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Increased calcium permeability is not responsible for the rapid lethal effects of amphotericin B on *Leishmania sp.*

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The mode of action of the polyene antibiotic amphotericin B (AmB), the drug of choice for the treatment of systemic fungal infections and visceral leishmaniasis, is still unclear. An increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), toxic in many cases, has been postulated as a possible lethal mechanism for AmB. Cell permeabilization to ethidium bromide (EB) was used as a criterion of viability. Kinetics of the DNA-EB fluorescent complex formation was studied in ergosterol-containing *Leishmania* promastigotes. Intracellular Ca^{2+} concentration was measured using quin-2 fluorescence in parallel aliquots. It is shown in this work that AmB can act as an efficient Ca^{2+} ionophore. However, the rapid permeabilization effect induced by AmB on these cells was not dependent on an increase in $[Ca^{2+}]_i$. On the contrary, it was found that leishmanicidal effect of AmB was enhanced in the absence of external calcium. Furthermore, A23187 a Ca^{2+} ionophore did not provoke cell permeabilization to EB.

Amphotericin B; Polyene antibiotic; Calcium; *Leishmania*; Quin-2; Ethidium bromide

1. INTRODUCTION

The polyene antibiotic amphotericin B (AmB) is an efficient drug against fungi and parasitic protozoa but it is also toxic to mammalian cells [1,2]. Cell-walled fungi containing ergosterol in its plasma membranes are more susceptible to lower concentrations of AmB than mammalian cells containing cholesterol [3–5]. A common mechanism of AmB action, involving the formation of aqueous pores of about 8 Å diameter, has been proposed for both types of membranes [6,7]. It was thought that the formation of such channels would disrupt the plasma membrane selective properties leading to leakage of potassium ions and essential metabolites and thus to cell death [8,9]. However, changes in the potassium concentration in yeast cells have been shown to be dissociated from the lethal effects exerted by AmB [10,11]. AmB can form two types of channels in ergosterol-containing liposomes and membrane vesicles prepared from *Leishmania sp.* [12,13]. One of them is responsible for the permeability to small monovalent cations, whereas the second type of channel made membranes permeable to glucose [12,13] and divalent cations such as Ca^{2+} [14]. In this respect, the question arises as to whether the fungicidal and leishmanicidal effects of AmB may be related to a selective increment of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), as it has been proposed by other authors to explain the lethal effects of a variety of pore-forming proteins and toxic compounds, including AmB, on mammalian cells [15,16]. In order to investigate this proposition in an ergosterol-containing cell membrane system, we have employed *Leishmania sp.*, a pathogenic protozoa which is the causative agent of leishmaniasis, a tropical disease second in importance only to malaria. Parallel experiments were carried out to study the relationship between the kinetics of *Leishmania* cell death with the changes in $[Ca^{2+}]_i$ induced by AmB.

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2. MATERIALS AND METHODS

Promastigotes of *Leishmania braziliensis yaracuyensis* were cultivated in LIT medium at room temperature, as previously described [17]. Parasites in the exponential phase were washed with a buffer solution (pH 7.6) containing Tris-HCl (75 mM), NaCl (140 mM) and KCl (11 mM), and resuspended in the same buffer with 10 mM glucose at a concentration of $2–3 \times 10^8$ parasites/ml.

The kinetics of *Leishmania* cell death was followed by spectrofluorometry with the nucleic acid-binding compound ethidium bromide (EB) to detect permeabilized cells. Promastigotes were incubated in the presence of 50 μM EB and fluorescence was registered continuously at 365–580 nm excitation-emission wavelengths at room temperature with constant stirring. After stabilization of signal (about 5 min) AmB (dissolved in DMSO) was added to the cuvette. Maximal permeabilization was always determined by adding digitonin at 30 μg/ml. This measurement of viability was validated using more conventional indexes, such as Trypan blue exclusion, in a cell culture system (MA-104) in which cell death had been induced by rotavirus infection (to be reported elsewhere).

Intracellular Ca^{2+} was measured using quin-2 fluorescence in aliquots of the same suspension [18,19]. *Leishmania* were incubated with 50 μM quin-2/AM in buffer containing 1 mM Ca^{2+} for 1 h at room temperature, washed twice by rapid centrifugation, resuspended in buffer without quin-2/AM and fluorescence registered at 335–493 nm excitation-emission wavelengths. Intracellular Ca^{2+} con-

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centration was determined by the digitonin-EGTA method of Tsien et al. [19], using an apparent K_d for the quin-2/Ca complex of 115 nM. No appreciable spectroscopic interference was found with concentrations of AmB less than 5 μM at the concentrations of quin-2 used.

