The Interaction of Amphotericin B with Cell Membrane of Rat Thymocytes

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Abstract. Amphotericin B (AB) at micromolar concentrations increases cell membrane permeability and induced swelling of rat thymus lymphocytes Potassium efflux is a precondition for AB to induce swelling of the cells The rate constants for potassium loss and volume changes were proportional to the 1 24th and the 2nd power of the antibiotic concentration respectively The reflection coefficients for nonelectrolytes with different hydrodynamic radii were determined, and the equivalent radius of the amphotericin pore in the thymocyte cell membrane was estimated to be 4 1 ± 0 3 Å at polyene concentrations varying between 2 5 μmol/l and 80 μmol/l It is suggested that channel formation by AB in cell membranes is actually able to modulate immune responses

Key words: Amphotericin B — Thymocytes — Plasma membrane — Pore size

Introduction

The polyene antibiotic amphotericin B (AB) is an important drug widely used as an effective antifungal agent At the same time AB has been shown to enhance both humoral and cell-mediated immunity (Bolard 1986) The mode of AB action is usually explained by its ability to form ion channels in the plasma membrane Thymocytes are extremely sensitive to AB and are used as a cellular model to investigate AB interaction with lymphoid cells (Henry-Toulme et al 1989 a, b) The molecular mechanism underlying this immunomodulation properties remains poorly understood It is not clear i) if the ion channel formation by AB leads to this immunostimulatory effects or whether AB acts by alternative way, ii) what is the size of AB-induced water pore in the thymocyte membrane To find answers to these questions we studied both the influence of AB on thymocyte membranes and the polyene-induced pathway in these membranes, and compared them with artificial systems

Materials and Methods

Amphotericin B was a generous gift of Squibb France A stock solution of the antibiotic in dimethylsulfoxide (1 or 4 mg/ml) was freshly prepared before experiments The concentration of the stock solution was found to have no effect on the results The media were prepared from analytical grade or high purity reagents

Thymocytes were isolated from the thymi of 100–150 g weighing rats according to standard methods (Hunt 1990) The cells were washed two times by standard solution containing 145 mmol/l NaCl, 8 mmol/l Tris-HCl buffer (pH 7 4), and resuspensed to 5% (v/v, 120 ± 20 million cells per ml) The cell suspension used usually contained less than $5 \pm 2\%$ damaged cells (determined by trypan blue exclusion)

The experimental chamber usually contained 4 ml standard solution with or without nonelectrolytes The cell suspension was added to obtain a final concentration of 1 5 million cells per ml Gentle stirring ensured homogeneity of the suspension during recordings The kinetics of potassium leakage from the cells after the addition of the antibiotic was measured by a valinomycin electrode Changes of the cell suspension turbidity (indicating swelling or shrinking) were registered by measuring the transmittance ($T, \%$) at 610 nm The optical density of cell suspension which is proportional to the cell volume (Hempling 1972) was calculated as $D = \log(100/T)$

The observed time-dependences of potassium leakage (K_k) and volume change (K_d) were characterized by rate constants Their values were obtained from experimental time dependences of potassium ion concentration ([K]) and optical density (D) after substracting the lag period from the initial sigmoidal shape of the kinetic curve by fitting the following equations

$$D = D_{\lim} \{1 - \exp(-K_d \ t)\} \quad (1)$$

$$[\mathrm{K}] = [\mathrm{K}]_{\lim} \{1 - \exp(-K_k \ t)\} \quad (2)$$

where $D_{\lim}$ and $[\mathrm{K}]_{\lim}$ are the limiting values of D and [K] respectively Best fits were calculated by nonlinear least square method using minimum random search algorithm (Eler 1972)

Osmolality was measured by a freezing point depression osmometer (OMKA 1-II-05) All experiments were carried out at 37 ± 1 °C

Results and Discussion

Amphotericin B at concentrations as low as in the micromolar range induced increase of cell membrane permeability and caused swelling of rat thymus lymphocytes Thymocyte volume changes induced by different amounts of AB are shown in Fig 1 The curves were usually sigmoidal in shape Their lag-periods increased from 30 s till 10 min when AB concentration decreased from 5 μmol/l up to 1 25 μmol/l

Fig 2 shows the dependence of the rate of cell swelling (determined as the slope of transmittance versus time, %/min) on the AB concentration On a double logarithmic scale the curve can be fitted by two linear functions (insert, Fig 2) with slopes $2\ 06 \pm 0\ 18$ for low and $0\ 40 \pm 0\ 06$ for high AB concentrations

