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The polyene antibiotic amphotericin B acts as a Ca^{2+} ionophore in sterol-containing liposomes

H. Ramos [1], A. Attias de Murciano [1], B.E. Cohen [1] and J. Bolard [2]

[1] *Centro de Biologia Celular, Facultad de Ciencias, Universidad Central de Venezuela, Apartado Postal 47860 Caracas 1041 (Venezuela) and* [2] *Laboratoire de Physique et Chimie Biomoléculaire (U.A. CNRS 198), Tour 22, Université Pierre et Marie Curie, Paris (France)*

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Amphotericin B (AmB) was shown to induce a Ca^{2+} influx across ergosterol- and cholesterol-containing large unilamellar liposomes, by following spectrophotometrically the formation of the Arsenazo III-Ca^{2+} complex. At equivalent antibiotic concentrations the Ca^{2+} influx was much more extensive through ergosterol-containing membranes (almost 100% with 1 μM AmB, 160 μM lipid) than through cholesterol-containing membranes (below 0.5 μM the influx of Ca^{2+} was negligible). In the presence of ergosterol-containing membranes the initial rate of Ca^{2+} influx had the same linear dependence on the ratio antibiotic/lipid whatever the lipid concentration, which was not the case in cholesterol-containing membranes. These results suggest that the channels responsible for the AmB-induced Ca^{2+} permeability across cholesterol- and ergosterol-containing liposomes have different structures.

The polyene macrolide antibiotic amphotericin B (AmB) is toxic for fungal and animal cells. Despite the large number of studies devoted to the elucidation of its mechanism of action, no really satisfactory comprehensive explanation has yet been derived. Membrane permeability inducement has been considered as important. AmB-induced K^+ permeability has received considerable attention but does not seem to be related with the toxicity (for a review, see Refs. 1 and 2). Ca^{2+} movements have been much less addressed: they were only shown to be induced by nystatin, another polyene antibiotic, in *Saccharomyces cerevisiae* cells [3] but not in erythrocytes [4].

As far as studies on model membranes are concerned, it is known that when AmB or nystatin is added to one side of planar bilayer membranes, single conductance channels are observed but are selective for univalent cations, being virtually impermeable to divalent cations [5]. It was recently shown [6] that the induction by AmB of K^+ and H^+/OH^- currents in small unilamellar vesicles occurs via two different mechanisms, since such currents are affected differently by the presence of cholesterol or ergosterol. With large unilamellar liposomes containing ergosterol, it also appeared that two types of active channels are observed, depending on AmB concentration and time of incubation. One of them has the characteristics of an ionic channel, that is, a high permeability to univalent salts as compared with water or non-electrolytes such as urea [7]. The second type of structure is permeable to glucose and has a reflection coefficient to urea $\ll 1$ [7]. It was of some interest to determine the selectivity of these channels to Ca^{2+}, specially since it is known that AmB at a ratio AmB/lipid higher than 10^{-2} can induce some leakage of divalent cations through the membrane of cholesterol-containing multilamellar liposomes (Co^{2+}, Ni^{2+}, etc.) [8] or small unilamellar liposomes (Mn^{2+}) [9]. In the present study, we have therefore determined the Ca^{2+} movements induced by AmB across sterol-containing large unilamellar liposomes, monitoring Ca^{2+} influx by the metallochromic Arsenazo III entrapped in the vesicles [10].

Egg phosphatidylcholine (PC), cholesterol, Arsenazo III and amphotericin B were obtained from Sigma Chemical Co. and used without further purification. Ergosterol (Sigma) was purified as described previously [7]. Stock solutions of 1 mM amphotericin B were prepared in dimethylsulfoxide and stored in the dark below 0 °C for no longer than a week.

Large unilamellar liposomes (LUVs) were formed by the reverse-phase evaporation method as described by

Correspondence: B.E. Cohen, Centro de Biologia Celular, Facultad de Ciencias, Universidad Central de Venezuela, Apartado Postal 47860, Los Chaguaramos 1041, Caracas, Venezuela.

