ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1994, p. 1079–1084
0066-4804/94/$04.00+0
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Vol. 38, No. 5

Effect of Ketoconazole on Lethal Action of Amphotericin B on *Leishmania mexicana* Promastigotes

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Received 11 August 1993/Returned for modification 11 October 1993/Accepted 4 March 1994

The effect of ergosterol depletion by ketoconazole on the leishmanicidal activity of the pore-forming antibiotic amphotericin B (AmB) was investigated. *Leishmania mexicana* promastigotes were lysed within minutes by the addition of micromolar concentrations of AmB (0.5 μM) but became insensitive to AmB after growth in the presence of ketoconazole (0.25 μM, 90 h). Lipid chromatographic analysis indicated that under such conditions, ketoconazole depleted the major *Leishmania* sterols, dehydroepisterol and ergosterol. Plasma membrane vesicles prepared from ketoconazole-treated promastigotes exhibited a much reduced enhancement of their salt permeability after the addition of AmB at concentrations as high as 5 μM. This finding clearly indicates that upon ketoconazole treatment, the capacity of pore formation by the antibiotic is substantially impaired. The reduction of desmethyl sterols by ketoconazole was accompanied by a significant increase of 14-α-methyl sterols, but exogenous cholesterol remained unchanged. This ability of *Leishmania* promastigotes to incorporate cholesterol from the external medium may explain why ketoconazole-treated cells exhibited a much decreased but significative response to AmB when they were exposed to high AmB concentrations (2.5 or 5.0 μM). Parallel measurements by using a fluorescence energy transfer method indicated that binding of AmB to ketoconazole-treated *Leishmania* promastigotes and heat-transformed leishmanias was also decreased but to different extents, a finding that may be related to the differences in their sterol content. The results obtained clearly indicate that the specific interaction of AmB with desmethyl sterols, such as dehydroepisterol, ergosterol, and even exogenous cholesterol, is an absolute requirement for the lethal action exerted by this polyene antibiotic on *L. mexicana* promastigotes.

The parasitic protozoans of the genus *Leishmania* are the causative agents of several forms of cutaneous, mucocutaneous, and visceral leishmaniasis in many parts of the world (9). The mainstay of antileishmanial chemotherapy is parenteral administration of pentavalent antimony in the form of sodium stibogluconate (Pentostam) or meglamine antimonate (Glucantime). Amphotericin B (AmB), a pore-forming polyene antibiotic (3), has also been widely used for the treatment of visceral leishmaniasis in spite of its toxic side effects (9). 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 (10). However, there is not yet a clear understanding of the cellular basis of the leishmanicidal action of AmB.

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, which is the main sterol of mammalian host cells (reviewed in references 3 and 5). According to this theory, the formation of these pores causes the plama membrane to become nonselectively leaky to ions and essential metabolites, and as a result, cell death ensues. Other effects of AmB, such as the lipid peroxidation observed in various cell types, may also play a role in its mechanism of action (22).

In parasitic protozoa such as *Leishmania* spp., the sterol hypothesis of AmB action (21) has been supported by the finding that isolated membrane vesicles prepared from their plasma membranes are made permeable by AmB to monovalent salts and glucose (7, 8), at the same concentrations which are known to be leishmanicidal in vitro (20). However, a direct evaluation of such a hypothesis by determining the AmB response of sterol-depleted leishmanias has been precluded by the lack of viable sterol mutants of these organisms.

In a previous investigation (19), we found that the transformation by heat of *Leishmania mexicana* promastigotes led to a drastic reduction of the parasite ergosterol/phospholipid ratio at the membrane phase, but such a process did not reduce the AmB sensitivity of the transformed cells. On the contrary, an enhancement of the rate of response of such cells to AmB was observed (19). However, no clear conclusion about the absolute requirement for ergosterol in the AmB mechanism of action can be derived from these experiments, because heat transformation of leishmanias involves changes in their overall lipid composition (1, 12, 25), a process that by itself can modulate the interaction between AmB and sterol molecules at the membrane level (4, 6, 15).

