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Amphotericin B Kills Unicellular Leishmanias by Forming Aqueous Pores Permeable to Small Cations and Anions

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Abstract. The polyene antibiotic amphotericin B (AmB) is known to form two types of ionic channels across sterol-containing liposomes, depending on its concentration and time after mixing (Cohen, 1992). In the present study, it is shown that AmB only kills unicellular *Leishmania* promastigotes (LPs) when aqueous pores permeable to small cations and anions are formed. Changes of membrane potential across ergosterol-containing liposomes and LPs were followed by fluorescence changes of 3,3′ dipropylthiadicarbocyanine ($DiSC_3(5)$). In KCl-loaded liposomes suspended in an iso-osmotic sucrose solution, low AmB concentrations ($\leqslant 0.1$ μM) induced a polarization potential, indicating K^+ leakage, but no movement of cations and anions was allowed until AmB concentrations greater than 0.1 μM were added. In agreement with these data, it was found that AmB altered the negative membrane potential held across LPs in a manner consistent with the differential cation/anion selectivity exhibited by the channels formed in liposomes. Thus, LPs suspended in an iso-osmotic sucrose solution did not exhibit any AmB-induced membrane depolarization effect brought about by efflux of anions until 0.1 μM or higher AmB concentrations were added. By contrast, LPs suspended in an iso-osmotic NaCl solution and exposed to 0.05 μM AmB exhibited a nearly total collapse of the negative membrane potential, indicating Na^+ entry into the cells.

The concentration dependence of the AmB-induced permeability to different salts was also measured across vesicles derived from the plasma membrane of leishmanias (LMVs), by using a rapid mixing technique. At concentrations above 0.1 μM, AmB induced the formation of aqueous pores across LMVs with a positive cooperativity, yielding Hill coefficients between 2 to 3. Measured anion selectivity across such aqueous pores followed the sequence: SCN > NO3 > Cl > I > Br > acetate (SO_4^{2-} being impermeable). Cell killing by AmB was followed by fluorescence changes of the DNA-binding compound ethidium bromide (EB). At low concentrations ($\leqslant 0.1$ μM), AmB was found to be nonlethal against LPs but, above this concentration, leishmanias were rapidly killed. The rate and extent of such an effect were found to be dependent on the type of cation and anion present in the external aqueous solution. For both NH_4^+ and Na^+ salts, the measured rank order of AmB cell killing followed the same sequence that was determined for AmB-induced salt permeation across LMVs. Further, replacement of either extracellular Na^+ by choline or Cl^- by SO_4^{2-}, or its partial substitution by sucrose, in iso-osmotic conditions, led to a complete inhibition of the killing effect exerted by otherwise lethal AmB concentrations. Finally, it was shown that tetraethylammonium (TEA^+), an organic cation that is known to block AmB-induced salt permeation across LMVs was able to retard the time lag observed for EB incorporation across LPs, indicating that this parameter can be taken to represent the time taken for salt accumulation inside the parasites. The present results thus indicate clearly that low AmB concentrations ($\leqslant 0.1$ μM) were able to form across LPs, cation channels that collapsed the parasite membrane potential but are not lytic. At high concentrations ($\geqslant 0.1$ μM), a salt influx via the aqueous pores formed by the antibiotic was followed by osmotic changes leading to cell lysis. This last stage is supported by electron microscopy observations of the changes of parasite morphology immediately upon addition of AmB, which indicated that the typical elongated promastigote cell forms became rounded and the flagella swells and round up. The present work is the first demonstration of the in vitro

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sensitivity of *Leishmania* promastigotes to osmotic lysis by AmB.

Key words: Amphotericin B — Permeability — Anions — Aqueous pores — Leishmania

Introduction

The polyene antibiotic amphotericin B (AmB) is the compound most frequently used in the treatment of systemic mycosis (for a recent review, *see* Brajtburg, et al., 1990) and visceral leishmaniasis (Croft, 1988), in spite of its toxic side effects. Clinical visceral leishmaniasis has a high mortality if untreated. Recent studies have shown a significant improvement of the therapeutic index of AmB against visceral leishmaniasis by the use of a delivery system in which the antibiotic is bound to liposomes (Croft, Davidson & Thornton, 1991). However, the cellular basis of the leishmanicidal and fungicidal action of AmB remains unclear and understanding this process is a necessary prerequisite for the development of less toxic compounds.

The selective toxicity of AmB against sensitive organisms appears to be the result of the capacity of this polyene antibiotic to form aqueous pores by binding more strongly to ergosterol, the principal fungal sterol, than to cholesterol, the main sterol of mammalian host cells (*see* review by Bolard, 1986). In sterol-containing model membranes such as planar lipid bilayers and liposomes, AmB is known to form aqueous pores of 4A radius, which are permeable to monovalent cations and anions, and small nonelectrolytes including glucose (Andreoli, 1974; De Kruijff et al., 1974; Finkelstein, 1987). According to the pore theory of AmB action, the formation of aqueous pores causes the plasma membrane to become nonselectively leaky to K^+ ions and essential metabolites and eventually cell death ensues. In parasitic protozoa such as *Leishmania spp.*, the pore theory of AmB action, has been supported by the finding that a total depletion of desmethyl sterols by ketoconazole effectively reduced the lytic activity of AmB on these cells, by depriving the polyene antibiotic of its capacity to form aqueous pores in the cell membrane (Ramos et al., 1994).

However, the pore hypothesis of AmB action has been challenged by several investigators on the basis that the AmB permeabilizing effects on sensitive cells were shown to be dissociated from the lethal effects (Chen, Chou & Feingold 1978; Brajtburg et al., 1980*a*, 1984; Beggs, 1994). Thus, at low concentrations, AmB induced K^+ leakage from erythrocytes but no hemolysis occurred until higher concentrations were used (Brajtburg et al., 1980*a*). Likewise, in mammalian L cells, the K^+-permeabilizing effect exerted by AmB occurred at concentrations lower than those required for the toxic effect (Brajtburg et al., 1984). In *Candida albicans*, addition of concentrations of AmB as low as 0.2 μg/ml were able to elicit a K^+ efflux as large as that produced by 1.6 μg/ml AmB, but the fungicidal activity of the former concentration was very modest compared with the lethality of the latter concentration (Beggs, 1994). This separation between K^+ leakage and lethality have led to the conclusion that the formation of aqueous pores by AmB is not central to its lethal action, and that other mechanisms such as chemical degradation of the membrane (Brajtburg et al., 1984; Sokol-Anderson et al., 1986) or a physicochemical detergentlike membrane damage may be primarily responsible for the fungicidal action of AmB (Beggs, 1994).

