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Concentration- and time-dependence of amphotericin-B induced permeability changes across ergosterol-containing liposomes

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The effect of amphotericin B on the permeability properties of liposomes prepared by reverse-phase evaporation was examined by using an osmotic method. This study has revealed that the magnitude and type of the alterations in permeability induced by amphotericin B in liposomes made of egg phosphatidylcholine and ergosterol depend not only on the amphotericin B concentration in the external aqueous solution but also on the time elapsed after mixing. Thus, low amphotericin B concentrations (from 0.2 to 1.2 μM) led to, (1) an small increment of the total extent of shrinkage of liposomes suspended in non-electrolytes such as urea or salts like KNO_3, (2) an enhancement of urea and salt permeabilities at the same time scale at which volume changes were measured (ms to s), (3) a maximal blocking by tetraethylammonium of amphotericin B-induced urea permeability and (4) an enhancement of glucose permeability but only after liposomes were incubated with amphotericin B for some minutes before mixing. The high amphotericin B concentration regime (beyond 1.2 μM) led to, (1) a decrease of the total extent of shrinkage of liposomes immediately after rapid mixing of liposomes with urea solutions containing amphotericin B and (2) a 50% reduction of the tetraethylammonium blocking of amphotericin B-induced urea permeability. These results are explained by assuming that amphotericin B may form in ergosterol-containing liposomes two types of active channel differing in internal diameter.

The use of polyene macrolides such as amphotericin B as antibiotic agents is based on their ability to alter the selective permeability properties of cell membranes (reviewed in Refs. 1–3). All polyene antibiotics require the presence of cholesterol or ergosterol in the membrane of sensitive cells to express its activity [4–6]. Various lipid model membrane systems have been used to investigate the type and extent of the permeability changes induced by amphotericin B in the lipid barrier. As a result of these studies, it was proposed that the action of amphotericin B in natural and sterol-containing lipid model membranes can be accounted for by way of the formation of aqueous pores of about 8 Å in diameter [7,8]. On the other hand, it has been shown by Ermishkin et al. [9] that addition of amphotericin B to cholesterol-containing black lipid membranes led to the formation of single ionic channels. The conductance of these single channels is blocked by small non-electrolytes and several tetraalkylammonium ions such as tetraethylammonium [10].

It is also known that much higher concentrations of amphotericin B are required for measuring an increase of the permeability of erythrocytes to large anions such as phosphate or lactate than for the corresponding enhancement of potassium, chloride or glycerol permeabilities [11]. Deuticke

et al. [11] have noted that the action of amphotericin B on erythrocytes appears to be a time-dependent process, a phenomena originally observed in microbial [5], epithelial [12], black lipid membranes [13] and small unilamellar liposomes [14].

Recently, it was shown [15] that external addition of amphotericin B to liposomes prepared by the reverse-phase evaporation method [16] led to a differential enhancement of the permeability to water, urea and salts of these membranes. It was argued in this work that the structures formed by one-sided addition of amphotericin B to liposomes behaved more like ionic channels than to the aqueous channels formed by two-sided addition of amphotericin B to black lipid membranes [15]. In the present work a concentration and time dependence of the effects of amphotericin B on liposomal permeability to different solutes is reported. These findings suggest that both types of structure, ionic and aqueous channels may be formed by one-sided addition of amphotericin B to ergosterol-containing liposomes depending on antibiotic concentration and time elapsed after mixing.

Egg phosphatidylcholine and amphotericin B were obtained from Sigma Chemical Co. and used without further purification. Ergosterol (Sigma) was recrystallized at least three times by adding cold methanol to a saturated ergosterol solution in chloroform. Stock solutions of 1 mM amphotericin B were prepared in dimethylformamide and stored in the dark below 0°C for no longer than a week. The concentration of the stock solution of amphotericin B was determined by using the extinction coefficient $\epsilon = 12.3 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [17].

Liposomes were prepared by the reverse-phase evaporation method as described by Szoka and Papahadjoupoulos [16]. Liposomes formed by this technique are referred to as reverse-phase evaporation vesicles (REV). REV made of egg phosphatidylcholine and ergosterol (84 : 16, molar ratios) were found by negative-staining electron microscopy to be predominantly unilamellar eventhough oligolamellar vesicles were also observed. REV diameters were estimated by measuring a total of 410 vesicles in four areas of grid. About 70% of vesicles obtained have a diameter between 0.05 to 0.2 μm. The ideal osmotic behaviour of REV has been described previously [15].

