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Amphotericin B toxicity and lethality: a tale of two channels

B.E. Cohen *

Laboratory Cell Biology and Genetics, NIDDK, NIH, Bethesda, MD 20892, USA
Institute of Experimental Biology, Faculty of Sciences, Central University of Venezuela, P.O. Box 47860, Caracas 1041, Venezuela

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Abstract

In spite of its toxic side effects, Amphotericin B (AmB) is the most effective drug in the treatment of visceral Leishmaniasis and systemic fungus infections. However, the toxic side effects of this drug can be substantially reduced when delivered as a complex with liposomes and other lipidic systems. Nonetheless, the mechanisms of AmB antifungal activity and of toxicity to the host are not yet fully understood. The classical pore model of AmB action postulates that a single type of channel permeable to monovalent cations and anions is responsible for the AmB fungicidal and leishmanicidal action, as well as for its toxic side effects. Thus, the loss of membrane cation selectivity, caused by AmB aqueous pores has been proposed as the primary cause of fungal cell death. The hypothesis has been that fungal cell death results from intracellular acidification associated with K^+ leakage. However, this mechanism of AmB action has been challenged by several investigators on the basis that low AmB concentrations cause K^+ leakage from sensitive cells which is dissociated from the lethal effects produced by higher AmB concentrations. Our own studies indicate that the formation of aqueous pores by AmB in sterol-containing liposomes is always preceded by the formation of non-aqueous channels. At low AmB concentrations or in the absence of sterols, AmB non-aqueous channels do not evolve to form aqueous pores, nor is the K^+ leakage they produce lethal to cells. It is only when a 'critical' concentration of AmB is reached at the membrane that non-aqueous channels interact with ergosterol or cholesterol to form transmembrane aqueous pores. The pore diameter of the channels formed by AmB is critical for AmB toxicity or lethality because non-aqueous channels are only permeable to urea and monovalent cations, whereas AmB aqueous pores are permeable to monovalent cations and anions (including H^+ and OH^-) and divalent cations such as Ca^{2+}. In fact, *leishmanias* are killed rapidly by colloid osmotic lysis due to a net salt influx across the AmB aqueous pores. Fungal cells are protected from osmotic lysis by the presence of a cell wall, but an increased H^+/OH^- permeability across AmB aqueous pores leads to an elevation of intracellular pH which then results in membrane damage. In host mammalian cells, non-aqueous channels appear to be responsible for some of the toxic but reversible side effects produced by AmB. However, more acute and damaging effects such as those exerted by AmB in kidney tubular cells may be caused by increased salt, Ca^{2+} and/or H^+ permeability across aqueous pores. A sustained collapse of pH and Ca^{2+} gradients is a mechanism which is also exhibited by molecular inducers of programmed cell death (apoptosis) in eucaryotic cells. © 1998 Elsevier Science B.V. All rights reserved.

* Correspondence address. Department of Anatomy and Cell Biology, Uniformed Services University School of Medicine, Building B, Room B2200, 4301 Jones Bridge Rd., Bethesda, MD 20819, USA. Tel.: +1 301 2953200; fax: +1 301 2951715.

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1. Introduction

The polyene antibiotic Amphotericin B (AmB) is a natural product of *Streptomyces nodosus*, an actinomycete which was isolated from soil collected from the Orinoco river in Venezuela (Gold et al., 1956). Amphotericin B is a member of a complex group of macrocyclic compounds that are well known because at very low concentrations they induce a lethal effect against fungus and parasitic protozoa such as *Leishmania* sp. (Gale, 1984; Bolard, 1986; Brajtburg et al., 1990). Early studies on the mechanism of action of amphotericin B (AmB) and nystatin revealed that these compounds were able to induce changes in ion, water and non-electrolyte permeability across sterol-containing planar lipid bilayers, which were consistent with the formation of aqueous pores of 4-Å radius (Finkelstein et al., 1973; Andreoli, 1974). Solutes larger than glucose were excluded from the AmB aqueous pores but glucose itself and other small non-electrolytes such as urea, glycerol and ethyleneglycol permeated with reflection coefficients varying from near to unity in the case of glucose to about 0.5 or less in the case of urea and glycerol (Finkelstein et al., 1973; Andreoli, 1974).

The mechanism of action of AmB against fungi is also based on a specific interaction with ergosterol present in the plasma membranes of these organisms (Lampen, 1969). Thus, according to the pore hypothesis of AmB action, the formation of AmB/ergosterol aqueous pores causes the plasma membrane to become leaky to K^+ and eventually cell death ensues (Lampen, 1969; Gale, 1984). However, the pore hypothesis of AmB action has been challenged by several investigators on the basis that the AmB-induced K^+ leakage from fungi is dissociated from the lethal effects (Chen et al., 1978; Brajtburg et al., 1984; Sokol-Anderson et al., 1986; Beggs, 1994). Thus, in *Candida albicans*, AmB concentrations as low as 0.16–0.2 μM cause a leakage of 98% of the internal potassium, but no killing effects were detected (Sokol-Anderson et al., 1986; Beggs, 1994). The observation of a separation between K^+ leakage and lethality in the cellular effects of AmB have therefore raised doubts about the conclusion that aqueous pore formation by this antibiotic is the central event in its lethal action against fungi and other sensitive cells. However, such a dissociation between an AmB-induced permeabilizing event and lethality may simply reflect the antibiotic capacity to form two types of channels, differing in intrinsic toxicity for sensitive cells.