The complex time and concentration dependence of the interaction between AmB and membrane sterols in the process of formation of aqueous pores has been studied (Cohen, 1986; Cohen & Gamargo, 1987; Cohen, 1992). It was shown in this work that addition of AmB to liposomes — containing or not sterols, always led to the formation of nonaqueous prepore structures; after a certain time, depending on the AmB concentration, such structures interact with the sterols in the membrane to form aqueous channels, having an enlarged diameter (Cohen, 1992). Thus, the channels formed in liposomes by low AmB concentrations are permeable to urea (Cohen, 1986) and monovalent cations (Hartsel et al., 1991, 1994) but not to anions or glucose (Cohen & Gamargo, 1987; Cohen, 1992; Hartsel et al., 1994). In the light of these results, the question arises as to whether the observation of separate stages for AmB action on sensitive cells may simply reflect the AmB capacity to form two types of channels, differing in its permeability properties.

In the present work, we have tested the pore hypothesis of AmB action by using *Leishmania mexicana* promastigotes (LPs), a protozoan parasite which contrary to fungal cells do not have a cell wall. It is shown that at low concentrations, AmB enhanced the cation permeability across ergosterol-containing liposomes and LPs, leading to a collapse of the parasite membrane potential but the leishmanicidal effects of AmB may be related specifically to an osmotic lysis mechanism due to the formation — at higher concentrations — of aqueous pores, simultaneously permeable to both cations and anions.

Materials and Methods

Materials

Analytical quality reagents were used whenever possible. Ethidium bromide (EB), egg phosphatidylcholine (egg-PC), ergosterol, valinomycin and amphotericin B (AmB) were purchased from Sigma. The fluorescent probe 3,3′-dipropylthiadicarbocyanine ($DiSC_3(5)$) was purchased from Molecular Probes (Eugene, Oregon). For all experiments,

a 1 mM stock solution of AmB in dimethylsulphoxide was used. The AmB stock solution was used on the same day it was prepared.

Preparation of Liposomes

Large unillamelar liposomes (LUV) were formed by the reverse-phase evaporation method (Szoka & Papahadjopoulos, 1979). Briefly, 2 ml of an aqueous solution containing 100 mM KCl were mixed with 6 ml diethylether containing 20 μmoles of a lipid mixture (egg-PC + 16 mol% ergosterol). 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).

Growth of *Leishmania* Promastigotes

Promastigotes of *Leishmania mexicana* strain NR were cultivated in LIT medium at room temperature, as previously described (Cohen et al., 1986). Growth was estimated from turbidimetric measurements at 560 nm. The cells in the exponential phase were harvested and washed twice by centrifugation at 1,000 × *g* for 10 min, with a Na/K buffer solution (pH = 7.6) containing Tris-HCl 75 mM, NaCl 140 mM and KCl 11 mM (Buffer A). The cell suspensions were kept under ice until further use.

Measurements of Membrane Potential Changes

AmB-induced membrane potential changes across liposomes (LUV) and *Leishmania* promastigotes were measured by using the fluorescence changes of 3,3′-dipropylthiadicarbocyanine ($DiSC_3(5)$) (Rink et al., 1980; Loew et al., 1985). Fluorescence measurements were done with an SPEX Fluorolog II spectrophotometer equipped with a magnetic stirrer at 620- and 670 nm, excitation-emission wavelengths, respectively. In a typical protocol, ergosterol-containing liposomes prepared in a 100 mM KCl solution (*see above*) were added to a fluorescence cuvette containing 200 mM sucrose solution (final lipid concentration 0.05 mM). $DiSC_3(5)$ (stock 0.6 mM in ethanol) was then added at a final concentration of 2 μM. The fluorescence of the positively charged $DiSC_3(5)$ is rapidly quenched whenever it concentrates into the liposomes by the formation of a negative diffusion potential inside. Thus, by adding valinomycin (1 μM), a maximum quenching was always obtained as a result of the movement of internal K^+ into the external sucrose medium driven by its concentration gradient.

For the corresponding membrane potential measurements across *Leishmania* promastigotes, cells (25×10^6 cells/ml) were transferred to a fluorescence cuvette containing 2 ml of the appropriate buffer solution but always containing 10 mM glucose. Gentle stirring ensured the homogeneity of the suspension during recordings. Upon addition of $DiSC_3(5)$ (3 μM final concentration), the fluorescence signal slowly decreased indicating uptake of the probe into the cells (time constant 3–5 min), driven by the existence of a negative membrane potential across LPs (Glaser, Utz & Mukkada, 1992; Vieira, Slotki & Cabantchick, 1995). Thus, by disrupting the cell membrane upon addition of digitonin (50 μg/ml), a maximal increase of fluorescence was always obtained. In all cases, the traces show fluorescence changes which were normalized to 100 arbitrary units. All fluorescence measurements were carried out at room temperature.

Kinetics of Cell Death

The kinetics of cell death induced by AmB against *Leishmania* promastigotes was followed by using fluorometry with the DNA-binding compound ethidium bromide (EB) (Cohen et al., 1990; Ruiz et al., 1991). Fluorescence measurements were performed on a SPEX Fluorolog II spectrophotometer at 365–580 nm excitation-emission wavelengths. Promastigotes at a final concentration of 25×10^6 cells/ml were incubated for 5 min with gentle stirring in the fluorescence cuvette with 2 ml of different buffered solutions but always containing 10 mM glucose and EB (50 μM). After signal stabilization was achieved, AmB was added and dissolved in dimethylsulfoxide. Maximal EB incorporation was always obtained by adding digitonin (50 μg/ml). All solutions used were buffered with 75 mM TRIS (pH = 7.6) and contain 150 mM NaCl (BNa^+), 150 mM KCl (BK^+), 150 mM choline chloride (Bcholine), and 100 mM sucrose + 100 mM NaCl (Bsucrose). The osmolarity of all solutions was always adjusted to 390 ± 5 mOsm using an Advanced instrument SW2 osmometer (Boston, MA).