A Durrum stopped-flow apparatus was adapted to monitor the relative intensity of 450 nm light scattered at 90°. In a typical experiment, liposomes (1 mM lipid concentration) were mixed with a 600 mosM urea or salt solution by using a 1 : 4 ratio drive syringe. Amphotericin B dissolved in dimethylformamide was added to the hyperosmotic solution before mixing so that organic solvent final concentration was less than 0.5% by volume. Control experiments indicated that at this concentration dimethylformamide had no effect on light-scattering changes. The temperature of all experiments was 30 ± 1°C.

Concentration dependence of amphotericin B-induced permeability changes across liposomes. A typical stopped-flow oscilloscope trace of light scattered by liposomes after mixing with an hyperosmotic urea solution is shown in Fig. 1a (control). The initial rapid upward portion of the trace depicts the liposomes shrinking due to water movement to the external solution in response to the osmotic gradient. The second slower and downward portion represents the swelling of liposomes as urea enters the vesicles with its concomitant water movement [18].

The effect of one-sided addition of amphotericin B at increasing concentrations on these biphasic scattered light changes is shown in Figs. 1a and 1b. It can be observed that between 0.2 μM to 1.2 μM (Fig. 1a) the extents of shrinkage of liposomes suspended in urea exhibited small increments with raising amphotericin B concentrations but at higher amphotericin B concentrations (Fig. 1b), the maximum shrinkage decreased abruptly.

A bimodal effect of amphotericin B on the extent of shrinkage was not observed when liposomes were mixed with an hyperosmotic KNO_3 solution (Figs. 1c and 1d). It can be seen that raising the amphotericin B concentration from 0.2 μM to 1.2 μM led to small increments in the extent of shrinkage but above 1.2 μM amphotericin B (Fig. 1d) no significant decrease of the maximum shrinkage with respect to the control curve (C) was observed.

The traces shown in Figs. 1a and 1c also show that low amphotericin B concentrations were able to induce an increase of the rate of swelling exhibited both by liposomes suspended in urea or KNO_3, after its maximum shrinkage. However,