2. Studies of the concentration and time dependence of the AmB-induced permeability changes across liposomes revealed the formation of two different types of channels

It has been known for some time that the permeability alteration induced by AmB across a variety of cells is concentration and time dependent (Deuticke et al., 1973 and references therein). In order to investigate the existence of possible functional intermediates in the spontaneous insertion process leading to the formation of AmB aqueous pores, we have carried out systematic studies on the concentration and time dependence of the AmB-induced permeability changes induced across liposomes by using a rapid mixing technique (Cohen, 1986; 1992). It was first found that when 0.2 μM AmB was added to ergosterol-containing liposomes, the polyene antibiotic did not exert any enhancement of the glucose permeability unless such vesicles were previously pre-incubated with AmB for 2–4 min. By contrast, no incubation of liposomes with AmB was required in order to measure an increase of urea permeability by the same AmB concentration (Fig. 1, left). Under these conditions, the reflection coefficient of urea across AmB-treated liposomes remained at a constant value of 0.8, up to an AmB concentration of 0.8 μM (Fig. 1, right). Beyond this concentration, AmB not only decreased by half

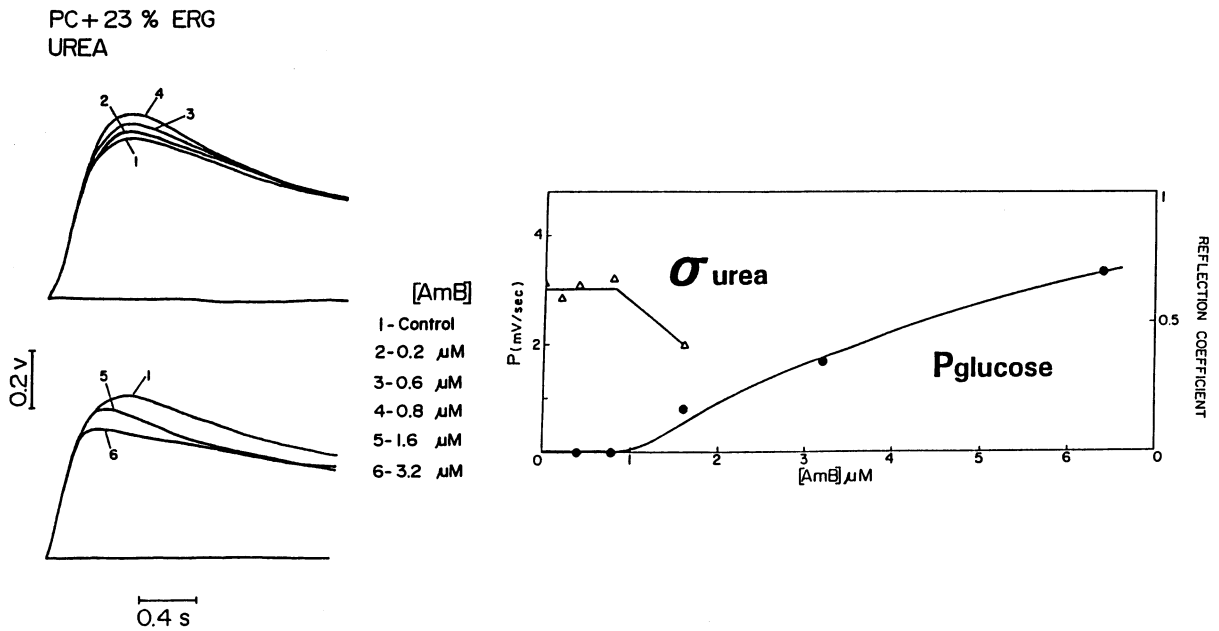

Fig. 1. Dose-dependent formation of two different types of channels by AmB across ergosterol-containing liposomes. (Left) The effect of increasing AmB concentrations on the volume changes of liposomes suspended in an hyperosmotic urea solution. (Right) The corresponding urea reflection coefficient (σ_{urea}) and glucose permeability ($P_{glucose}$) at increasing AmB concentrations. Liposomes were prepared as described previously (Cohen, 1992) from egg-PC and ergosterol (84:16, molar ratios) in a 60 mOsm buffer phosphate solution (pH 7.0). At time zero, they were mixed (1:4, volume ratios) with a 600 mOsm urea or glucose solution using a rapid mixing stopped-flow apparatus. Volumetric changes of the liposomes were determined by following 90° light scattering changes at a constant temperature (20°C). Values of σ_{urea} at different AmB concentrations were determined by measuring the ratio between the initial volume flow (J_v) induced by the hyperosmotic urea and glucose solutions ($\sigma_{urea} = (J_v^{urea}/J_v^{glucose})_{t\to 0}$). Glucose permeabilities at increasing AmB concentrations ($P_{glucose}$) were determined by the 'maximum slope' method as described previously (Cohen, 1986; 1992).

the reflection coefficient of urea but also induced a progressive enhancement of the glucose permeability across liposomes (Fig. 1, right). This set of data indicates clearly that the formation of aqueous pores by AmB is preceded by the formation of non-aqueous pre-pore structures. It also indicates that the AmB aqueous pore has an increased pore radius compared to those channels which formed at lower concentrations or shorter times after mixing.

