce an osmotic gradient of +550 ΔmOsm against the 15 mM K_2HPO_4 external buffer. The direction of the fluorescence change in each case shows that $K^+ > Cl^-$ selective channels are formed by AmB in the presence of an osmotic gradient.

rather than K_2SO_4 as the internal salt. The generation of these currents in the quenching direction indicates a strong $K^+ > Cl^-$ selectivity as previously seen with eggPC [5].

Fig. 3 shows a comparison between stress-induced activity against eggPC, POPC and the 20:1 acyl chain phospholipid, DEPC, at +550 ΔmOsm. It is readily apparent that synthetic POPC membranes are even more sensitive than eggPC whereas DEPC is relatively insensitive.

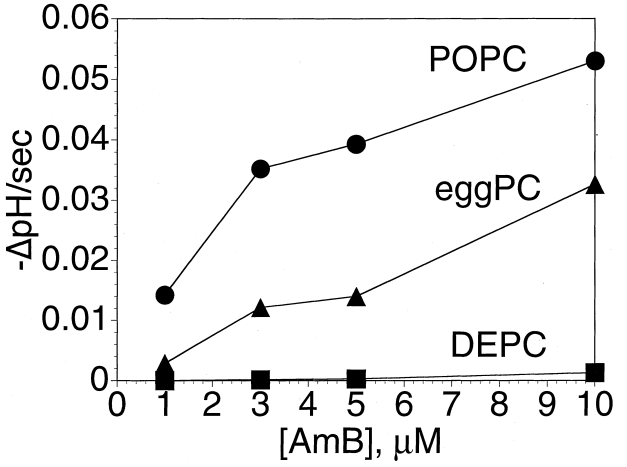

Fig. 3. The effect of lipid composition on K^+ currents. Conditions are the same as Fig. 1 at +550 ΔmOsm. Synthetic sterol-free POPC vesicles are even more sensitive than eggPC, whereas slightly thicker bilayers of DEPC (by ~3 Å) are practically insensitive.

The preceding data show that a relatively minor perturbation (increase of LUV internal sucrose), can cause a transition from a largely inactive AmB channel state to a very active state.

4. Discussion

Amphotericin B has long been a tantalizing target for a molecular understanding of ion channel function and selectivity. Since AmB seems to form multimeric aggregates in this process, it is more like a multisubunit biological protein channel than, e.g. gramicidin with its unusual wide single-helical pore. From a practical standpoint, AmB represents an important class of antimicrobial substances, membrane-active drugs, for which microbial resistance develops very infrequently. Substances such as the membrane-active antimicrobial peptides, e.g. magainins, offer hope of new antibiotics in an era of alarming increase in antibiotic resistance. However, even some of the basic features of the membrane channel/disruption formed from AmB have remained refractory and only one X-ray structure (a heavy atom derivative) of AmB has ever been solved, that being over 25 years ago [16].

The sterol-selective disruption/toxicity of AmB has formed the basis of a long-lived model for AmB as a heteromeric sterol-containing pore. However, there has been ample evidence that AmB can form sterol-free membrane pore/disruptions under some conditions [4,6,7]. Often these are dismissed as being the result of a general detergency effect found with any amphiphile at sufficiently high concentrations[10]. In addition, the use of natural lipids or mixtures in many experiments opens up the possibility for channels forming with small amounts of contaminating sterol. The data in Figs. 1–3 here for the synthetic POPC LUV's conclusively proves that specific AmB channels can form in the absence of sterols and that they can form at concentrations ($\geq 5.0 \times 10^{-7}$ M) where a gross detergency effect is unlikely. With POPC, [AmB]/[lipid] ratios as low as 4×10^{-4} show measurable K^+ currents. If vesicles are assumed to

have a 1000 Å diameter, the surface area occupied by one lipid is 60 $Å^2$ and membrane thickness is 45 Å, this threshold corresponds to about 34 AmB/vesicle.