In the present work, we have used an alternative approach to reduce the ergosterol content of *Leishmania* promastigotes and to investigate its consequences on AmB action. The present method is based on the use of ketoconazole, an azole derivative, which is known to act in leishmanias and other cells by inhibiting the cytochrome P-450-dependent C-14 demethylation of lanosterol (2, 11, 13, 23). For this purpose, *L. mexicana* promastigotes were grown in the presence of increasing ketoconazole concentrations, and the changes of cell viability in the presence of AmB were determined. Changes in

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the sterol composition content in control and ketoconazole-treated promastigotes were also determined. The extent of AmB binding to ketoconazole-treated *Leishmania* promastigotes and heat-transformed promastigotes was also determined. Finally, we investigated the capacity of the polyene antibiotic to form aqueous pores across vesicles prepared from the plasma membranes of *Leishmania* promastigotes grown in the presence or absence of ketoconazole.

MATERIALS AND METHODS

Materials. Ethidium bromide (EB) and AmB were purchased from Sigma Chemical Co. (St. Louis, Mo.). Ketoconazole was obtained from Janseen (Beerse, Belgium), and TMA-DPH [1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate] was obtained from Molecular Probes (Eugene, Oreg.). An AmB stock solution (1 mM) was dissolved in dimethyl sulfoxide and used on the day that it was prepared.

Growth of *L. mexicana* promastigotes. Promastigotes of *L. mexicana* NR were cultivated at 25°C in LIT medium supplemented with 5% fetal bovine serum (GIBCO), as previously described (19). Growth was estimated from turbidimetric measurements at 560 nm. When the promastigotes reached the exponential phase of growth (10^7 cells per ml), ketoconazole was added, and growth was allowed to continue for 90 h. In all experiments, ketoconazole was added as an aqueous solution titrated to pH 2.4 with HCl and sterilized by filtration through 0.22-μm-pore-size Millipore filters. Promastigotes were then harvested and washed twice by centrifugation at $1,000 \times g$ for 10 min, with a buffer solution (pH 7.6) containing 75 mM Tris-HCl, 140 mM NaCl, and 11 mM KCl (buffer A).

Transformation of promastigotes by elevation of temperature. *L. mexicana* promastigotes in the stationary phase of growth (about 5 days after inoculation) were harvested and washed twice with buffer A. One-half of the culture was transferred into medium 199 (pH 7.2) to a density of 2×10^7 to 3×10^7 cells per ml. The cells were incubated at 37°C for 6 h in a CO_2 chamber (25). The other half was kept for control experiments. Observation of cells by light microscopy after 6 h of incubation indicated that most cells became round and nonmotile, with a shortening of the flagellum.

Analysis of free sterols of ketoconazole-treated *Leishmania* promastigotes. *L. mexicana* promastigotes grown in ketoconazole-containing LIT medium for 90 h, as described above, were harvested and washed twice in buffer A. Total lipids were extracted by suspending a cell pellet in chloroform-methanol (2:1, vol/vol) at 4°C for 12 h. The mixture was centrifuged at $10,500 \times g$ for 15 min and then filtered by using Whatman paper (no. 2). Filter paper was washed several times with chloroform-methanol, and the wash fluid was pooled and evaporated to dryness in a rotary evaporator. After saponification as described by Kates (17), the petroleum ether extracts, containing the neutral lipids, were dried with Na_2SO_4 (anhydrous), filtered with Whatman paper, and reduced in volume in the rotary evaporator. The free sterol content was analyzed by gas-liquid chromatography with a VARIAN model 3700 chromatograph equipped with glass columns (4 m by 4 mm) of 3% OV-1 and N_2 gas carrier (24 ml/min).