Szoka and Papahadjoupoulos [11]. Briefly, 2 ml of an aqueous phase containing 20 mM K-Mops buffer (pH 6.8), 0.7 mM Arsenazo III and 150 mM KCl were mixed with 6 ml of diethyl ether containing 20 μmol of lipid (egg PC + 20 mol% ergosterol or egg PC + 20 mol% cholesterol). Sterol-free LUV were also prepared. The resulting two-phase system was sonicated by 5–10 s in a sonicator (Braun-Sonic 2000) until the mixture become a one-phase dispersion. LUVs were formed after the organic solvent was removed under reduced pressure in a rotary evaporator [11]. This preparation of liposomes was filtered through a polycarbonate membrane (Nuclepore, pore size 0.4 μm) without loss of lipid. Non-trapped Arsenazo III was removed by passing vesicles down a Sephadex G-50 column (1 × 10 cm) equilibrated with buffer A composed of 20 mM K-Mops (pH 6.8) and 150 mM KCl and eluted with this same buffer. The vesicle preparation thus obtained was diluted with buffer A to a phospholipid concentration of 2 mM or less. The composition of such liposomes was found to be identical to the lipid composition in the mother solution (that is, 80 : 20 molar ratios for PC : ERG and PC : CHOL), indicating that under the present conditions, incorporation of ergosterol (ERG) and cholesterol (CHOL) into the membranes was maximal. Lipid phosphorus was determined as reported in Ref. 12 and sterol content using the enzymatic test 'C system' provided by Boehringer.

Liposomes made of PC and ergosterol or cholesterol were found by negative-staining electron microscopy to be fairly homogeneous in size, being predominantly unilamellar even though oligolamellar vesicles were also observed. The vesicles were seen to be smooth and rounded. The diameters of the LUVs were estimated to have an average diameter of 0.1 ± 0.04 μm (n = 286) for PC + ERG LUV and 0.14 ± 0.06 μm (n = 466) for PC + CHOL LUV. In agreement with these results, the corresponding LUV internal volumes as determined by measurements of the amount of entrapped Arsenazo III (after adding Triton X-100 and Ca^{2+}, see below) gave values of 4.9 ± 0.14 μl/μmol for PC + ERG LUV and 7.7 ± 1.4 μl/μmol for PC + CHOL LUV.

Spectrophotometric absorbance measurements of Arsenazo III-calcium complex formation were performed on an Aminco (model DW-2a) by using the dual wavelength mode. For this purpose, the changes in absorbance of the samples at 577 nm were measured with the absorbance at 587 nm as reference (isobestic point of the spectrum of Arsenazo III-calcium complex).

In Figs. 1a and 1b are shown the time course of the differential changes of ΔA, exhibited by Arsenazo III-encapsulated liposomes, after adding Ca^{2+} (3 mM) and AmB at increasing concentrations. It can be seen in Fig. 1a that raising the AmB concentration from 0.5 μM to 10 μM led to increasing increments in the initial rate of calcium influx across ergosterol-containing (PC + ERG)