It was also demonstrated that the two types of channels formed by AmB across liposomes are equilibrium structures that differ in cation/anion selectivity (Ramos et al., 1996). For this purpose, we have measured membrane diffusion potentials using fluorescent probes. This method is based on the observation that the fluorescence of 3-3′-dipropylthiadicarbocyanine ($DiSC_3(5)$) is rapidly quenched whenever it concentrates into liposomes by the formation of an inside negative diffusion potential. Thus, by adding valinomycin to KCl-containing liposomes suspended in a sucrose solution, K^+ moves out of the vesicles and a maximum quenching was obtained. Upon addition of AmB, two types of behavior were observed: at 0.1 μM AmB or lower concentrations, there is a quenching of the probe fluorescence that reached equilibrium within a few seconds. However, beyond this concentration, the initial quenching of the fluorescence probe is reversed by an enhancement which occurred more rapidly at increasing concentrations (see Ramos et al., 1996; Fig. 1). On this basis, it was concluded that, at concentrations lower than 0.1 μM, the AmB channels formed by AmB are permeable only to monovalent cations such as K^+ ions, whereas

beyond this concentration, they became permeable to K^+ and Cl^- ions. In fact, as it was shown for the AmB-induced urea permeability (Cohen, 1986), the effectiveness of TEA to block the K^+ channels formed by low AmB concentrations decreased upon the formation of aqueous pores (Valdivieso, 1992), a finding which is consistent with an increase of the pore radius of the aqueous structures formed.

A dose-dependent formation of the two types of AmB channels has also been observed in liposomes prepared with cholesterol (Cohen, 1992; Hartsel et al., 1994). However, for such liposomes, somewhat greater AmB concentrations are required to induce the formation of aqueous pores, as indicated by a requirement of 0.5 μM AmB for the reversal of the membrane potential changes monitored by $DiSC_3(5)$ fluorescence (Valdivieso, 1992; Hartsel et al., 1994). By contrast, when liposomes were prepared in the absence of sterols, the AmB channels formed were only permeable to cations and no reversal of membrane polarity was ever observed in spite of adding AmB at concentrations as large as 10 μM (Valdivieso, 1992). These results clearly indicated that the presence of either ergosterol or cholesterol is strictly required for the formation of aqueous pores permeable to salts (Cohen, 1992).

3. Formation of two types of channels by AmB across *Leishmania* promastigotes

Differential alterations of the cell membrane potential by increasing AmB concentrations—a distinctive characteristic of the formation of two types of channels by AmB, were also measured in *Leishmania* promastigotes (LPs) (Ramos et al., 1996). Thus, it was shown that when 0.05 μM AmB or higher concentrations were added to LPs suspended in an iso-osmotic NaCl solution, the cell membrane potential collapsed (Fig. 2, bottom). This finding indicated that low AmB concentrations were able to increase Na^+ permeability leading to membrane depolarization. On the other hand, when LPs were suspended in a sucrose-containing iso-osmotic solution, membrane depolarization by efflux of anions also occurred. However, such a depolarization did not occur until AmB concentrations were higher than 0.1 μM AmB (Fig. 2, top). These findings are thus fully consistent with the differential cation/anion selectivity that was exhibited in liposomes by the two types of AmB channels.

4. *Leishmania* promastigotes are killed by AmB at concentrations greater than 0.1 μM

Leishmania mexicana promastigotes (LPs) can be grown in a liquid medium with a mean genera-

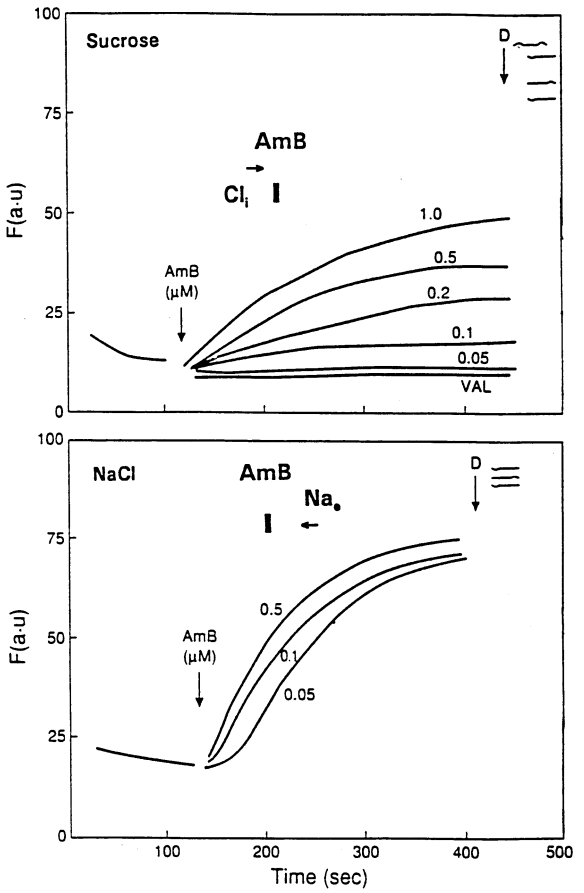

Fig. 2. Concentration and time-dependence of the AmB-induced fluorescence changes of $DiSC_3(5)$ across *Leishmania* promastigotes. Cells (25×10^6 cells/ml) were suspended in iso-osmotic solutions of sucrose (A) and NaCl (B), containing 10 mM glucose. Maximal $DiSC_3(5)$ incorporation was determined by adding digitonin (arrow D). Taken from Ramos et al. (1996).