Preparation of *Leishmania* Membrane Vesicles (LMVs)

LMVs were isolated and purified at 4°C as described previously (Cohen et al., 1986). Briefly, after washing the cells with 10 mM HEPES buffer containing 400 mM mannitol, 10 mM KCl and 3 mM magnesium acetate (Buffer B), the ice-cold pellet of cells was mixed in a mortar on ice with glass beads of 75–150 μm (Sigma) in a 4:1 ratio (w/w). This mixture was ground by gentle rotation for 5–10 min until no more than 2–3 intact cells per field could be seen under the microscope. After separation by differential centrifugation of glass beads, unruptured cells and mitochondria, a fraction enriched in plasma membrane vesicles was isolated by centrifugation at 40,000 × *g* for 50 min. The enzymatic characteristics and osmotic properties of LMVs prepared by this method were reported previously (Cohen et al., 1986).

Salt Permeability Measurements across LMVs

Volume changes of LMVs were measured by following the 90° light scattering changes at 450 nm in a Durrum (D-110) stopped-flow spectrophotometer (Cohen et al., 1986). A calibration curve was obtained by suspending LMVs (1.2 mg protein/ml) in hyperosmotic glucose solutions (80–800 mOsm) prepared in the same buffer B (*see above*) but without mannitol. In this range, a linear relationship was obtained between light scattering and the volume changes (Cohen et al., 1986). Permeability coefficients to different salts induced by AmB were measured by the ''maximum slope'' method as described (Cohen, 1986, 1992). For this purpose, LMVs (1.2 mg protein/ml) were rapidly mixed in a 1:4 volume ratio with a hyperosmotic salt solution (600 mOsm) prepared in the same buffer B but without mannitol. All measurements were done with water continuously circulating through the drive syringes and mixing cuvette at constant temperature (20 ± 2°C).

Electron Microscopy

A sample of promastigotes (10^8 cell/ml) cultivated as described previously was incubated during 10 min at a concentration of 0.5 μM. A small aliquot of glutaraldehyde 2.5% solution (v/v) was then added to the promastigote suspension. The cells were collected at 1,000 × *g* for 7 min and refixed with 2.5% glutaraldehyde in normal buffer A for 2 hr. Fixed cells were washed three times in normal buffer and post fixed in 1% OsO4 in the same buffer in the cold for 1 hr. Dehydration was carried out in a graded series of ethanols and embedded in Epon. Ultrathin sections were obtained with LKB Ultramicrotome, stained with uranyl acetate and lead citrate and observed in a H-500 transmission electron microscope.

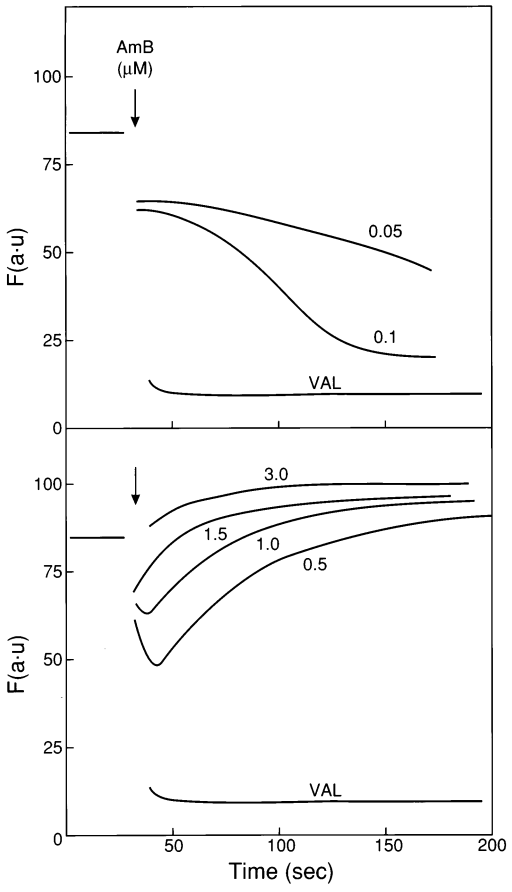

Fig. 1. Time-dependence of the AmB-induced fluorescence changes of $DiSC_3(5)$ across ergosterol-containing liposomes. Large unilamellar liposomes (LUV) were prepared in 100 mM KCl and suspended in a 200 mM sucrose solution. Calibration of polarization changes was always performed by adding valinomycin (1 μM). AmB (dissolved in DMSO) was added at the concentrations indicated (in μM).

Results

Formation of Anion Impermeable and Anion Permeable Channels by AmB across Ergosterol-containing Liposomes

Figure 1 shows the effect of increasing AmB concentrations on the membrane potential of eggPC-ergosterol liposomes as measured by the fluorescence changes of the membrane probe $DiSC_3(5)$ (*see* Materials and Methods). The extent of the fluorescence quenching of the membrane probe after adding valinomycin to KCl-loaded liposomes suspended in an iso-osmotic sucrose solution can be observed in Fig. 1 (top). This ionophore rapidly formed a negative potential inside the liposomes by allowing the movement of K^+ ions out of the liposomes, down its concentration gradient. Upon addition of AmB, two types of behavior were observed. At low AmB concentrations (Fig. 1, top), there is a progressive quenching of the probe fluorescence, that reached a maximal value at 0.1 μM AmB. Beyond this concentration, there is a rapid reversal of the initial quenching leading in a few seconds to an overall enhancement of fluorescence. Thus, it can be seen in Fig. 1 (bottom), that the initial quenching of the fluorescence is now superseded by a rapid enhancement which occurred at shorter times, as the AmB concentration increased. Clearly, the AmB channels formed at low concentrations are permeable only to K^+ ions. However, those AmB channels formed at high concentrations (>0.1 μM) became permeable within minutes to both K^+ and Cl^-. A similar behavior has been observed for other monovalent salts (Valdivieso, 1992).

Effect of AmB on *Leishmania* Membrane Potential

Figure 2*A* shows the fluorescence signals for $DiSC_3(5)$ in an iso-osmotic sucrose solution after addition of LPs (*see* Materials and Methods). The traces at the bottom of Fig. 2*A* show that addition of valinomycin (10 μM) or 0.05 μM AmB did not change the fluorescent signal. By contrast, by adding 0.1 μM AmB or higher concentrations, an increase of fluorescence was observed (Fig. 2*A*). This progressive depolarization of LPs by AmB occurred at the same concentration range at which liposomes treated with AmB became permeable to anions (Fig. 1*B*).