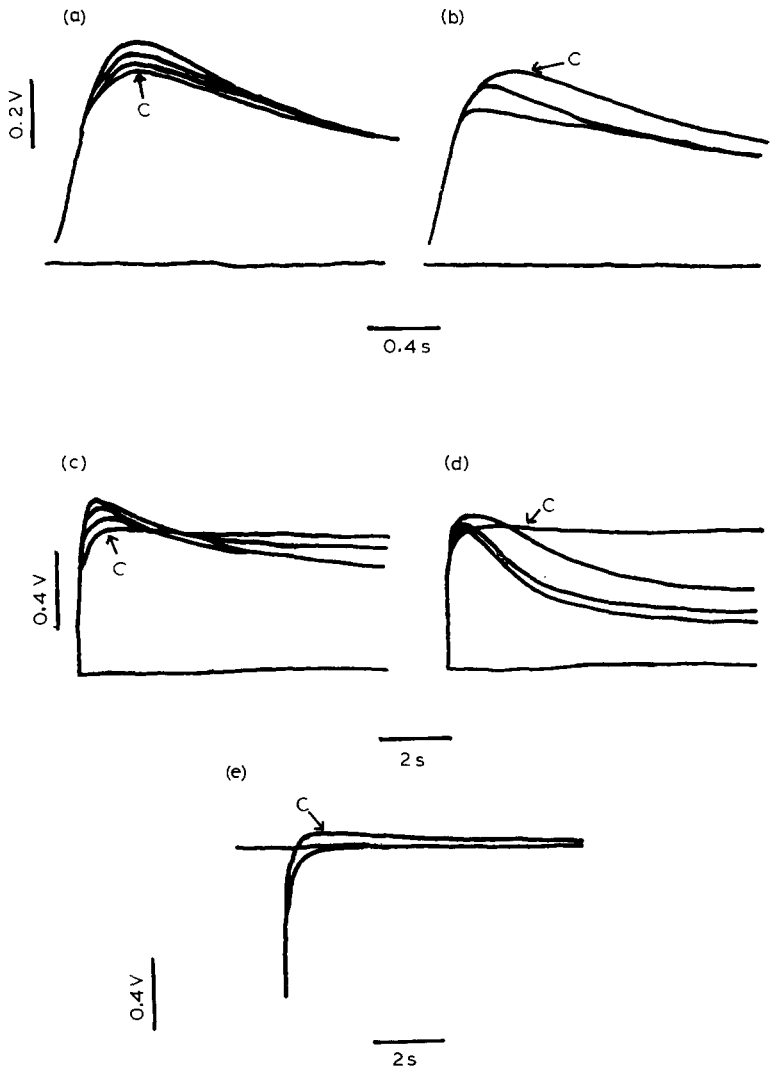

Fig. 1. Typical oscilloscope traces of the 90° light-scattering changes (in mV) of liposomes suspended in hyperosmotic solutions of different solutes. Liposomes (REV) were prepared from egg-phosphatidylcholine and ergosterol (77 : 23, molar ratio) in a 60 mosM phosphate buffer (pH 7) and rapidly mixed (1 : 4, volume ratio) with a 600 mosM nonelectrolyte or salt solution containing different amphotericin B concentrations. The final (after mixing) AmB concentrations (in μM) are given: (a) Urea. 0 (control); 0.2; 0.8; 1.2 (bottom to top); (b) Urea. 0 (control); 1.6; 3.2 (top to bottom); (c) KNO_3. 0 (control); 0.2; 0.6; 0.8 (bottom to top); (d) KNO_3. 1.6; 0 (control); 3.2, 6.4 (top to bottom); (e) K_2SO_4. (top to bottom): 0 (control); 6.4 μM. Curved traces show the first sweep of the oscilloscope and the horizontal lines indicate the steady-state light-scattering values. The steady-state value of liposomes suspended in KNO_3 solution without amphotericin B remained at the top of Figs. c and d but were omitted for the sake of clarity. Arrows (C) point to control traces.

when liposomes were mixed with a K_2SO_4 solution, no enhancement of the swelling rate was observed, even though amphotericin B concentrations as high as 6.4 μM were added (Fig. 1e). On the contrary, the small basal permeability to K_2SO_4 exhibited by liposomes not exposed to amphotericin B (control trace in Fig. 1e) was reduced even more after addition of the polyene antibiotic.

The relative increments in the magnitude of the swelling rates induced by low amphotericin B concentrations are much smaller when liposomes were mixed with urea (Fig. 1a) than with KNO_3 (Fig. 1c). Thus, it can be estimated by using the 'maximum slope' method of determining solute permeabilities [19] that the effect of 0.8 μM amphotericin B on the relative permeability change $(P_{AmB} - P_0)/P_0$ to KNO_3 is at least one order of magnitude greater than the corresponding value estimated for urea (note the difference between the ordinate scales of Fig. 2 and inset). These results appear, then, to indicate that the structures already formed a few seconds after mixing liposomes with low amphotericin B concentrations (under 1.2 μM) behave more like ionic channels than aqueous channels.

On the other hand, at amphotericin B concentrations higher than 1.2 μM (Figs. 1b and 1d),