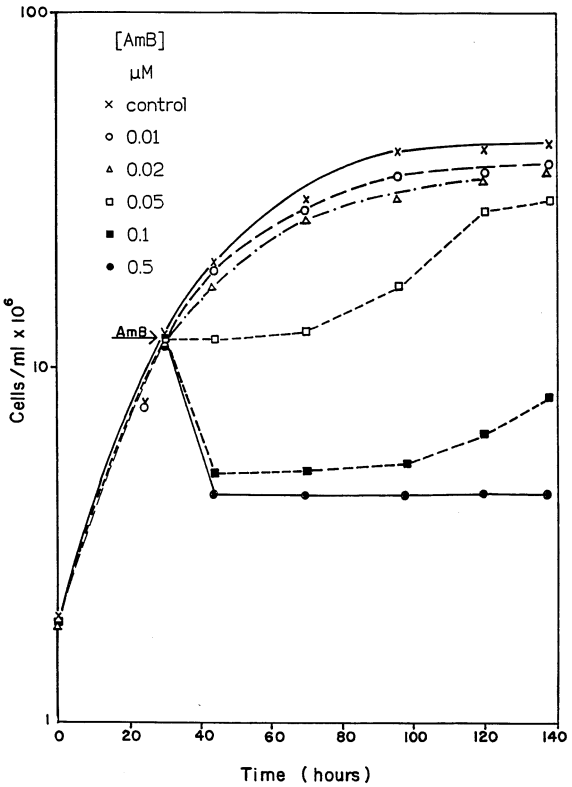

Fig. 3. Growth of *L. mexicana* promastigotes in a liquid medium as a function of AmB concentration. The number of parasites/ml as a function of time was followed by turbidimetry. For more experimental details see Ramos et al. (1995).

tion time of about 20 h (Fig. 3). When AmB (dissolved in DMSO) was added to this culture in the exponential phase of growth, there was an inhibition of the cell growth rate at a concentration of 0.05 μM (Fig. 3). This is the same concentration of AmB that was shown to alter membrane potential across LPs suspended in an iso-osmotic NaCl solution (Fig. 2, bottom). However, it can be observed in Fig. 3 that after a time interval of about 10 h with no growth, these LPs cells recovered and continued growth at the same rate as the control cells.

The results of Fig. 3 also show that by adding 0.1 μM AmB to LPs, an abrupt decrease of the number of cells occurred. In effect, direct microscopic observations carried out about 10 h after adding the polyene antibiotic indicated that after this time of drug exposure, very few cells remained intact in the culture. Instead, a large amount of cell debris was observed at the microscope, indicating that a substantial cell lysis by exposure to AmB has occurred.

A convenient method to study the rapid kinetics of cell death induced by AmB against *leishmanias* is to use fluorometry to follow the incorporation of ethidium bromide (EB) (Cohen et al., 1990). Thus, after a characteristic lag time lasting several minutes, *leishmanias* treated with AmB exhibited a very rapid incorporation of EB but only by adding AmB concentrations higher than 0.1 μM. In effect, observations by combined phase/fluorescence microscopy revealed that exposure of LPs suspended in an iso-osmotic NaCl solution to 0.1 μM or higher AmB concentrations led within minutes to changes in the typically elongated promastigote forms to rounded forms. This process is followed by cell lysis (Cohen et al., 1990).

5. *Leishmania* promastigotes are killed by colloid osmotic lysis due to a net salt influx across AmB aqueous pores

The lethal effect that is induced by AmB against LPs is driven by the rate of salt permeation across aqueous pores formed by the antibiotic in the LP plasma membrane. Thus, when the permeability properties of the AmB aqueous pores were measured directly by using vesicles (LMVs) prepared from the plasma membrane of LPs (Cohen et al., 1986; Cohen and Gamargo, 1987; Ramos et al., 1996), it was found that 0.1 μM and higher AmB concentrations were able to induce an anion selectivity across LMVs which followed the sequence: $SCN > NO_3 > Cl > I > Br >$ acetate (SO_4^{2-} being impermeable). This was the same order that was observed for AmB-induced rate of cell killing when LPs were suspended in different salt solutions. Furthermore, the shape changes of LPs and the subsequent osmotic cell lysis induced by AmB can be prevented completely if the external saline medium is replaced with solutes that can not pass through the aqueous pores. For example, substitution of external Na^+ by choline$^+$, Cl^- by SO_4^{2-} or the

partial replacement of NaCl by sucrose led to a complete inhibition of both the AmB-induced rounding up and incorporation of EB into LPs (Ramos et al., 1996).