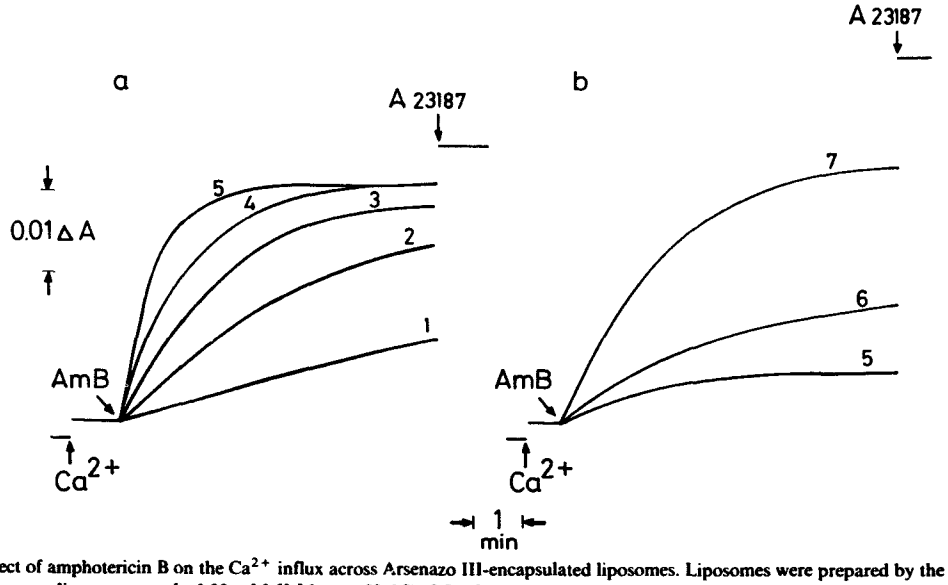

Fig. 1. The effect of amphotericin B on the Ca^{2+} influx across Arsenazo III-encapsulated liposomes. Liposomes were prepared by the reverse phase method [11] in a medium composed of 20 mM K-Mops (pH 6.8), 0.7 mM Arsenazo III and 150 mM KCl. After filtering and passing down a Sephadex column (see text) the absorbance changes of Arsenazo III-encapsulated liposomes were recorded as a function of time at the wavelengths 577–587 nm, in a single cuvette using a dual wavelength spectrophotometer. Arrows indicate additions of aliquots of $CaCl_2$ (3 mM), AmB (dissolved in DMSO, see below) and A23187 (5 μM). Final volume: 1.0 ml. All the experiments were carried out at room temperature. (a) Ergosterol-containing liposomes (1.66 mM lipid concentration). (b) Cholesterol-containing liposomes (2 mM lipid concentration). The final aqueous concentrations of AmB were as follows: 1, 0.5 μM; 2, 1.0 μM; 3, 2.5 μM; 4 5.0 μM; 5, 10 μM; 6, 25 μM; 7, 50 μM.

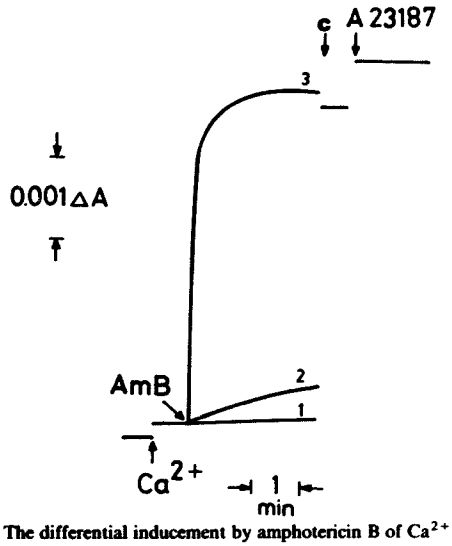

Fig. 2. The differential inducement by amphotericin B of Ca^{2+} influx across Arsenazo III-encapsulated liposomes, prepared with ergosterol on cholesterol. Arsenazo III-encapsulated liposomes were mixed with 3 mM $CaCl_2$ and the absorbance changes at the wavelength 577–587 nm were recorded. Arrows indicate the addition of AmB (50 μM at curve 1 and 5 μM at curves 2 and 3) and A23187 (5 μM). Arrow c at curve 3 indicates the magnitude of the ΔA value after the AmB-containing sample was passed down an small Sephadex G-50 column (0.6×8 cm), in order to remove any Arsenazo III released to the external aqueous solution. Curve 1, liposomes prepared without sterol (0.2 mM lipid concentration). Curve 2, cholesterol-containing liposomes (0.2 mM lipid concentration). Curve 3, ergosterol-containing liposomes (0.16 mM lipid concentration).

liposomes. The traces shown in Fig. 1a also indicate that the maximal absorbance ΔA change due to AmB-induced calcium permeability was about 85–90% of the total ΔA changes, as measured by addition of the calcium ionophore A23187.

The corresponding changes in Ca^{2+} permeability induced by AmB across cholesterol-containing (PC + CHOL) liposomes are shown in Fig. 1b. In this system, the initial rates of influx of AmB-induced calcium permeabilities were also observed to increase with raising antibiotic concentrations but at a much lower rate and extent in spite of the much higher concentration of antibiotic used.

It can be observed in Fig. 2 that the external addition of AmB did not induce any leakage of Arsenazo III entrapped inside the ergosterol-containing liposomes (arrow C at curve 3) or the cholesterol-containing liposomes (curve 2, results not shown). It follows that the measured rate of change of ΔA induced by AmB reflects the differential permeability of calcium ions across the liposome membranes. It can also be seen in Fig. 2 (curve 1) that liposomes prepared without sterol exhibited no changes in ΔA, even after adding AmB concentrations as high as 50 μM.