Kinetics of *Leishmania* cell death. The effect of ketoconazole on the kinetics of AmB-induced cell death was monitored by using fluorometry with the DNA-binding compound EB (19). Promastigotes grown with or without 0.25 μM ketoconazole (90 h) were incubated in the fluorescence cuvette at a final concentration of 25×10^6 cells per ml in buffer A containing 10 mM glucose for 5 min. EB (50 μM) followed by AmB (dissolved in dimethyl sulfoxide) was then added. Fluorescence measurements were done with an SPEX Fluorolog II spectrophotometer equipped with a magnetic stirrer at 365- and 580-nm excitation-emission wavelengths.

Isolation of *Leishmania* membrane vesicles. The preparation and permeability properties of membrane vesicles from *Leishmania* promastigotes have been described previously (7, 8). An identical procedure was used to prepare vesicles from the plasma membranes of *L. mexicana* promastigotes grown in a ketoconazole-containing LIT medium. For this purpose, ketoconazole (0.2 μM) was added to a culture in the exponential phase of growth. After cell growth for 90 h, promastigotes (10^{10} to 10^{11} cells) were harvested and washed twice by centrifugation ($1,000 \times g$ for 10 min with buffer A) (see above). After another wash with buffer B (10 mM HEPES [*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; pH 7.6], 400 mM mannitol, 10 mM KCl, 3 mM magnesium acetate), the ice-cold pellet of cells was mixed in a mortar on ice with glass beads (75- to 150-μm diameter; Sigma) in a 4:1 ratio (weight of beads to wet weight of pellet). This mixture was ground by gentle rotation for 5 to 10 min until no more than 2 or 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 $40,000 \times g$ for 50 min. The enzymatic characteristics and osmotic properties of *Leishmania* plasma vesicles prepared by this method have been reported previously (8). Protein concentrations were determined by the method of Lowry et al. (18).

Salt permeability changes of *Leishmania* membrane vesicles. Volume changes of *Leishmania* membrane vesicles occurring after exposure to an osmotic gradient of a salt were monitored by measuring the 90° light scattering changes (Durrum stopped-flow spectrophotometer, model D-110) (6–8). The effect of AmB on the salt permeability across such vesicles was determined by measuring the maximal rate of swelling after their maximum shrinkage (minimum volume) was reached (6). For this purpose, *Leishmania* vesicles (protein concentration, 1.2 mg/ml) prepared from cells grown in the absence or presence of ketoconazole (0.2 μM, 120 h) were rapidly mixed in a 1:4 volume ratio with a hyperosmotic KNO_3 solution (600 mosM) prepared in the same buffer A but without mannitol.

AmB binding by fluorescence energy transfer. TMA-DPH has been used as a specific marker of the plasma membrane of various types of cells (14). The fluorescence energy transfer to AmB from TMA-DPH was used to determine the binding of AmB to the plasma membrane cells. This method applied to monolayer kidney cells has already been described (16). Since the emission spectrum of TMA-DPH overlaps the absorption spectrum of AmB, an energy transfer can occur between the two molecules when AmB is near the probe. As AmB does not fluoresce, the energy transfer results in a quantifiable decrease of the fluorescence of TMA-DPH proportional to the quantity of AmB bound to the plasma membrane of *Leishmania* promastigotes. A 10^{-6} M TMA-DPH solution was prepared in phosphate-buffered saline from a 2×10^{-3} M stock solution in dimethylformamide. *Leishmania* promastigotes were injected into 2 ml of this solution in the cuvette of the JY 3D spectrofluorimeter (Jobin Yvon), at a final concentration of 5×10^6 cells per ml. Gentle stirring ensured the homogeneity of the suspension during recordings. Upon the addition of cells, the intensity increased and a steady state was reached after 2 min, indicating that TMA-DPH was incorporated into cell membranes. Further addition of AmB was done at this point.