The existence of a causal connection between aqueous pore formation by AmB and its lethal action on LPs was further demonstrated by the effect of TEA on the AmB-induced EB incorporation. Thus, TEA lengthened the time lag of AmB-induced EB incorporation, in direct proportion to its concentration (Ramos et al., 1996). Such an effect was anticipated since TEA was known to block AmB-induced aqueous pores by blocking the pore entrance (Borisova et al., 1979; Cohen, 1986; Cohen and Gamargo, 1987). However, the subsequent AmB-induced rate of EB incorporation across LPs was also blocked by TEA but in a saturable way ($K_i = 2$ mM). Since ethidium$^+$ and TEA$^+$ are cations impermeable across AmB aqueous pores, this finding indicated that at this stage such compounds are incorporated by diffusion across larger pores or fractures which are formed across the membrane by disruptive osmotic forces.

6. The sterol hypothesis and the formation of two types of channels by AmB across *Leishmania* promastigotes

The production of ergosterol in LPs is strongly inhibited by ketoconazole, an azole derivative which acts by blocking the cytochrome P_{450}-dependent C-14 demethylation of lanosterol (Berman et al., 1984). Such an effect by ketoconazole leads to a substantial accumulation of 14-α-methylsterols in the plasma membrane of LPs and as a consequence of this, to an inhibition of the leishmanicidal action exerted by AmB (Ramos et al., 1994). It was also found also that AmB was unable to increase the permeability of monovalent salts across membrane vesicles prepared from ketoconazole-treated LPs (Ramos et al., 1994). This set of results demonstrated an essential requirement for the presence of ergosterol in LPs plasma membranes for the formation of AmB aqueous pores and subsequent cell lysis.

However, we have also found that LPs which have been transformed by an elevation of temperature into amastigote-like forms (Hunter et al., 1982) are killed more rapidly by AmB than normal LPs, in spite of a 4-fold reduction in the cell ergosterol content (Ramos et al., 1990). Thus, measurements of ergosterol/phospholipid molar ratio in LPs cells indicated a decrease from 0.32 ± 0.06 in control cells grown at 25°C to 0.08 ± 0.01 in cells incubated for 6 h at 35 °C (Ramos et al., 1990).

In order to investigate what other changes at the membrane level occurred in LPs after this heat-induced transformation, we measured the total membrane binding of AmB by using a fluorescence energy transfer method (Ramos et al., 1994). The results obtained indicated that membrane bound AmB decreased both in heat-transformed and ketoconazole-treated LPs as compared to control cells. However, the amount of membrane bound AmB in heat-transformed cells was greater than in ketoconazole-treated LPs. In addition, we have also observed that a Scatchard plot of the binding of AmB to *leishmania* membrane vesicles (LMVs) was normal (non-co-operative) when LMVs were prepared from control cells but it changed to a co-operative type of binding when LMVs were prepared from heat-transformed cells (Ramos et al., 1990). Clearly, AmB molecules have a different form of insertion into the membrane of LPs when they are transformed by heat into amastigote-like cells.

The transformation of LPs by heat also leads to concomitant changes in the degree of saturation of the plasma membrane fatty acyl groups (Beach et al., 1982). These findings suggest a mechanism by which the lytic action by AmB on heat-transformed LPs with a reduced ergosterol content-can occur more rapidly than in control LPs. Thus such an effect may be due to changes in the bilayer lipid composition which may facilitate the initial insertion and formation of non-aqueous channels.

In order to test this hypothesis, we have investigated the effect of lipid composition on the formation of non-aqueous channels by AmB by measuring the corresponding urea permeability across liposomes (Fig. 4). Thus, it can be observed

in Fig. 4 that egg-PC liposomes, prepared in the absence of sterols, support a very limited enhancement of urea permeability by AmB. However, permeability increased in the presence of rising concentrations of ergosterol in the egg-PC liposome membranes. By contrast, when liposomes were prepared from saturated dimyristoyl-PC (DMPC), AmB was not only able to induce a significant permeation of urea in the absence of sterols but now the incorporation of ergosterol to these saturated liposomes led to a drastic reduction of urea permeability (Fig. 4). We can conclude from this data that the insertion of AmB molecules into the lipid bilayers to form non-aqueous channels not only can occur substantially in sterol-free saturated liposomes (above its transition temperature) but that the presence of ergosterol itself can also modulate the amount of channels formed depending on the acyl composition of the phospholipid chains. This set of data also provides an explanation for the observation that heat-transformed LPs, by incorporating an increased proportion of saturated acyl chains into its membrane phospholipids, can retain its susceptibility to AmB action, in spite of a drastic reduction of ergosterol content.

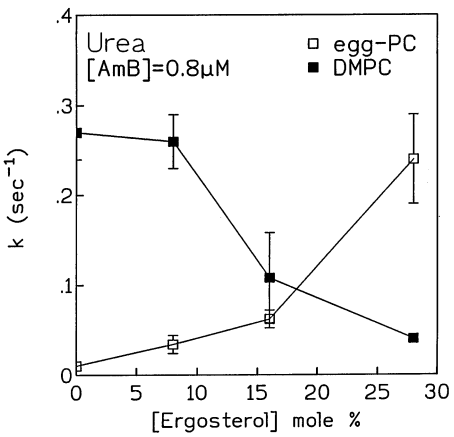

Fig. 4. The effect of membrane ergosterol content on the urea permeability induced by AmB across liposomes prepared from different phospholipids. Liposomes were prepared from egg-PC/ergosterol (□) or dimyristoyl-PC (DMPC)/ergosterol (■) at different molar proportions. All measurements were carried in the stopped-flow apparatus by mixing liposomes (prepared in 60 mOsm buffer phosphate) with a 600 mOsm hyperosmotic urea solution containing 0.8 μM AmB. Temperature = 30°C. For further experimental details see legend to Fig. 1.