How can these data be reconciled with the data of Brutyan and McPhie [10] and a long line of other planar bilayer experimentalists ([2,8,9] and references within [17])? These and other workers have consistently failed to observe single AmB channels in the absence of sterol. Firstly, AmB concentrations use to see single channel activity in planar bilayer studies are often exceedingly low ($< 10^{-7}$ M) and possibly sterol is necessary to stabilize channel formation at these low concentrations. Secondly, it has been shown that AmB single channels in cholesterol-containing bilayers were very short lived as compared to ergosterol [10]. It is certainly possible, then, that channels in sterol-free bilayers are even more short lived and/or rare and thus not easily detectable, especially at low [AmB]. In principle, our method can detect very small ion currents in the tens of picoampere/cm^2 range and we are sampling very large membrane surface areas (about 2–3 m^2/ml sample assuming 60 $Å^2$ area/lipid). Thirdly, membrane thickness is extremely important. Fig. 3 shows that a relatively small increase of $\sim$3 Å in overall bilayer thickness from $\sim$43 Å (eggPC, POPC) to 46 Å (DEPC) [18] is sufficient to practically eliminate sterol-free membrane activity. Planar bilayers formed by spreading in solvent could exceed this thickness due to hydrocarbon sequestered in the membrane interior. This precise and dramatic thickness dependence further suggests discrete sterol-free AmB (aggregate) channel structures must be responsible for the ion currents as compared to a general lytic/detergency effect. Fourth, AmB can readily form these sterol-free channels only under special circumstances; in the presence of osmotic stress [5] or with sonicated SUV [6,7,19]. In non-stressed LUV systems there was indeed very little activity for eggPC at concentrations of 10^{-5} M or less [5]. Finally, until single AmB channels are observed in the absence of sterol and their properties characterized and compared, it cannot be ruled out that there exist *both* sterol and non-sterol-containing pore structures and that the relative preponderance of each is a function of several physical, temporal and lipid composition factors, not unlike the models of Cohen[14] and Bolard's group [12].

If there exist sterol-independent channels, how can selectivity toward sterol-containing membranes and discrimination among sterols be rationalized? If there are multiple channel forms or differential stability as proposed above, there is no problem with observations of different sterol-dependence and selectivity of AmB channels under a variety of conditions. Even without invoking multiple channel structures, there are many cases in the literature of amphiphilic molecules, not thought to form barrel-stave channels, whose membrane activity can be modulated in either direction by sterols [20,21]. In addition, there is intriguing evidence that antibacterial selectivity of small basic amphipathic peptides may be due to the greater sensitivity of sterol-free membranes to their action [22,23].

Potentially, the most interesting aspect of the osmotic stress-induced AmB channels is the possibility of using this property to isolate and characterize the active channel AmB structure in a simple model system. Weakliem et al. [24] have formulated a two-dimensional 'micellization' theory to explain the osmotic stress induced sensitization of SUV composed of cholesterol, hydrogenated soy phosphatidylcholine, disteroyl phosphatidylglycerol and AmB (termed AmBisome) which is certainly consistent in principle with our results. Recently, Fujii et al. [19] have extended this study and correlated the onset of membrane activity with steady state CD and absorption spectra of an aggregated form of AmB in an iso-osmotic AmBisome system. The threshold for AmB activity in this AmBisome system was about 16 AmB/vesicle; about half the number observed in this report for sterol-free POPC vesicles (34 AmB/vesicle) consistent with a sensitizing effect for sterol. Studies are now under way in our laboratory to attempt to correlate kinetically the absorption and CD species observed in our sterol-free LUV spectra with the onset of membrane activity.

In addition, there is a potential pharmacological relevance to these results. One of the most devastating side effects of AmB is the serious and sometimes permanent nephrotoxicty. While it is clear that AmB damages membranes of fungi and to a lesser extent mammalian cells, the specific sites and mechanisms of nephrotoxicity are not entirely clear and have been recently reviewed [25,26]. A recent report from this laboratory [5] coupled with several other in vivo observations may provide some explanation

and guidance for future research. It is known that the filtrate within the medullary thick ascending limb of the loop of the nephron (Henle) is significantly hypo-osmotic relative to the peritubular fluids. Since this segment is only weakly permeable to water, there must be considerable osmotic stress on the cells of the tubules and presumably their membranes. In accordance with the enhanced toxicity of AmB to stressed membranes in vitro, it has been demonstrated in the perfused rat kidney that this is precisely the region most damaged by AmB [27]. Furthermore, AmB accumulates to a much greater extent in sodium depleted perfused rat kidneys as compared to kidneys perfused with excess sodium [28]. Sodium loading can also, to a limited extent, prevent chronic AmB toxicity in rats and humans (see refs. 96, 97, 169, 170–172, 173 in [25]). It is possible that these effects are due to reduction (or increase) of the relative osmotic gradient in the ascending loop and corresponding reduction or increase in the accumulation and membrane-activity of AmB. In accordance with this model, two less toxic derivatives, AmB methyl ester (AME) and nystatin were shown to be nearly inactive (a least at physiologically low concentrations) against osmotically stressed membranes in sharp contrast to AmB [5]. It has been shown in the rhesus monkey that AME is distributed at 24 h at 3–10×*higher* concentrations in the kidney relative to AmB yet its renal toxicity is lower [29]. Selective activity against osmotically stressed membranes may be a valuable marker for toxic AmB derivatives and preparations (and other drugs exhibiting renal toxicity, such as cyclosporin) or in the design of novel antifungal substances.

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