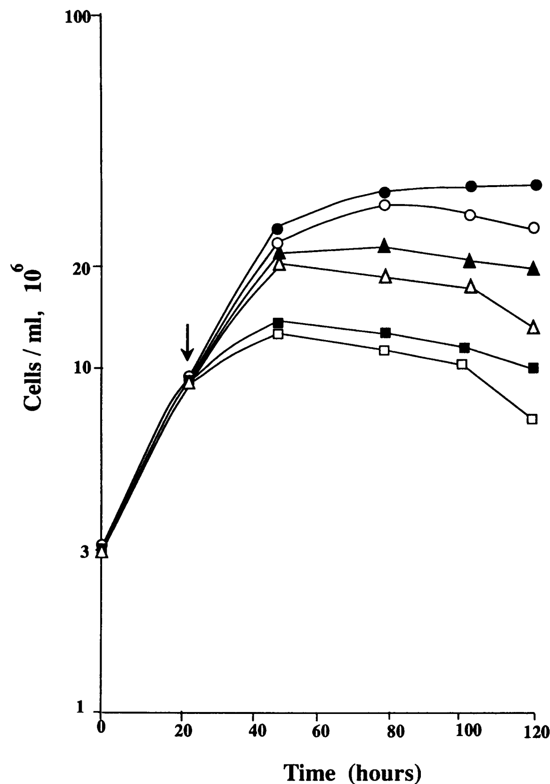

FIG. 1. Effect of ketoconazole on growth of *L. mexicana* promastigotes. Promastigotes were grown in a liquid LIT medium (see Materials and Methods), and the number of parasites per milliliter as a function of time was monitored by turbidimetry. Ketoconazole was added (arrow) at the following concentrations: 0 μM (●), 0.2 μM (○), 0.4 μM (▲), 0.8 μM (△), 1.0 μM (■), and 2.0 μM (□).

The final concentration of AmB varied from 0.05 to 0.8 μM. All experiments were done at 25°C.

Calculation methods. A nonradiative singlet-singlet transfer occurred between the fluorescent TMA-DPH probe and AmB. AmB, which is the acceptor, does not emit fluorescence; all the fluorescence comes from the donor. The efficiency of energy transfer was calculated from the quenching of the energy donor by the equation $E = \Delta F/F_0$, where F_0 is the initial intensity emitted in the absence of the acceptor and ΔF corresponds to the extinction of fluorescence due to the binding of AmB to the membrane. No correction for photon reabsorption of AmB was used, because at the AmB concentration used no significant photon reabsorption occurred.

Fluorescence measurements. Fluorescence emitted by TMA-DPH embedded in the cell membrane was recorded with an excitation monochromator set at 365 nm. For kinetic recording, the emission wavelength was 427 nm, which corresponds to the maximal intensity of the emission of TMA-DPH in membranes.

Fluorescence was also observed microscopically by using an inverted microscope (Axiovert 10; Zeiss, Suresnes, France) and a SIT camera (Lhesa, Cergy-Pontoise, France). Micrographs were made with a video graphic printer (Sony).

RESULTS

Effect of ketoconazole on the growth of *Leishmania* promastigotes. *L. mexicana* promastigotes were cultured in a liquid LIT medium supplemented with 5% fetal bovine serum (Fig. 1). In this medium, leishmanias have a mean generation time of about 20 h. The addition of 0.2 μM ketoconazole to such cells in the exponential phase of growth produced a slight inhibitory effect on the cell growth rate. Figure 1 shows that a ketoconazole concentration of 0.4 μM was clearly leishmanistatic. At higher ketoconazole concentrations (0.8, 1.0, and 2.0 μM [Fig. 1]), cell growth stops more rapidly and lysis begins to take place. However, no clear leishmanicidal effect was apparent before 96 h of azole exposure.

Direct observation by light microscopy of ketoconazole-treated leishmanias (data not shown) indicated that such cells became rounded and lost their mobility.