7. Formation of two types of channels by AmB in fungal cells

The lethal action of AmB against fungus is known to exhibit a relatively slow kinetics compared to LPs cells. Thus, it takes about 2 h and a pre-incubation step lasting 3–5 h in order for EB to enter 90% of *C. albicans* cells with 2.5 μM AmB (O'Gorman and Hopfer, 1991). In effect, using a conventional tube dilution assay an incubation time of 24 h is usually required to evaluate the activity of AmB against *C. albicans* (Pfaller et al., 1990). Under these conditions, these authors have demonstrated that the minimum inhibitory concentration ranges from 0.18 to 0.5 μM. By contrast, incorporation of EB into *L. mexicana* is already completed 30 min after adding 0.5 μM AmB (Ramos et al., 1996). Such a slow fungicidal effect by AmB reflects the presence in such organism of a cell wall that protects them against osmotic lysis. Thus, in protoplasts prepared from *C. albicans*, a rapid K^+ leakage followed by lysis, can be measured by adding only 0.25 μM AmB (Sokol-Anderson et al., 1986). This finding indicates that the permeability changes accompanying the formation of AmB aqueous pores are not limiting the killing process of this antibiotic in fungi.

However, the specific changes of membrane permeability induced by the formation of AmB aqueous pores in fungal cells, which eventually lead to cell death, are not yet completely clear (see Brajtburg et al., 1990). In this respect, in one of the first cellular studies on the action of polyene antibiotics on fungal cells (Lampen, 1969), it was suggested that the leakage of K^+ was accompanied by an influx of protons. The resulting intracellular acidification was considered responsible of the fungicidal effect. Some years later, it was demonstrated that AmB and nystatin inhibited the proton-coupled transport of maltose in *Saccharomyces cerevisae*, by increasing the proton permeability of the yeast membranes (Palacios and Serrano, 1978). On this basis, Palacios and Serrano (1978) argued that the primary effect of AmB (and nystatin) on fungal cells was the dissipation of the proton gradient, and that protons could be the species whose permeability would be

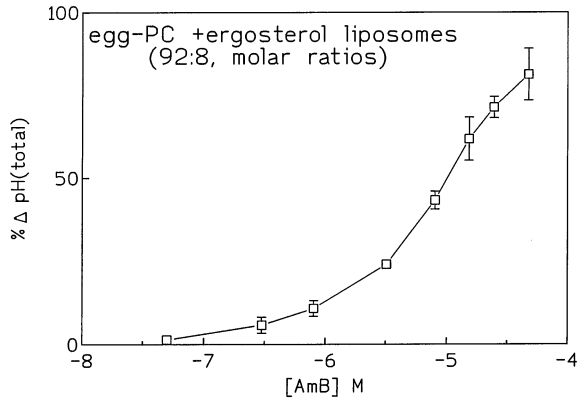

Fig. 5. The effect of increasing AmB concentrations on the cation–proton exchange across liposomes. Cation–proton exchange induced by AmB was measured at 25°C by a modification of the procedure described by Deamer and Nichols (1983). Liposomes were prepared as described previously (Cohen, 1992) from egg-PC and ergosterol (92:8, molar ratios) in a 40 mM aspartic acid buffer solution (pH 4.0) containing 250 mM K_2SO_4. Aliquots of liposomes (2 ml at 1 mM lipid concentration) were added to a 3-ml glass vial provided with continuous magnetic stirring The external pH was adjusted to pH 7.0 with KOH 1 N. After obtaining a stable external pH, appropriate amounts of a stock solution of AmB (in dimethylformamide) were added and the cation–proton exchange was determined recording continuously the acidification of the external solution. Total exchangeable protons were determined in an identical sample by adding Triton X-100 (0.2%).

firstly increased by AmB due to its smaller size and greater mobility in solution (Palacios and Serrano, 1978).

Recently, the time course of intracellular acidification and K^+ efflux induced by AmB in *C. albicans* was followed by in vivo ^{31}P NMR and K^+ atomic absorption (Rabaste et al., 1995). These authors found that most of the H^+ movement occurred after K^+ leakage was completed, a finding that contradicts the former conclusion that the primary effect of AmB was to increase the proton permeability of fungal cells.

We have also carried out in our lab, studies on the effect of AmB on the pH gradient held across ergosterol-containing liposomes (Fig. 5). The results obtained indicate that at low concentrations ($< 0.1\ \mu M$), the non-aqueous channels formed by AmB exhibit a low ability to increase the proton permeability across the liposome membranes. Previous studies in ergosterol-containing small unilamellar liposomes (SUV) have also indicated that the K^+-selective but anion impermeable channels which are formed by AmB across such liposomes exhibited a low H^+ permeability (Hartsel et al., 1991). However, as the results shown in Fig. 5 indicate, at AmB concentrations greater than 0.1 μM, the pH gradient abruptly collapses, thus reflecting an increased K^+/H^+ exchange across the AmB aqueous pores formed. Such dose-dependent effects of AmB action on ergosterol-containing liposomes are entirely consistent with the observation that K^+ leakage preceded intracellular acidification in fungal cells treated with increasing AmB concentrations (Rabaste et al., 1995). Nonetheless, it is important to note that the fungal cells metabolically regulate its pH gradient and as a consequence of this, variations in intracellular pH induced by AmB can be slower than expected even at higher doses of AmB.