When LPs were suspended in an iso-osmotic NaCl solution, AmB depolarized cells to a large extent at concentrations as low as 0.05 μM (Fig. 2*B*). This abrupt collapse of the membrane potential by AmB was attributed to a rapid Na^+ influx across LPs induced by formation of AmB cation channels. Thus, when Na^+ in the external solution was replaced iso-osmotically by choline, low AmB concentrations (≤0.1 μM) did not depolarize cells (*data not shown*), but at higher concentrations, membrane depolarization was comparable to that observed in sucrose-suspended LPs (Fig. 2*A*). Choline is a cation that is known to be impermeable across the aqueous channels formed by AmB in liposomes (B.E. Cohen, *unpublished observations*).

The Effect of Different Anions on the Salt Permeability of *Leishmania* Membrane Vesicles

Figure 3 shows the effect of increasing AmB concentrations on the KNO_3 and KCl permeabilities across vesicles prepared from the plasma membranes of leishmanias (LMVs). It can be seen in Fig. 3 that for both salts, the onset of AmB-induced relative salt permeability ($\Delta P/P_0$) exhibit a threshold concentration near 0.1 μM AmB. At higher concentrations, a sigmoidal enhancement of salt permeability was observed. Thus, by fitting

this data to the Hill equation, cooperativity coefficients lying in the range 2–3 were calculated (Table).

In the Table are listed calculated values for maximal relative permeabilities ($\Delta P_{max}/P_0$) induced by AmB for different potassium salts and corresponding values of antibiotic concentrations required for half maximal permeabilities (C_{50}). It can be seen in the Table that the rank order of AmB-induced salt permeabilities across LMVs follows the sequence: $SCN^- > NO_3^- > Br^- > I^- > Cl^- >$ acetate$^-$. AmB did not enhance LMVs permeability to K_2SO_4. On the other hand, at the reference concentration of 0.8 μM (Table), the corresponding rate constants ks calculated for the permeation of KNO_3 and NH_4NO_3 across LMVs yield values of 0.22 ± 0.05 sec^{-1} (4) and 0.8 ± 0.1 sec^{-1} (3), respectively.

The Effect of AmB at Increasing Concentrations on the Incorporation of Ethidium Bromide across *Leishmania* Promastigotes

The lethal action exerted by AmB against LPs was determined by using ethidium bromide-DNA fluorescence, as a measure of cell viability (Ruiz et al., 1991). As reported previously (Cohen et al., 1990), this effect depended on AmB concentration and exhibited a characteristic lag time. When promastigotes were suspended in a normal buffer A solution (*see* Materials and Methods) and 0.5 μM AmB was added, the lag time measured for EB incorporation was 5.4 ± 0.4 min (3). Increasing the concentration of AmB reduced the lag time down to a limiting value (Fig. 4A) and increased the velocity of EB incorporation (Fig. 4*B*). It can be observed in Fig. 4*B* that the threshold concentration for AmB-induced EB incorporation into LPs was about 0.1 μM. Beyond this concentration, a sigmoideal, cooperative enhancement was observed reaching a maximal value at about 1 μM AmB.

The data shown in Fig. 4 also indicate that the rate of EB incorporation into LPs was greater when the NaCl in the external media was replaced by $NaNO_3$ (Fig. 4*B*). In addition, it can be observed in Fig. 4*A* that the differences in lag time for EB incorporation for promastigotes suspended in nitrate or chloride salts ($NO_3^- < Cl^-$), were smaller at increasing AmB concentration, reaching a maximal value at 1 μM.

The Effect of Permeable and Impermeable Solutes on the Kinetics of Killing of *Leishmania* Promastigotes by AmB

Figure 5*A* shows the effect that iso-osmotic replacement of NaCl in the external solution by another salt or solute had on EB incorporation into LPs treated with 0.5 μM AmB. It can be observed in Fig. 5*A* that no significant changes in the time lag or rate of EB incorporation were

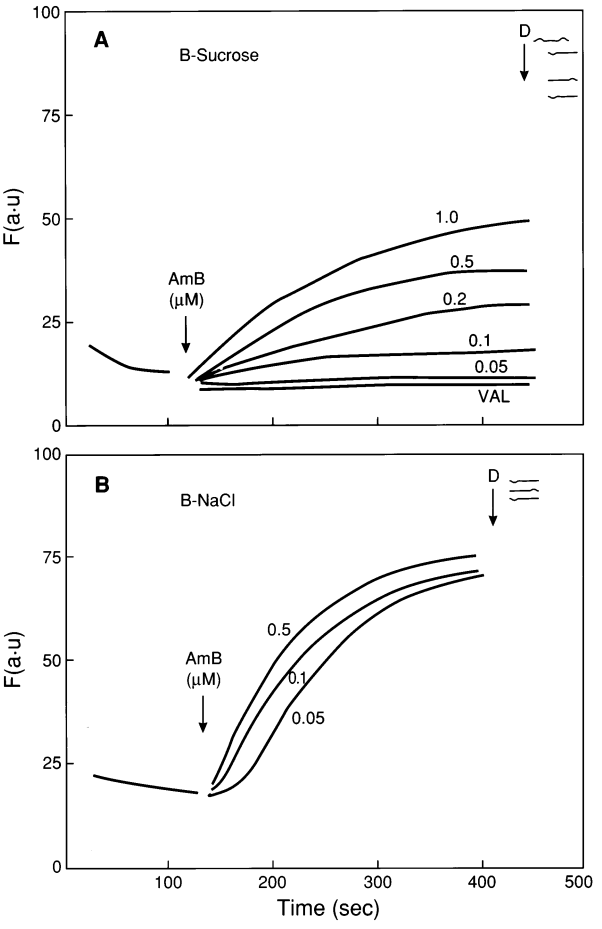

Fig. 2. Time-dependence of the AmB-induced fluorescence changes of $DiSC_3(5)$ across *Leishmania* promastigotes. Fluorescence changes were recorded as described in Materials and Methods. Cells (25×10^6 cells/ml) were suspended in iso-osmotic buffered solutions of sucrose (*A*) and NaCl (*B*), containing 10 mM glucose. Maximal $DiSC_3(5)$ incorporation was determined by adding digitonin (arrow ↓ D).

induced by AmB after replacement of NaCl by KCl in the corresponding external solution. By contrast, replacement of Na^+ ions by choline led to a complete inhibition of the AmB-induced incorporation of EB (Fig. 5*A*). On the other hand, replacement of the external NaCl by sucrose, a molecule which is also impermeable across the AmB aqueous channels (Andreoli, 1974), also blocked completely the AmB-induced EB incorporation (Fig. 5*A*).