The enhancement by AmB of Ca^{2+} permeability across sterol-containing liposomes was also examined as a function of the AmB/lipid concentration ratio. It can be seen in Figs. 3a and 3b that AmB-induced calcium permeability increased linearly with the AmB/lipid concentration ratio, independently of the type of sterol

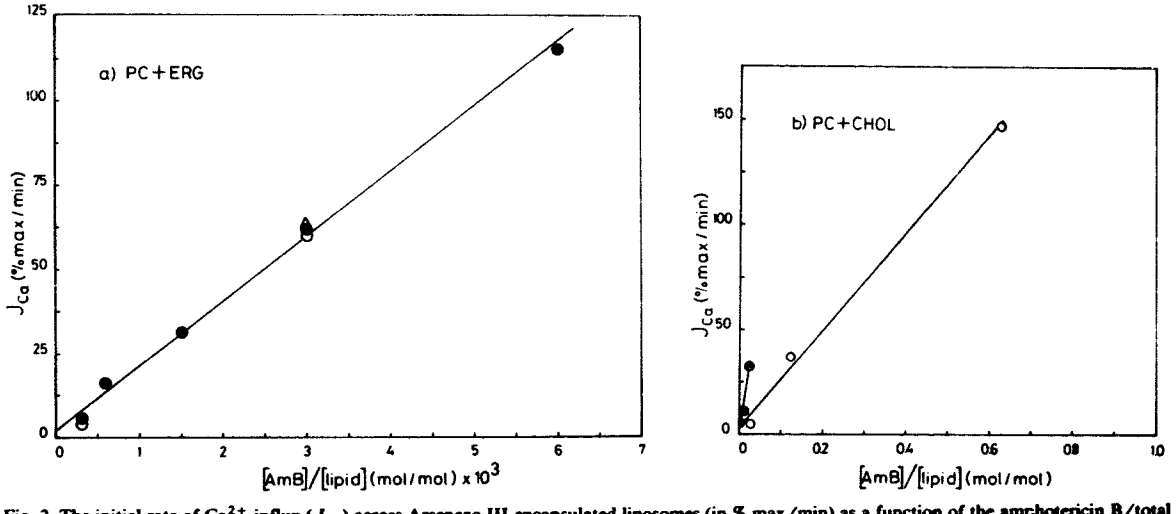

Fig. 3. The initial rate of Ca^{2+} influx (J_{Ca}) across Arsenazo III-encapsulated liposomes (in % max/min) as a function of the amphotericin B/total lipids ratio (in mol/mol). (a) Ergosterol-containing liposomes. (b) cholesterol-containing liposomes. The initial rates of Ca^{2+} influx (% max/min) across liposomes are expressed as per cent of the total changes at 577–587 nm induced by the calcium ionophore A23187 (5 μM). Symbols denote measurements carried out at different final lipid concentrations as follows: (a) PC+ERG: ●, 1.66 mM; ○, 0.166 mM; △, 0.0166 mM; (b) PC+CHOL: ●, 2.0 mM; ○, 0.2 mM.

present. However, it can be seen that the initial ratio of calcium influx induced by a certain concentration of AmB decreased significantly when PC + CHOL LUV were treated with the same AmB concentration but at much lower lipid concentrations (Fig. 3b). In contrast, PC + ERG LUV (Fig. 3a) exhibited the same linear dependence whatever the lipid concentration used.

Our results confirm the observation that AmB can induce the permeability of divalent cations across multilamellar liposomes containing cholesterol, although to a limited extent compared with ergosterol-containing liposomes. More interesting are the results obtained with PC + ERG LUV since they indicate that the AmB-induced permeability to calcium occurs in the same concentration range and time after mixing at which the formation by AmB of channels permeable to glucose has been observed [7,13]. We can therefore think that AmB-induced Ca^{2+} influx across liposome membranes occurs through aqueous channels with an enlarged diameter compared to those channels responsible of K^+ leakage.

The difference in the dose-response relationship in the presence of cholesterol and ergosterol indicate that the nature of the species responsible for the permeability changes are different. In the presence of cholesterol, the species inducing permeability are not formed below $5 \cdot 10^{-7}$ M, which could be related to the formation of aggregated AmB above this concentration. On the contrary, in the presence of ergosterol the formation of the permeabilizing species depended only on the AmB/lipid ratio, whatever the lipid concentration, and can therefore occur at AmB concentrations where only monomeric AmB is present.

Differences in the nature of the interaction of AmB with LUV containing cholesterol or ergosterol have recently been reported by circular dichroism [14]. Such a different behaviour of the CD spectra had not been observed with SUV [15]. It seems therefore difficult to limit the origin of the selective toxicity of AmB for fungi to a greater affinity of the antibiotic for their membranes. The present results suggest a difference in the nature of the permeabilizing species. We are currently evaluating the biological significance of this differential inducement by AmB of Ca^{2+} permeability across membranes.

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