3. RESULTS AND DISCUSSION

The AmB-induced permeabilization of parasites to EB is shown in fig.1a. This effect depended on AmB concentration and exhibited a characteristic lag time (lag phase). Increasing the concentrations of AmB reduced the lag time down to a limiting value (fig.1b) and increased the velocity of EB entry (fig.1c). Observation by combined phase/fluorescence microscopy revealed that AmB-treated parasites first became rounded, their motility became reduced and then became permeable to EB. After a certain time, depending on AmB concentration cell lysis can be observed. In another ergosterol-containing cell membrane system such as *Saccharomyces cerevisiae* protoplasts, AmB induced the same rapid lethal effect as measured by EB permeabilization (not shown).

AmB increased $[Ca^{2+}]_i$ in *Leishmania* almost immediately after addition (fig.2a). Measurement of $[Ca^{2+}]_i$ with quin-2 fluorescence gave an average basal value of 33 nM, rather low as compared to cells of higher eucaryotes [20]. Although little is known about Ca^{2+} homeostasis in pathogenic protozoa [21], this figure may indicate fine regulation of $[Ca^{2+}]_i$ in *Leishmania*. The increase in $[Ca^{2+}]_i$ was due to extracellular

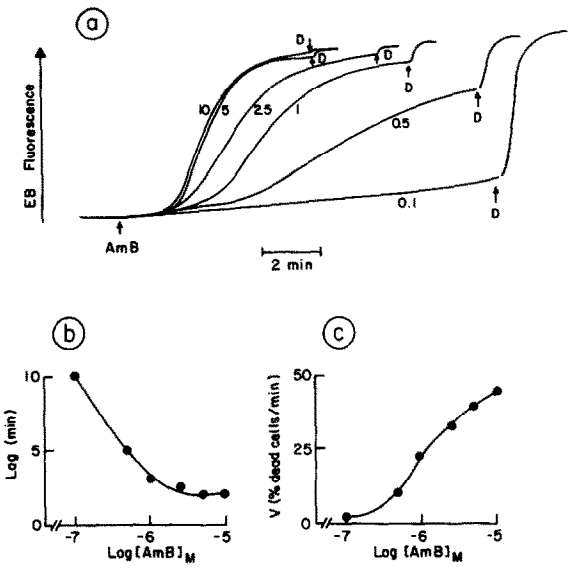

Fig.1. Permeabilization of *Leishmania* promastigotes to EB by amphotericin B (AmB). (a) Effect of different concentrations of AmB (in μM) on loss of cell viability as determined by EB-nucleic acid fluorescence. (b) The time lag for the onset of permeabilization was taken from fig.1a and plotted as a function of the logarithm of molar AmB concentration. (c) The rate of cell permeabilization (V) as a function of log molar AmB concentration. This parameter was calculated from the slopes in the straight part of the traces in 1 min and referred to 100% cell permeabilization induced by digitonin (D).

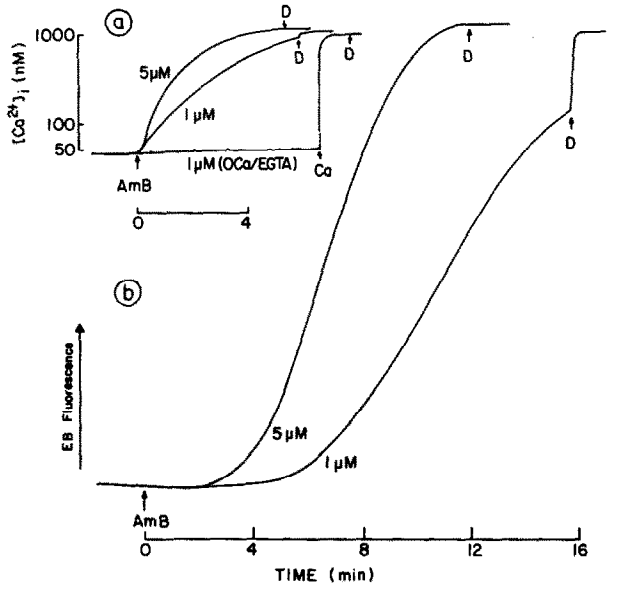

Fig.2. AmB induced an increase in $[Ca^{2+}]_i$ which preceded cell death in *Leishmania*. Parallel aliquots from the same preparation were incubated with either quin-2/AM for measuring $[Ca^{2+}]_i$ or EB for measuring loss of viability in response to AmB. Redrawn for original traces obtained with identical time scales.