Figure 5*B* shows the effect of a gradual iso-osmotic replacement of NaCl by sucrose on the initial rate of EB incorporation across LPs. It can be observed that % blocking of AmB-induced EB uptake into promastigotes increased with rising sucrose concentrations. Total blocking of EB incorporation into LPs was obtained after replacement of about 50 mM sucrose by NaCl (Fig. 5*B*). This sucrose concentration can be taken as a measurement of the external solute impermeants which is required to equilibrate the intracellular impermeants.

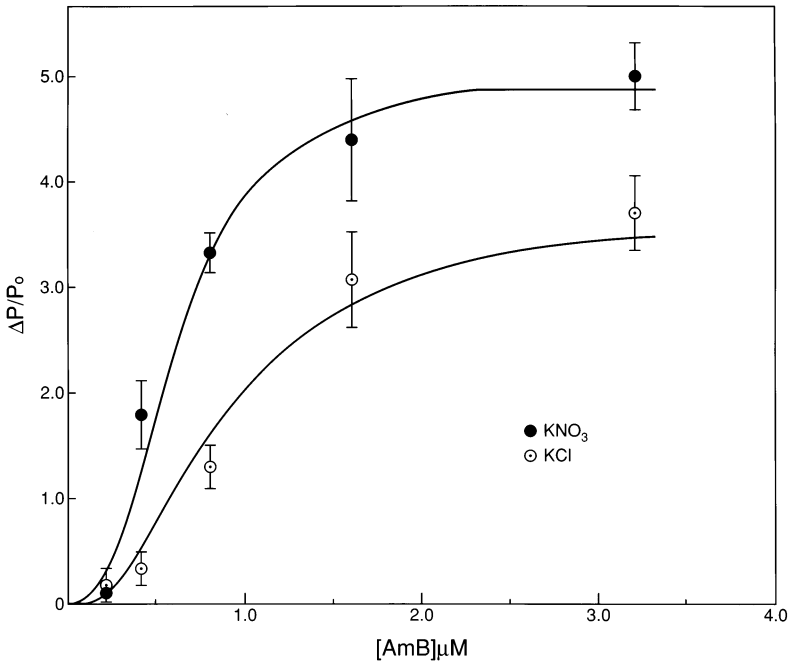

Fig. 3. Permeabilization of KCl and KNO_3 across *Leishmania* membrane vesicles as a function of the AmB concentration. Volume changes were measured by light scattering by mixing LMVs with hyperosmotic 600 mOsm solutions of KNO_3 and KCl, containing increasing AmB concentrations (*see* Materials and Methods). The corresponding relative permeabilities ($\Delta P/P_0$) for salt permeation were calculated from the ''maximum slopes'' attained by the vesicles after the minimum volume was reached. The sigmoid curves shown were calculated by fitting the experimental points to the Hill cooperativity equation (*see* Table). Experimental points plotted are the average of 3–5 separate determination (±SD). (●) KNO_3; (○) KCl.

Table. Fitting by using the Hill's equation[1] of the permeability changes to different salts induced by increasing AmB concentrations across *Leishmania* membrance vesicles

Salt	*n*	C_{50} (μM)	$\Delta P_{max}/P_0$	p_{rel}
KSCN	1.7 ± 0.5	0.7 ± 0.1	6.7 ± 2.4	5.8
KNO_3	1.6 ± 0.3	0.7 ± 0.1	4.3 ± 0.6	3.8
KCl	2.4 ± 0.4	0.8 ± 0.2	3.0 ± 0.8	1.0
KI	2.9 ± 1.3	0.6 ± 0.1	3.0 ± 0.9	1.4
KBr	nm	nm	nm	1.8
Kacetate	nm	nm	nm	0.1

[1] Permeability data (*P*) as shown in Fig. 3 were fitted to the Hill equation: $\Delta P = \Delta P_{max} C^n / (cte + C^n)$, where *n* is the cooperativity index; *C* is the *AmB* concentration; C_{50} is the concentration required for half-maximal effect. ΔP_{max} is the maximal permeability change induced by the antibiotic. P_{rel} are permeability values measured at 0.8 μM AmB and calculated with respect to KC1. All measurements were carried at a constant temperature (20°C). ± SD (2–3 separate determinations). nm = not measured

The Effect of Different Anions on the Kinetics of Cell Killing by AmB

The effect of different anions on the kinetics of AmB-induced EB incorporation across LPs was also measured (Fig. 6). It can be observed in Fig. 6*A* that the duration of the time lag for promastigotes suspended in Na^+ salts of SCN^-, NO_3^- and Cl^- were 3.1, 3.8 and 5.5 min, respectively. For promastigotes suspended in NH_4^+ salts of SCN^-, NO_3^- and Cl^- the corresponding time lags were 1.4, 3.0 and 5.2 min (Fig. 6*B*). In a similar way, Fig. 6*A* and *B* show that the rate of EB incorporation across promastigotes follows the sequence: $SCN^- > NO_3^- > Cl^-$ for both Na^+ and NH_4^+ salts.

Figure 6*A* and *B* also show that there was no AmB-induced incorporation of EB when promastigotes were suspended in iso-osmotic solutions of either ammonium or sodium sulfate. Control experiments carried out in the absence of AmB indicated that incubation (2–3 hours) of promastigotes into iso-osmotic solutions of the different ammonium or sodium salts tested did not induce any incorporation of EB into cells, including incubation in the very permeable ammonium thiocianate, although under the latter condition, cells became rounded as seen under the light microscope.

Blocking by TEA of AmB-induced Incorporation of EB across *Leishmania* Promastigotes

We have previously shown that the ionic compound tetraethylammonium (TEA) at millimolar concentrations blocked the aqueous channels formed by AmB across LMVs (Cohen & Gamargo, 1987). To investigate the

relationship between the formation of aqueous pores by AmB and the incorporation of EB into LPs, we studied the effect of different TEA concentrations on the time lag and rate of EB incorporation across promastigotes. For this purpose, LPs were suspended in the iso-osmotic NaCl solution with a constant amount of AmB (0.5 μM) and increasing concentration of TEA (Fig. 7). It can be observed in Fig. 7 (open circles) that raising the concentration of TEA up to 10 mM led to a linear reduction of the time lag for AmB-induced EB incorporation. However, the corresponding rates of EB incorporation (filled circles) exhibited a saturation kinetics. In fact, from this data, a $K_i = 2.0$ mM for the inhibitory effect of TEA on AmB-induced rate of EB incorporation across LPs was calculated.