Free sterols in control and ketoconazole-treated promastigotes. *L. mexicana* promastigotes yielded three groups of sterols which were identified by their chromatographic properties (Table 1). One group consisted of 14-α-desmethyl sterols and included ergosterol, cholesta-5,7,24-trien-3-β-ol (cholestatrienol), and ergosta-5,7,24(28)-trien-3-β-ol (5-dehydroepisterol). Dehydroepisterol is the most abundant component (Table 1), but the amounts of all three were below detection levels in promastigotes exposed to ketoconazole. The second group consisted of endogenous, 14-α-methyl sterols and included 4,14-dimethyl zymosterol, obtusifoliol, and lanosterol. The first two sterols were not detected in control promastigotes, but they accumulated in substantial amounts in the presence of ketoconazole, specially 4,14-dimethyl zymosterol. Finally, a third group included cholesterol, the mammalian sterol which is also present in *L. mexicana* promastigotes (Table 1). As indicated by the data shown in Table 1, the amount of cholesterol found in control promastigotes remained relatively unchanged after ketoconazole treatment. This result is consistent with an external origin of this sterol (11). In fact, as demonstrated by Goad et al., the cholesterol found in *L. mexicana* promastigotes grown in a cholesterol-containing medium was neither biosynthesized nor metabolized (11). On the other hand, the presence in control *Leishmania* promastigotes of cholestatrienol (Table 1), a desmethyl sterol of the cholestane series, has been associated with the occurrence of C-24 alkylation at a late stage in the biosynthesis

TABLE 1. Effect of ketoconazole on free sterol composition in *L. mexicana* promastigotes

Ketoconazole concn (μM)	% ± SD[a]						
	Ergosterol	Cholestatrienol	5-Dehydroepisterol	4,14-Dimethylzymosterol	Obtusifoliol	Lanosterol	Cholesterol
None (control)	8.2 ± 2.5	1.4 ± 0.4	80.4 ± 2.9	NM	NM	2.0 ± 1.1	8.1 ± 0.9
0.5	NM[b]	NM	NM	74.8 ± 3.3	10.4 ± 1.2	2.5 ± 0.6	7.1 ± 1.7
1.0	NM	NM	NM	79.9 ± 2.3	10.5 ± 0.7	2.8 ± 0.9	6.7 ± 0.5
2.0	NM	NM	NM	73.7 ± 1.5	9.9 ± 1.3	3.8 ± 0.9	7.7 ± 3.3

[a] Results, expressed as the percentages of total free sterols ± standard deviations, are the averages of two experiments.
[b] NM, not measurable.