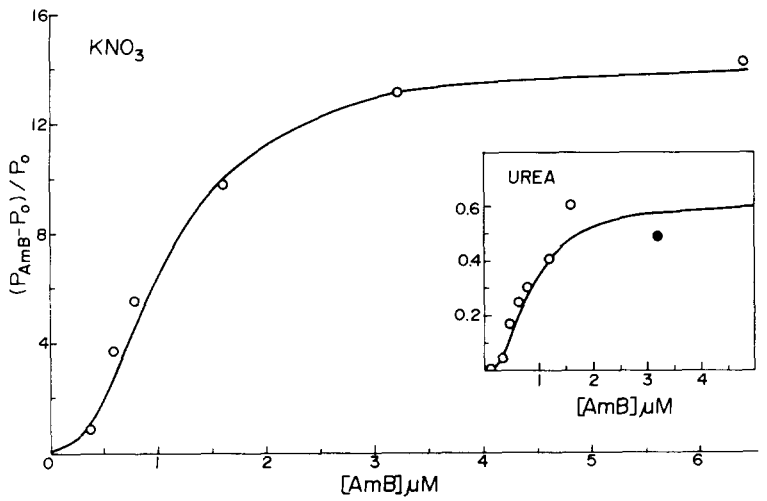

Fig. 2. Relative changes in the maximum slope after the minimum volume ($(P_{AmB} - P_0)/P_0$) for liposomes suspended in KNO_3 or urea (inset) plotted vs. the amphotericin B (AmB) concentration (μM) in the external aqueous solution. The sigmoid curves were traced by fitting experimental points to the equation of Hill. The calculated values for the cooperativity order, n, were: $n = 2.3$ ($r^2 = 0.99$) for KNO_3 and $n = 2.2$ ($r^2 = 0.99$) for urea. All experimental points (an average of five determinations at each concentration) were used in these calculations, with the exception of the data obtained for urea-suspended liposomes treated with 3.2 μM amphotericin B (filled circle, inset) (see text). The maximal standard deviation was less than twice the size of the symbols. The rest of experimental details are the same as in Fig. 1.

the rate of swelling of liposomes mixed with KNO_3 (Fig. 1d) increased markedly, whereas the corresponding changes for urea-suspended liposomes remained small or even decrease at the highest amphotericin B concentration used (3.2 μM). At amphotericin B concentrations higher than 1.2 μM, the observed swelling rates of urea-suspended liposomes no longer reflect solute permeability changes, because the extents of shrinkage at the minimum volume have decreased (Fig. 1b) and under these conditions the 'maximum slope method' [19] of determining solute permeabilities underestimates the true permeability changes induced by amphotericin B. This behaviour may be taken as an indication of a decrease of the urea reflection coefficient across liposome membranes, possibly due to the formation of channels of amphotericin B with an enlarged diameter.

The hypothesis that a second type of active amphotericin B channel with an enlarged diameter may be forming rapidly at high amphotericin B concentrations was tested by using tetraethylammonium, an efficient blocker of the single channels formed by low amphotericin concentrations in black lipid membranes [9]. Borisova et al. [10] have proposed that the blocking efficiency of structurally related compounds on the amphotericin B single channel's conductance was greater the closer the size of the molecule to the pore diameter.

It can be seen in Fig. 3 that blocking by tetraethylammonium of amphotericin B-induced urea permeability across liposomes was maximal below 1.2 μM amphotericin B but decreased to nearly a half beyond this concentration value.

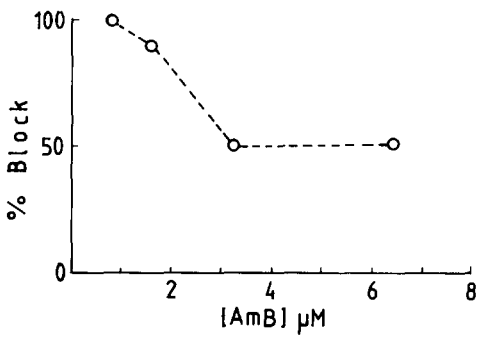

Fig. 3. Percentage blocking by tetraethylammonium of amphotericin B-induced urea permeability across liposomes as a function of the concentration of amphotericin B in the external aqueous solution. Liposomes (REV) were prepared from egg-phosphatidylcholine and ergosterol (84:16, molar ratio) in a 60 mosM phosphate buffer (pH 7). Liposomes were rapidly mixed (1:4, volume, ratios) with a 600 mosM urea solution containing 4 mM tetraethylammonium and amphotericin B at increasing concentrations. At each concentration of amphotericin B, blocking by tetraethylammonium was calculated by substracting the maximal slope after the minimum volume shown by liposomes exposed to tetraethylammonium from control values; these values were then normalized by multiplying them by the V/V_0 ratio, where V is the total extent of shrinkage of liposomes exposed to tetraethylammonium and V_0 is the corresponding control value. The experimental points shown are an average of at least five determinations. The maximal standard deviation was less than the size of the symbol.