Ca^{2+} entry since it was not seen in the presence of EGTA, and preceded the permeabilization to EB as observed in parallel experiments (fig.2b). Although the initial change in quin-2 fluorescence was due to Ca^{2+} entry, later changes also involved leakage of quin-2 from permeabilized cells. Loss of quin-2 from loaded parasites was evaluated by rapid centrifugation at different times after addition of AmB, followed by the measurement of fluorescence in supernatant and resuspended pellets. Leakage of quin-2 paralleled permeabilization to EB.

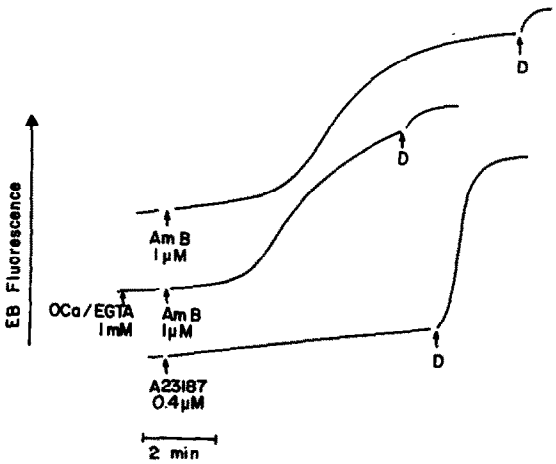

Fig.3. Induction of *Leishmania* cell death by AmB is not a Ca^{2+}-dependent process. Loss of cell viability in response to 1 μM AmB was measured by the entry of EB to the cells as in fig.1, in the presence or absence of extracellular Ca^{2+} (Ca-free medium with 1 mM added EGTA). Addition of Ca^{2+} ionophore A23187 (0.4 μM) did not induce cell permeabilization. Digitonin was added at D to completely permeabilize cells.

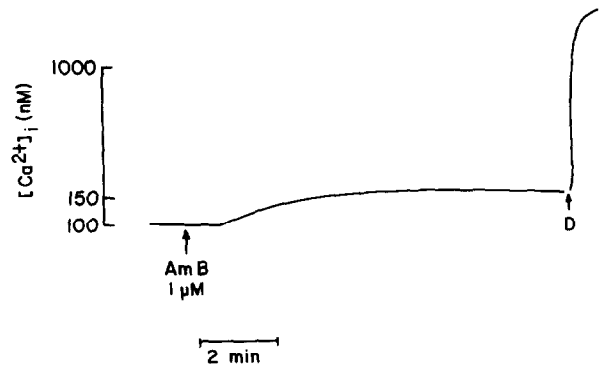

Fig.4. AmB increases $[Ca^{2+}]_i$ without cell death in a mammalian cell line (MA104). MA104 cells were grown to confluency in Eagle's Medium, trypsinized and resuspended in fresh medium. Cell suspensions were incubated with 50 μM quin-2/AM for 20 min at 37°C. Cells were washed twice by rapid centrifugation, resuspended in phosphate-buffered saline (PBS) and quin-2 fluorescence measured as in fig.2. Addition of digitonin released most of the accumulated quin-2 indicating lack of permeabilization by AmB.

The early increase in $[Ca^{2+}]_i$ due to the Ca^{2+} ionophoretic effect of AmB was not responsible for cell death. The absence of extracellular Ca^{2+} did not prevent the lethal effect of AmB; on the contrary, it appears to reduce the time for the onset of cell death (fig.3). Supporting this point, an increase in $[Ca^{2+}]_i$ by addition of the Ca^{2+} ionophore A23187 did not produce permeabilization to EB at least within 15 min of addition.

In mammalian derived cultured cells (MA104 kidney cell line) AmB also increased $[Ca^{2+}]_i$ (fig.4). However, in contrast to *Leishmania*, this increase was not followed by cell death since neither leakage of quin-2 nor increment in EB permeability was observed. In other mammalian cells, Schanne et al. [15] have shown that both AmB and A23187 induced a Ca^{2+}-dependent cell death but at much higher AmB concentrations (200 μM) and longer incubation times (2–3 h).

In conclusion, AmB provoked an increase in Ca^{2+} permeability both in cells with cholesterol-rich membranes (MA104) and cells with ergosterol-rich membranes. However, a rapid lethal effect seems to be associated only with cells containing ergosterol and is independent of the increase in $[Ca^{2+}]_i$.

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