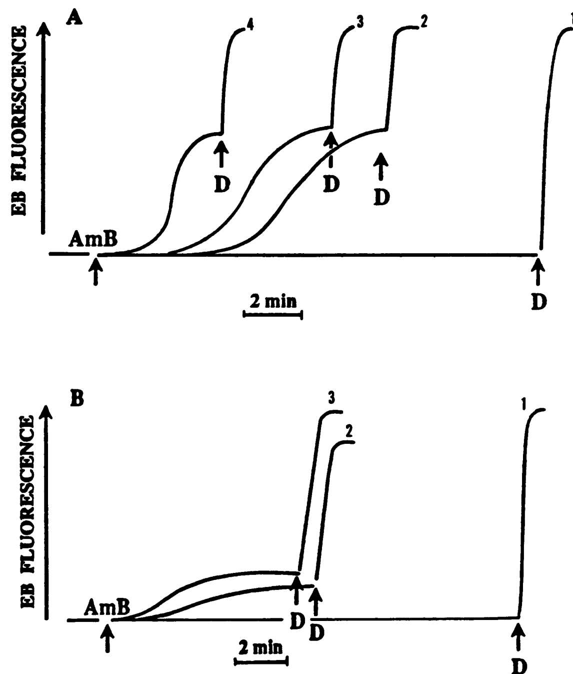

FIG. 2. Effect of AmB on kinetics of permeabilization to EB in ketoconazole-treated promastigotes. *L. mexicana* promastigotes were grown in an LIT medium with or without ketoconazole (0.25 μM). After 90 h of cell growth, promastigotes were harvested, washed with buffer A (see Materials and Methods), and suspended in the same buffer A containing 10 mM glucose and EB (50 μM). EB fluorescence changes were recorded as described in the text. Parasites were incubated at a final concentration of 25 × 10^7 cells per ml. (A) Promastigotes grown in a ketoconazole-free medium. Traces: 1, control, no AmB (dimethyl sulfoxide was added); 2, 0.5 μM AmB; 3, 1.0 μM AmB; 4, 5.0 μM AmB. (B) Promastigotes grown in a medium containing 0.25 μM ketoconazole. Traces: 1, 1 μM AmB; 2, 2.5 μM AmB; 3, 5.0 μM AmB. Arrows indicate the addition of digitonin (D) or AmB. Maximal EB incorporation was always determined by adding digitonin.

of *Leishmania* sterols and not with any cholesterol biosynthesis or metabolism (11).

The effect of AmB on the viability of ketoconazole-treated *Leishmania* promastigotes. *L. mexicana* promastigotes grown in the presence of ketoconazole were monitored by fluorometry with the DNA-binding compound EB (Fig. 2). When *L. mexicana* promastigotes grown in the absence of ketoconazole were exposed to 0.5 μM AmB, a leishmanicidal concentration (20), a sigmoidal incorporation of EB into the cells was observed (Fig. 2A, trace 2). This effect was always preceded by a lag phase, which at this AmB concentration lasted about 3 min. Increasing the concentration of AmB reduced the lag time and increased the velocity of EB entry (Fig. 2A, traces 3 and 4). By contrast, when 0.5 or 1.0 μM AmB was added to promastigotes grown for 90 h with 0.25 μM ketoconazole, no incorporation of EB was detected, at least during the time scale of our present measurements (Fig. 2B, trace 1).

Ketoconazole-treated promastigotes responded slightly to AmB when higher AmB concentrations were used (Fig. 2B, traces 2 and 3). It can be observed in Fig. 2B that the lag time of EB incorporation for promastigotes exposed to 5 μM AmB (Fig. 2B, trace 3) is very similar to that observed in control promastigotes exposed to equivalent AmB concentrations (Fig. 2A, trace 4), but the total extent of EB incorporation was a small fraction of the value measured in promastigotes not exposed to ketoconazole.

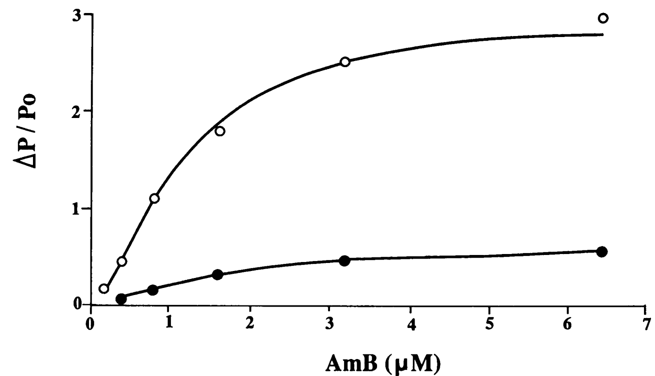

FIG. 3. Effect of AmB on permeability of KNO_3 across ketoconazole-treated *Leishmania* membrane vesicles. Membrane vesicles were prepared from promastigotes grown in the presence of ketoconazole (0.1 μM, 120 h). They were rapidly mixed (protein concentration, 1.2 mg/ml) with a hyperosmotic 600 mosM solution of KNO_3, and the volume changes were determined by light scattering as described previously (7, 8). ΔP/Po, relative permeability changes. ○, control; ●, ketoconazole (10^{-7} M).