Time dependence of amphotericin B-induced permeability changes across liposomes. When liposomes were incubated with amphotericin B for different time intervals before mixing them with hyperosmotic urea solutions, the measured total extents of shrinkage (V_t) at the minimum volume (proportional to the urea reflection coefficient) changed with the time of incubation (Fig. 4, top). It can be seen that the change of ΔV_t with incubation time varies with the concentration of amphotericin B. Thus, at 0.2 μM, amphotericin B takes about 2 min to induce a change in the total extent of shrinkage, this time being reduced at higher concentrations. At amphotericin B concentrations higher than 1.2 μM, reductions in V_t were already observed after the rapid mixing of amphotericin B-containing urea solutions with liposomes (Fig. 1b).

Finally, it was also found that liposomes incubated with amphotericin B for some minutes exhibited a significant permeability to glucose, whose magnitude increases with raising amphotericin B concentrations (Fig. 4, bottom). It can be seen that the time-dependent enhancement of glucose permeability is remarkably similar to the time dependence of the decrease in the measured V_t values for the same liposomes suspended in urea (top of Fig. 4).

In conclusion, we have presented evidence suggesting the formation of amphotericin B in ergosterol-containing liposomes of two types of structure: one with a predominantly ionic channel character, that is, a high value of the relative permeability coefficient of KNO_3 with respect to water and urea, a high reflection coefficient for urea and an elevated sensitivity of the urea permeability to ionic blockers such as tetraethylammonium. The second type of structure is less effectively blocked by tetraethylammonium and exhibited permeability properties to urea and glucose similar to the aqueous pores previously characterized by two-sided addition of amphotericin B to black lipid membranes [20,21]. Formation of these structures is found to be dependent both on the total concentration of amphotericin B and the time elapsed after mixing the antibiotic with liposome membranes.

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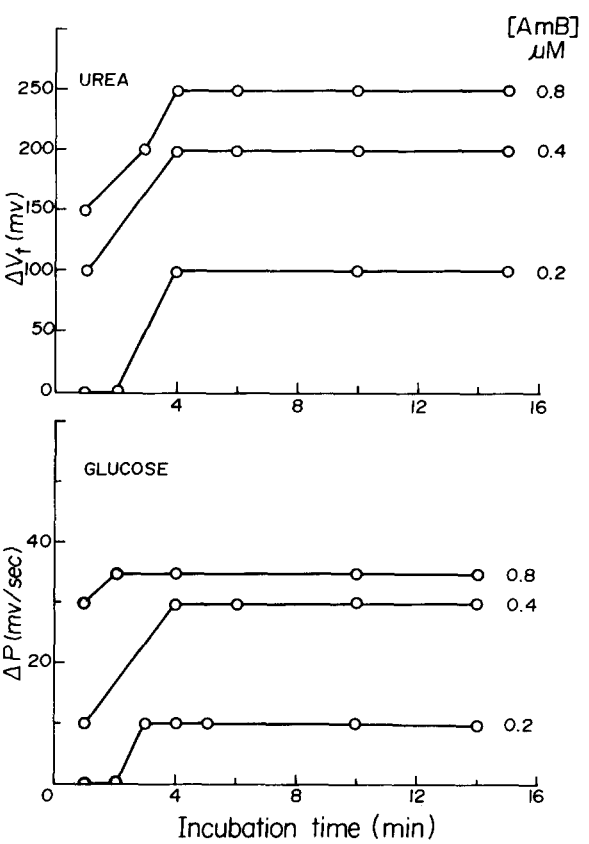

Fig. 4. The effect of the time of incubation of liposomes with amphotericin B (AmB) on urea and glucose permeability parameters. Liposome (REV) were prepared from egg-phosphatidylcholine and ergosterol (84:16, molar ratio) in a 60 mosM phosphate buffer (pH 7). To 1 ml aliquots of liposomes (1 mM lipid concentration) various concentrations of amphotericin B were added. After different time intervals, liposomes were rapidly mixed with a 600 mosM urea or glucose solution. Top. Ordinate: decrease in the total extent of shrinkage at the minimum volume (in mV) of liposomes suspended in urea. Abscissa: incubation time (in min). Bottom. Ordinate: relative changes in the maximum slope after the minimum volume of glucose-suspended liposomes ($P_{amB} - P_0$) in mV/s. Abscissa: incubation time (in min). In both parts of this figure, the concentrations of amphotericin B shown (in μM) are final (after mixing) concentrations. The maximal standard deviation was less than twice the size of the symbol. The rest of experimental details are as in Fig. 1.

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