The Effect of AmB on Cell Shape

Observations by electron microscopy of LPs showed the characteristic elongated shape of this stage of the parasite life cycle, with a long slender flagellum, a round nucleus and a single mitochondrium which contains the kinetoplast (Fig. 8, left). After 10 min of incubation with 0.5 μM AmB, few LPs could be seen intact at the electron microscope but those observed exhibited a marked change of shape (Fig. 8, right), that is cell bodies and flagella swell and round up; the mitochondria and the nucleus remain without visible alterations. A remarkable feature of the promastigotes exposed to AmB was the appearance throughout the cytoplasm of numerous membrane-bound large vesicles.

Discussion

The permeabilizing effects that are exerted by AmB on ergosterol-containing fungal and *Leishmania* membranes have been generally interpreted on the basis of the formation by the polyene antibiotic of a unique type of channels permeable to monovalent cations and anions, and nonelectrolytes up to the size of glucose (Andreoli, 1974; Finkelstein, 1987). However, several types of evidence have indicated that AmB can form two types of channels in ergosterol and cholesterol-containing membranes, depending on the antibiotic concentration (Cohen, 1986, 1992; Cohen & Gamargo, 1987; Hartsel et al., 1994). In the present work, we have shown that such channels also differed in their cation/anion selectivity (Fig. 1). Thus, at 0.1 μM or lower concentrations, AmB induced changes of membrane potential across ergosterol-containing liposomes indicating K^+ leakage, but not until higher antibiotic concentrations were added (>0.1 μM), could a differential enhancement of the permeability to both cations and anions be observed. Further, it was shown that 0.05 μM AmB added to LPs suspended in an iso-osmotic buffered NaCl solution (Fig. 2*B*), led to a

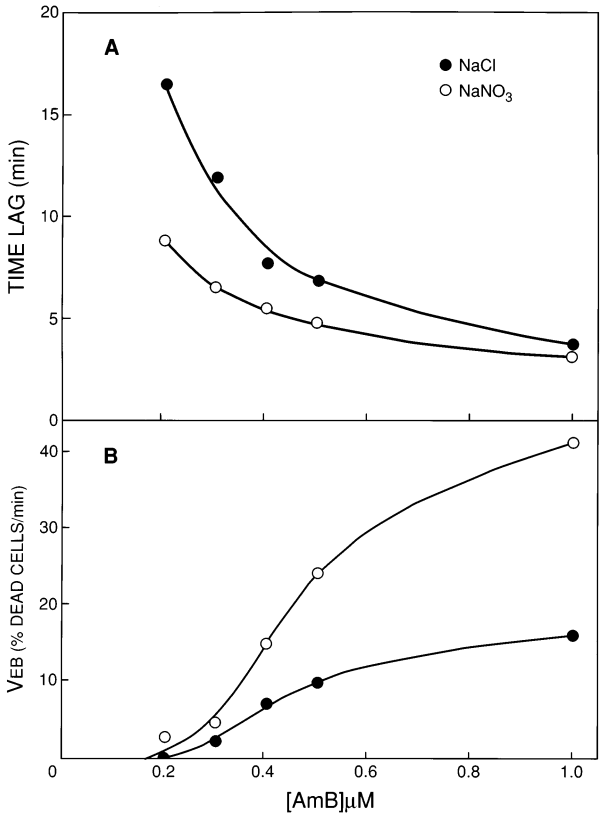

Fig. 4. Effect of AmB on the incorporation of ethidium bromide (EB) across *Leishmania* promastigotes. (*A*) time lag for the onset of EB incorporation as a function of the AmB concentration (in μM); (*B*) Rate (V) of EB incorporation after the time lag (percent total changes per minute) as a function of the AmB concentration (in μM). All points shown are the average values of experiments carried out in duplicate. LPs suspended in iso-osmotic buffered solutions of NaCl (●) and $NaNO_3$ (○).

collapse of the negative membrane potential that is maintained across such cells (Vieira et al., 1995). Since $[Na^+]_i$ at the cytoplasm of LPs ranges between 30 to 70 mM (LeFurgey, Ingram & Blum, 1990; Felibertt et al., 1995), this finding indicates that AmB at low concentrations was able to increase Na^+ permeability across LPs, leading to movement of positive charges into the cells and membrane depolarization. On the other hand, when LPs were suspended in a sucrose-containing iso-osmotic solution, membrane depolarization by AmB also occurred, but only by adding concentrations higher than 0.1 μM (Fig. 2*A*). Clearly, under these conditions, AmB increased anion permeability across LPs, negative charges moved out of the cells and as a consequence, membrane depolarized. These results are fully consistent with recent data by Vieira et al. (1995) indicating that the membrane potential of LPs does not depend on K^+, Na^+, or Cl^- permeabilities, but on an electrogenic H^+ pump.

One of the characteristics of AmB action of LPs is that at low concentrations (<0.1 μM AmB), it is leishmanistatic, as indicated by inhibition of cell growth (Ra-

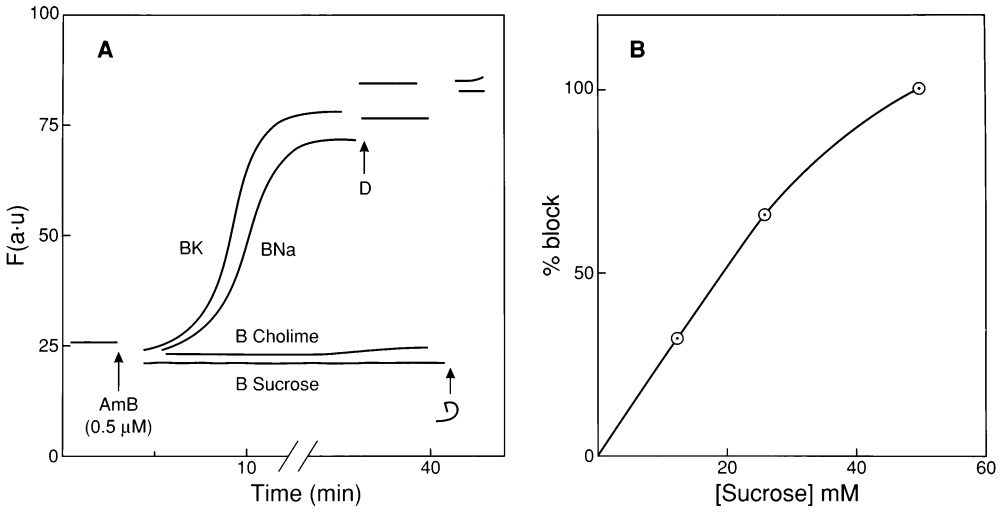

Fig. 5*A.* Effect of the medium composition on the AmB-induced incorporation of ethidium bromide (EB) across *Leishmania* promastigotes. EB fluorescence changes were recorded as described in Materials and Methods. LPs (3×10^7 cells/ml) were suspended in the following iso-osmotic buffered solutions: NaCl (BNa), KCl (BK), choline chloride (Bcholine) and sucrose (Bsucrose). AmB (arrow) was added at 0.5 μM. Maximal EB incorporation was always determined by adding digitonin (↑ D).