The molecular pathway by which AmB-induced intracellular acidification leads to fungal cell death is not known. However, studies in a variety of eucaryotic cells have revealed that alterations in cellular ionic gradients, including intracellular acidification, may activate several major pathways leading to loss of membrane integrity. Among such pathways can be included those leading to the degradation of membrane phospholipids and the generation of active oxygen species and free radicals (Buja et al., 1993). In this respect, there is strong evidence indicating a role of active oxygen species in modulating the lethal effects of AmB against fungi after aqueous pores are formed. Thus, it was shown by Sokol-Anderson et al. (1986) that while *C. albicans* protoplasts were less sensitive to lysis by AmB when incubated under low oxygen tension, K^+ leakage remained intact. A decreased susceptibility of various mutants of *C. albicans* to the killing action of AmB has also been associated with increased levels of catalase activity (Sokol-Anderson et al., 1988).

8. Magnesium ions protect *leishmanias* and fungal cells against the killing action of AmB

The lethal effects of AmB on fungi can also be prevented by adding Mg^{2+} ions (Brajtburg et al.,

1980; Gale, 1984). This result is another indication that the ultimate consequence of the sequence of permeability alterations induced by the formation of AmB aqueous pores is the killing of cells by membrane disruption. Thus, divalent cations such as Mg^{2+} are known to protect cells from the membrane damage caused by pore-forming hemolytic viruses and protein toxins by binding to negatively charged components present at the cell surface (Bashford et al., 1988). This binding leads to stabilization of the local fractures or holes produced by such toxic compounds in the membranes of sensitive cells, thus preventing leakage of large molecules (Bashford et al., 1988). Consistent with these results, we have found that addition of low Mg^{2+} concentrations into LP's after AmB aqueous pores are formed led to an abrupt halt in the incorporation of EB (Ramos et al., 1990). Such a protective effect by Mg^{2+} ions yielded an inhibitory constant than was much smaller for control LPs than for amastigote-like cells (Ramos et al., 1989), a finding that was not unexpected since amastigotes are known to exhibit a decreased amount of negative charge components at the membrane surface (Pimienta and de Souza, 1983).

9. The role of non-aqueous channels and aqueous pores in AmB toxicity and lethality against mammalian cells

There are some membrane active compounds such as the calcium ionophore A23187 or amiloride, a well known inhibitor of Na^+/H^+ exchange, which are known to trigger the so-called programmed or apoptotic cell death in certain types of eucaryotic cells by altering calcium or pH gradients across the plasma membranes.(Wertz and Hanley, 1996). In all these cases the lethal action is irreversible. This is not the case of some of the cellular effects of AmB on sensitive organisms (Fig. 3) or on host mammalian cells. In fact, there is evidence that the damage caused by AmB can be repaired, provided the damage is produced at low but not high concentrations (Malewicz et al., 1981; Brajtburg et al., 1980; Gale, 1984; Binet and Bolard, 1988). In this respect, the existence of a concentration beyond which the AmB-induced permeability alterations can not be repaired is consistent with the formation by the antibiotic of two distinct types of structures drastically differing in permeability properties. Thus, the more damaging effects of AmB on cells can be directly associated with the formation of aqueous pores capable of a sustained disruption of calcium (Ramos et al., 1989) or pH gradients (Fig. 5).

One of the side effects that most limit the clinical use of AmB is its nephrotoxicity (Sawaya et al., 1995). In fact, the decreased nephrotoxicity associated with the administration of the liposomal formulation of AmB as compared to free AmB (Lopez-Berestein et al., 1983) is at the basis of the observed improvement of the therapeutic index of this drug in the treatment of systemic fungal infections (Wassan and Lopez-Berestein, 1994). In this respect, our own studies on the effect of AmB on the salt permeability across brush border membrane (BBM) vesicles prepared from rat kidney proximal tubules have indicated that in such membranes AmB forms salt permeable aqueous pores at concentrations higher than 0.8 μM AmB (Fig. 6). The studies by Schell et al. (1989) using the same type of BBM vesicles have also indicated that at 2 μM AmB differentially increased the permeability of anions over cations. This is the same concentration range at which aqueous pores are formed in cholesterol-containing liposomes (Cohen, 1992). However, as we have shown in liposomes and *leishmanias*, the permeabilizing effect of AmB across BBM vesicles is not only concentration dependent but also time dependent, suggesting the formation of two types of AmB channels. Thus, pre-incubation of BBM vesicles with AmB previous to mixing leads to the disappearance of the constant time lag preceding salt permeability (Fig. 6). Furthermore, during this time lag interval, lower AmB concentrations are able to induce a significant increase of the Na^+ permeability across BBM vesicles (Capasso et al., 1986), a result that demonstrates that non-aqueous channels, permeable to cations, preceded the formation of aqueous pores.