The effect of AmB on the salt permeability of *Leishmania* membrane vesicles prepared in the presence or absence of ketoconazole. The lethal action of AmB on *L. mexicana* promastigotes has been associated with the disruptive permeabilizing effect that is originated by the formation at the parasite plasma membrane of sterol-dependent aqueous pores (7, 8). It was then anticipated that a reduction of ergosterol content at the membrane level as a result of ketoconazole treatment may directly affect the formation of such structures. In order to test this hypothesis, we prepared membrane vesicles (7, 8) from the plasma membranes of ketoconazole-treated and untreated *L. mexicana* promastigotes (see Materials and Methods) and determined the effect of AmB on the salt permeability of such membrane preparations.

When *Leishmania* membrane vesicles derived from promastigotes grown in a ketoconazole-containing medium were exposed to a hyperosmotic salt solution (see Materials and Methods), they exhibited an osmotic response comparable to that shown by untreated control vesicles. The corresponding relative permeabilities for the permeation of KNO_3 across such membrane vesicles are plotted against the external AmB concentration in Fig. 3. *Leishmania* vesicles prepared from untreated promastigotes exhibited a cooperative, sigmoidal enhancement of salt permeability in a relatively narrow AmB concentration range (Fig. 3). Thus, the AmB-induced enhancement of salt permeability increased abruptly from about 0.4 to 0.8 μM AmB (Fig. 3). When *Leishmania* membrane vesicles prepared from the corresponding ketoconazole-treated promastigotes were examined (Fig. 3), a much reduced and noncooperative response to AmB was observed. In effect, at 6.4 μM AmB, the maximal polyene concentration used in these experiments, the total AmB-induced salt permeability across ketoconazole-treated leishmania membrane vesicles was about 15% of that measured for vesicles derived from control promastigotes (Fig. 3). In other words, AmB-induced salt permeability across *Leishmania* membrane vesicles was inhibited by 85% as a result of the treatment with ketoconazole.

Study of the AmB binding to *L. mexicana* promastigotes. The binding of AmB to *L. mexicana* was monitored by

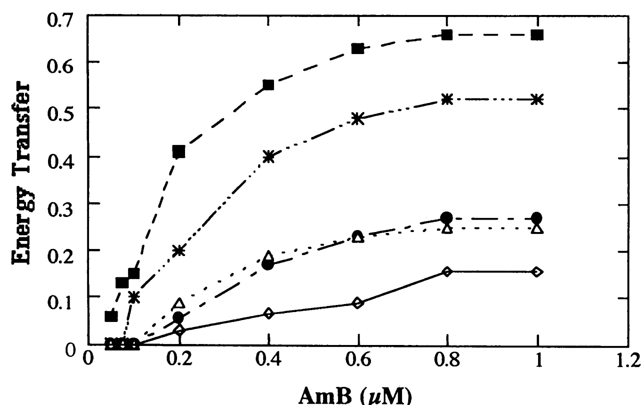

FIG. 4. Binding of AmB to ketoconazole-treated *L. mexicana* promastigotes and heat-transformed cells. AmB binding was determined by the transfer of fluorescence energy from TMA-DPH to AmB (see Materials and Methods). Promastigotes were grown for 90 h with no (control, ■), 0.5 μM (●) 1 μM (△), or 2 μM (◇) ketoconazole. *, heat-transformed cells. Results are expressed as the energy transfer efficiency as a function of AmB concentration.

measuring the transfer of fluorescent energy from TMA-DPH to AmB. Observation under the microscope of *Leishmania* promastigotes labelled with TMA-DPH revealed that only the cell membrane fluoresces (data not shown). Upon the addition of AmB, the fluorescence decreased normally (quenching) but then increased probably because of an AmB-induced internalization of TMA-DPH. The corresponding values for energy transfer were always calculated before such internalization occurred.