Fig. 5*B.* Effect of partial replacement of external NaCl by sucrose in the AmB-induced incorporation of ethidium bromide (EB) into *Leishmania* promastigites. Abcissa: sucrose concentration (in mM) that replaced NaCl in the iso-osmotic buffer; Ordinate: the initial rate of EB incorporation (% blocking of cell death).

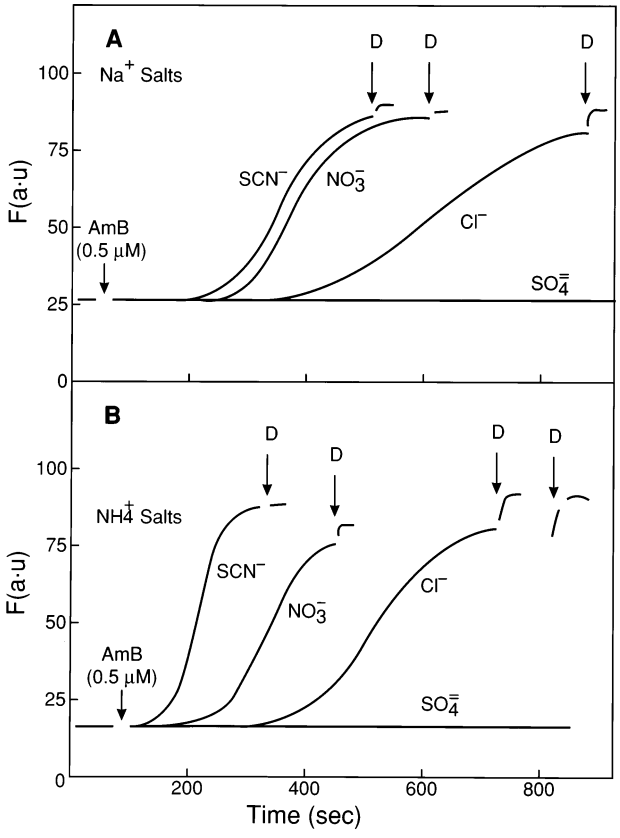

Fig. 6. The effect of different anions on the AmB-induced incorporation of *Leishmania* promastigotes to ethidium bromide. (*A*) Na^+ salts; (*B*) NH_4^+ salts. For experimental details *see* Materials and Methods.

mos et al., 1988) but at higher antibiotic concentrations, it is lytic (Ramos, Romero & Cohen, 1988; Ramos et al., 1995). In this respect, the present data conclusively demonstrated that it is the formation of AmB aqueous pores permeable to small cations and anions that leads to the leishmanicidal action. Thus, (i) the threshold concentration at which AmB was able to increase the permeability of KCl and KNO_3 across LMVs was 0.1 μM (Fig. 3), that is, the same concentration beyond which AmB-induced exerted a lethal effect against LPs, as measured by EB incorporation (Fig. 4) or cell lysis (Ramos et al., 1996), (ii) the AmB-induced salt permeation across LMVs (Table) followed the same rank order that was determined from the time lags and subsequent rate of incorporation of EB into AmB-treated LPs suspended in different salts (Fig. 6), (iii) the AmB-induced incorporation of EB into LPs was prevented completely by replacement of external NaCl by choline chloride, K_2SO_4 or Na_2SO_4 (Fig. 5*A*) or by its partial replacement by sucrose (Fig. 5*B*).

Since the aqueous channels formed by AmB have been shown to be impermeable to choline, sulfate or sucrose (Andreoli, 1974; Cohen, 1986; Finkelstein, 1987), it can then be concluded that the formation of such structures across LPs leads to an increase of the intracellular salt content and, as a consequence of this, water moves into the cells and cells lyzed. This colloid osmotic mechanism for the lytic action of AmB on LPs is similar to that previously established in erythrocytes, where AmB-induced hemolysis can also be prevented by

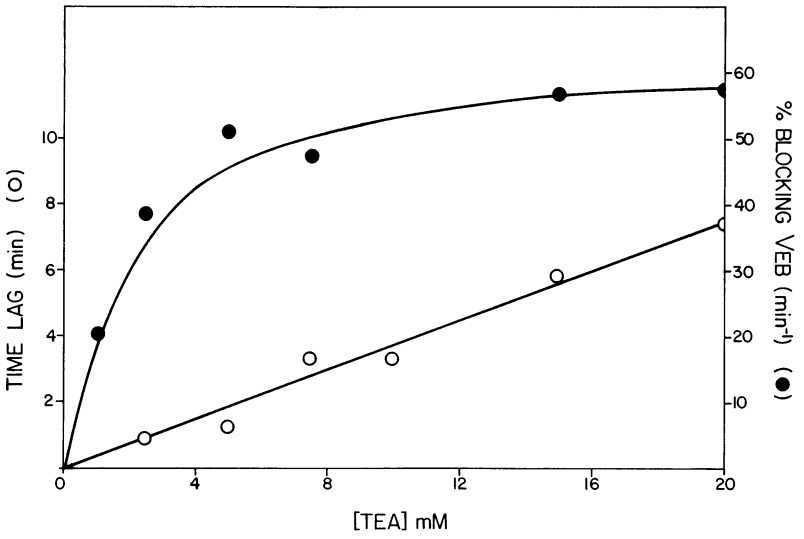

Fig. 7. The effect of TEA^+ on the AmB-induced incorporation of EB across *Leishmania* promastigotes. (○) time lag for the onset of EB incorporation (in min); (●) % rate of EB incorporation (V_{EB}) after the time lag (percent total changes per minute). Abscissa: TEA concentration (in mM).