Such in vitro findings may be used to explain how AmB affects kidney function in vivo. Thus,

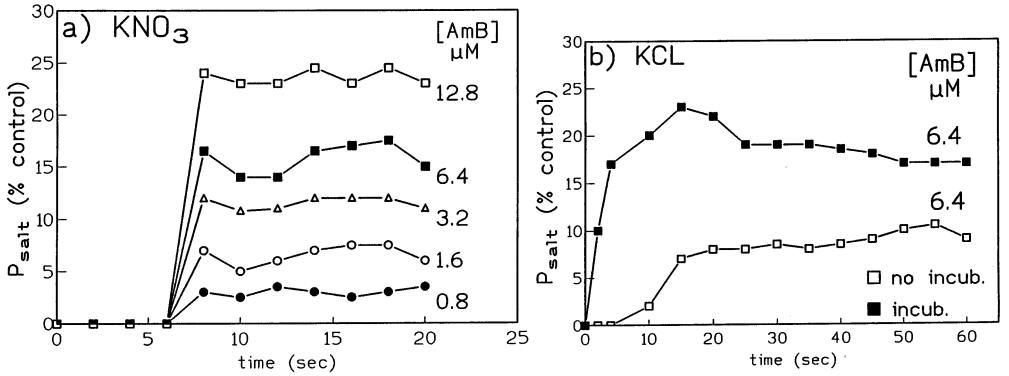

Fig. 6. The effect of AmB on the salt permeability across brush border membrane (BBM) vesicles. BBM vesicles were prepared from rat proximal kidney tubules as described previously (Leal-Pinto et al., 1987). (a) KNO_3 permeabilities induced by increasing AmB concentrations. At time zero, BBM vesicles (1 mg protein/ml in 25 mM NaCl) were rapidly mixed with a 600 mOsm KNO_3 hyperosmotic solution containing AmB at increasing concentrations. (b) (■) BBMs were pre-incubated with AmB for 5 min (3.2 μM final concentration) before mixing with hyperosmotic 600 mOsm KCl (□). Non-incubated vesicles were mixed with hyperosmotic KCl containing 3.2 μM AmB. The effect of AmB on salt permeabilities was calculated by subtracting volume changes in the presence of AmB from control values. All measurements were carried out at 30°C. For the rest of experimental details see legend to Fig. 1.

the observed decrease in the glomerular filtration rate by AmB is an effect that may be initiated by the formation of K^+ permeable non-aqueous channels across smooth muscle cells, leading to membrane depolarization and calcium influx through voltage-gated Ca^{2+} channels (Sawaya et al., 1995). In fact, an AmB-induced cell depolarization originated by increasing cation but not anion permeability has been demonstrated in some cell systems (Reuss, 1978). Cells are known to recover from these disturbances after some time. However, more acute effects during prolonged therapy such as tubular acidosis may be due to an increased H^+/OH^- permeability across AmB aqueous pores. In this respect, earlier studies have demonstrated that H^+ efflux across turtle bladder was affected by AmB (Steinmetz and Lawson, 1970). Recently, it has been shown that cultured kidney cells can even recover from losses of intracellular K^+ induced by high AmB concentrations, provided that they were incubated at neutral pH (Walev and Bhakdi, 1996). When such AmB-treated cells were incubated at low pHs of 5.5—6.0, the recovery mechanisms failed completely, leading to irreversible membrane damage. Since renal cells located in the distal epithelium are normally exposed to acidic urine, the formation of AmB aqueous pores under such conditions would contribute to intracellular acidification by increasing H^+/OH^- permeability.

10. Conclusions

The leishmanicidal and fungicidal effects exerted by AmB are due to the formation of aqueous pores permeable to small cation and anions, including H^+ and OH^-. Thus, salt permeation across aqueous pores formed by AmB across *Leishmania* promastigotes leads to rapid cell lysis via an osmotic mechanism. Fungal cells are protected from osmotic lysis by a cell wall but an increased H^+/OH^- permeability across AmB aqueous pores leads to an elevation of intracellular pH which then results in membrane damage.

The formation of AmB aqueous pores is always preceded by the formation of non-aqueous channels. Such pre-pore structures make cells permeable to monovalent cations (with the exception of H^+), collapse the cell membrane potential and may lead to a temporal inhibition of cell growth and other cellular effects in the absence of killing. It is only when a 'critical' concentration of AmB is reached in the membrane that non-aqueous

channels interact with ergosterol($[AmB]_e > 0.1$ μM) or cholesterol($[AmB]_e > 0.5$ μM) to form aqueous pores.

Finally, the toxic effects which are exerted by AmB on kidney cells and other cells can now be understood as possibly due to specific permeability changes induced either by the formation of non-aqueous AmB channels or aqueous pores, depending on the AmB concentration that is reached at a particular site of action. However, the more acute and disrupting effects of AmB appear to be induced by the formation of AmB aqueous pores, which are permeable to protons, salt and Ca^{2+}. In this respect, the observation than liposomal AmB is less nephrotoxic but as effective as free AmB in the treatment of patients with systemic fungal infections or visceral Leishmaniasis may be simply accounted for by a greater reduction in the formation of the more toxic aqueous pores in cholesterol-containing host cells than in ergosterol-containing parasite or fungal cells.

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