In Fig. 4 such energy transfer values are plotted as a function of the AmB concentration added. In all cases, a saturation of AmB binding to leishmanias was reached at approximately 0.8 μM AmB (Fig. 4). When promastigotes were heat shocked to induce heat-transformed cells (19), the efficiency of energy transfer was lower than that of untreated promastigotes (Fig. 4). On the other hand, for promastigotes grown in the presence of 0.5 to 2.0 μM ketoconazole for 90 h (Fig. 4), the efficiency of the fluorescence transfer was even lower than that in the heat-transformed cells, reflecting a much reduced binding of AmB to the membrane. At very low AmB concentrations, the depletion of membrane ergosterol by ketoconazole did not allow the binding of AmB to the membrane.

DISCUSSION

The results presented here indicate that the depletion by ketoconazole of Δ-5,7-desmethyl sterols, such as ergosterol and dehydroepisterol, in *Leishmania* promastigotes (Table 1) led to a drastic loss of the lytic effect induced in this parasite by low AmB concentrations. Thus, when *Leishmania* promastigotes were grown for 90 h in the presence of ketoconazole, washed, and resuspended in a buffer containing AmB and EB (Fig. 2), no fluorescence enhancement, indicative of EB incorporation, was observed. On the other hand, we have observed that the capacity of pore formation by AmB across ketoconazole-treated membrane vesicles was also substantially impaired compared with that of untreated membranes. In fact, such ketoconazole-treated vesicles exhibited a much-reduced salt permeability when they were exposed to increasing AmB concentrations (Fig. 3).

Since a reduction in the ergosterol content of *L. mexicana* did not always lead to the anticipated loss of AmB sensitivity (19), we thought that it would be of some importance to compare AmB binding in ketoconazole-treated promastigotes with the corresponding binding in leishmanias whose ergosterol content was reduced by growth at an elevated temperature (Fig. 4).

As shown in Fig. 4, the extent of AmB binding in heat-transformed leishmanias is significantly greater than that in ketoconazole-treated promastigotes. Although a proportion of such AmB binding can be ascribed to the interaction of the polyene antibiotic with other nonsterol membrane components, i.e., membrane phospholipids, it is evident that transformation by heat did not totally deplete leishmanias of their desmethyl sterols compared with treatment with 0.5 μM ketoconazole or higher azole concentrations. Thus, the extent of AmB binding exhibited by the *Leishmania* promastigotes treated with ketoconazole decreased as a function of the azole concentration (Fig. 4), a result that is consistent with the progressive accumulation of lanosterol and the drastic reduction in ergosterol and dehydroepisterol (Table 1).

The observation that heat-transformed leishmanias responded slightly faster to the lytic action of AmB (19) can be ascribed to the accompanying changes in the lipid composition of the transformed cells (1, 12, 25), which may facilitate aqueous pore formation. However, the presence of a small amount of ergosterol appears to be essential. Thus, we have demonstrated by using liposomes (6) that in the complete absence of ergosterol (or cholesterol), AmB can indeed form permeable channels by interacting with saturated membrane phospholipids, but only when sterols are present do such structures progress to form the enlarged AmB aqueous pores.

Finally, the present observation of a lack of complete protection by ketoconazole from the effect exerted by AmB at concentrations greater than the minimal leishmanicidal concentration (Fig. 2B, traces 2 and 3) is explained by the presence of exogenous cholesterol in promastigotes even after treatment with ketoconazole (Table 1). However, changes in membrane lipid packing (24) resulting from the ketoconazole-induced accumulation of 14-α-methyl sterols (2, 11, 13) may also facilitate aqueous pore formation (Fig. 3) and subsequent membrane lysis.

In summary, it can be concluded that a total depletion of desmethyl sterols in *Leishmania* promastigotes brought about by the use of saturating ketoconazole concentrations effectively reduces the lethal activity of AmB by depriving the polyene antibiotic of its capacity to form lethal aqueous pores in the cell membrane.

ACKNOWLEDGMENTS

This work was supported by the Commission of European Communities, grant no. c/1-0517-F (ED).

We thank J. Urbina for providing laboratory facilities for lipid analysis.

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