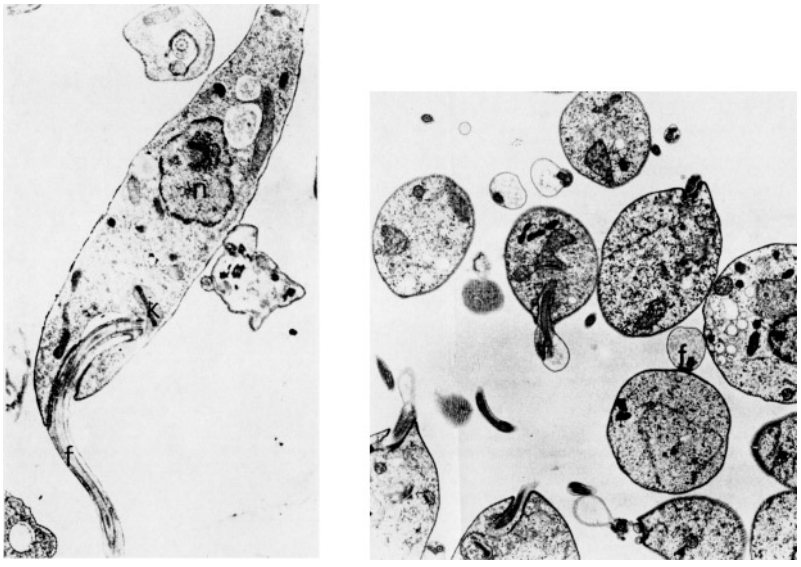

Fig. 8. (Left) General view of untreated *Leishmania mexicana* promastigote forms showing an elongated shape with a long flagellum, kinetoplast, nucleus and mitochondria. Magnification ×7,000. (Right) Promastigotes incubated for 10 min with 0.5 μM AmB. Magnification ×5,000.

replacement of external NaCl by choline chloride or sucrose (Brajtburg et al., 1980*a*).

The existence of a causal connection between aqueous pore formation by AmB and its lethal action on LPs was further supported by the measured effect of TEA^+ on the AmB-induced EB incorporation (Fig. 7). As it was anticipated from the known blocking effects of TEA^+ on the aqueous channels formed by AmB (Borisova et al., 1979; Cohen, 1986; Cohen & Gamargo, 1987), TEA^+ blocked AmB-induced salt influx across LPs and so retards the initial lag phase during salt and water moves into the cells. The nonsaturable characteristic of such a blocking effect was not unexpected since TEA^+ is known to be impermeable across the AmB aqueous pores (Cohen, *unpublished results*). By contrast, the subsequent AmB-induced incorporation of EB across LPs was inhibited by TEA^+ in a saturable way (K_i = 2 mM) (Fig. 7). Since ethidium is an organic cation larger than TEA^+ or $choline^+$ both of them being impermeable through AmB channels, such an EB incorporation across AmB-treated LPs possibly originate from a local fracture of the membrane, occurring as a result of the increased turgor

pressure generated by water movement. Such fractures would have a greater diameter than the AmB aqueous pores and so they can be also permeable to TEA^+, resulting in a saturable inhibition of EB incorporation. It is interesting to note that the AmB-induced incorporation of EB across LPs has been shown to be strongly inhibited by Mg^{2+} (Ramos et al., 1989), a phenomenon which appears to depend on the amount of negatively charged components present in the cell surface membrane (Ramos et al., 1989). Such a protective role of Mg^{2+} against the leishmanicidal action of AmB is common to the membrane damage exerted by pore-forming hemolytic viruses and toxins against a variety of cells (Bashford et al., 1986), including the lethal action of AmB on fungi (Brajtburg et al., 1980*b*).

As far as the nature of the aqueous pores formed by AmB in LPs is concerned, the observed positive cooperativity for salt permeation across LMVs is similar to that measured for a variety of solutes permeating across AmB-treated erythrocytes, where cooperativity values of 2–3 were calculated (Deuticke, Kim & Zollner, 1973). Likewise, the anion selectivity sequence across AmB aqueous pores measured in this work (Table) is also comparable to measurements in erythrocytes where a greater AmB-induced permeability to SCN^- than Cl^- was originally determined (Deuticke et al., 1973). The AmB-induced salt permeation across LMVs as listed in the Table coincides with Eisenman's sequence I for anion selectivity (Wright & Diamond, 1977). This result is consistent with the formation of enlarged hydrated aqueous pores by AmB. However, such a selectivity cannot be quantitatively ascribed to the diffusion of anions within the AmB aqueous channels. The reason is that anions such as SCN^- and NO_3^- are chaotropic ions that may shift the equilibrium of the AmB species dissolved in water from the self-associated to monomeric, and so they may also affect the rate of formation of AmB channels (Cohen, 1992).

Finally, it is important to note that the consequences of the exposure of LPs to a hypo-osmotic stress have been studied previously (Darling & Blum, 1990; Darling, Burrows & Blum, 1990). These authors have shown that the typical elongated LPs rapidly rounded up upon exposure to decreasing osmolarities (Darling & Blum, 1990). Such changes of shape were shown to be accompanied by a transient increase of cell volume and by a release of alanine and other compounds to the external medium, indicating the cell ability to regulate volume under hypo-osmotic stress (Darling et al., 1990).

In this study, LPs exposed to leishmanicidal AmB concentrations have also been observed to become shorter and more rounded (Fig. 8, right), a behavior which appears to be an early response to the AmB-induced water influx originated by net salt influx. In addition, the observed proliferation of vesicles throughout the cytoplasm of promastigotes treated with AmB may be part of a regulatory volume decrease (RVD) mechanism, activated under the osmotic stress. Nevertheless, the rate and extent of the cation and anion permeability induced by AmB and its concomitant massive water influx rapidly overcomes the capacity of leishmanias to osmoregulate and cells lysed.

In summary, we have demonstrated that low AmB concentrations are able to form across ergosterol-containing liposomes and LPs, prepore structures that enhance cation permeability and collapsed the parasite membrane potential but are not lytic. By contrast, at high AmB concentrations, cell lysis occurred as a direct consequence of the formation by AmB of aqueous pores permeable to small cations and anions. The toxic effects exerted by AmB on the clinically important *Leishmania* amastigote forms residing in the parasitophore vacuole of host macrophages, can also be based on the formation of AmB aqueous pores. The oncotic pressure of the proteins present inside the macrophage vacuole would not be sufficient to balance the significant turgor pressure brought about by the net intracellular salt accumulation induced by the antibiotic.

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