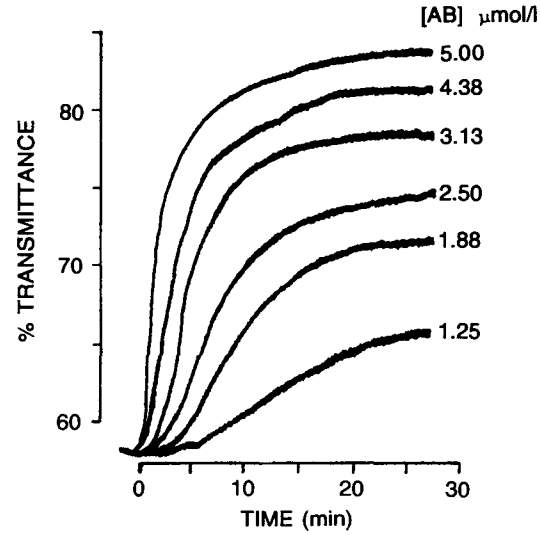

Figure 1. Effects of various concentrations of amphothericin B upon the turbidity of rat thymocyte suspension. The polyene antibiotic was added at time zero up to the indicated concentrations. Typical original recordings are represented. For details see Methods.

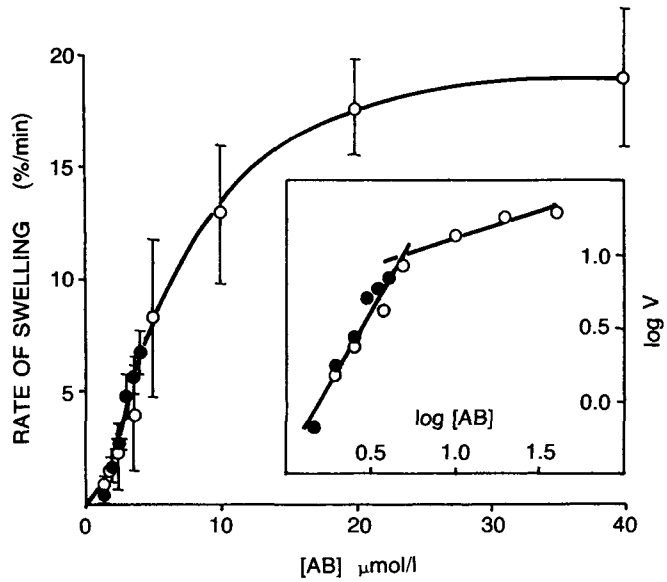

Figure 2. The rate of thymocyte swelling as a function of amphothericin B concentration. Insert: The same curve in double logarithmic scale. The polyene concentration in stock solution was 1 mg/ml (filled circles) or 4 mg/ml (empty circles).

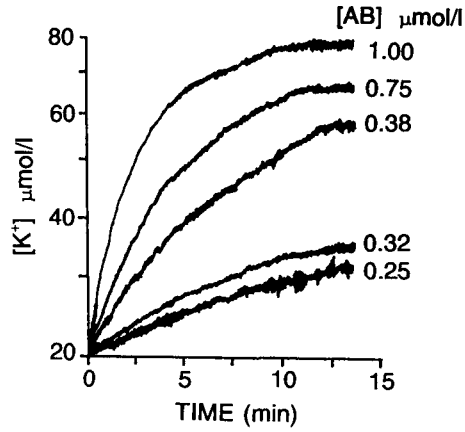

Figure 3. Effects of various concentrations of amphothericin B upon potassium ion release from thymocytes. The polyene antibiotic was added at time zero up to the concentrations indicated.

The observed swelling of cells is a consequence of an AB induced facilitation of membrane permeability for small ions. Low concentrations of AB induced a strong potassium leakage from thymocytes (Fig. 3). The shape of the time-dependence curves was also sigmoidal. But the lag-period in this case was shorter than that for cell swelling at all AB concentrations used.

To compare the rate of swelling with that of potassium leakage the corresponding rate constants were calculated (see Methods). As can be observed from Fig. 4, these dependences were linear in double logarithmic scale. The slope was 1.25 ± 0.04 for potassium leakage and 1.94 ± 0.09 for cells swelling. These values were similar to those obtained in erythrocyte experiments (1.5–2.5 according to Deuticke et al. 1973) but they were smaller than those obtained for artificial membranes (4–10, Cass et al. 1970). This finding indicates that more than one molecule of AB is actually required to build a water pore in the thymocyte membrane.

Fig. 4 shows also that the concentration dependence for potassium leakage is shifted toward lower AB concentrations as compared to the curve for cell swelling. Thus, we can conclude that potassium efflux is a precondition for the AB to induce swelling of thymocytes.

The swelling itself suggests that there is an osmotic gradient across the AB modified cell membrane. This gradient can be compensated for or even reversed by addition of sucrose into the basic salt solution (Fig. 5). When the sucrose concentration was raised up to 125 ± 5 mmol/l no cell sweelling was observed at any of the AB concentrations tested (Fig. 6). This result allows conclusion that an osmolarity value of $\sim 125 \pm 20$ mosm/kg corresponds to the oncotic pressure across the thymocyte membranes upon addition of AB.

It should be noted that at sucrose concentrations below 125 mmol/l biphasic

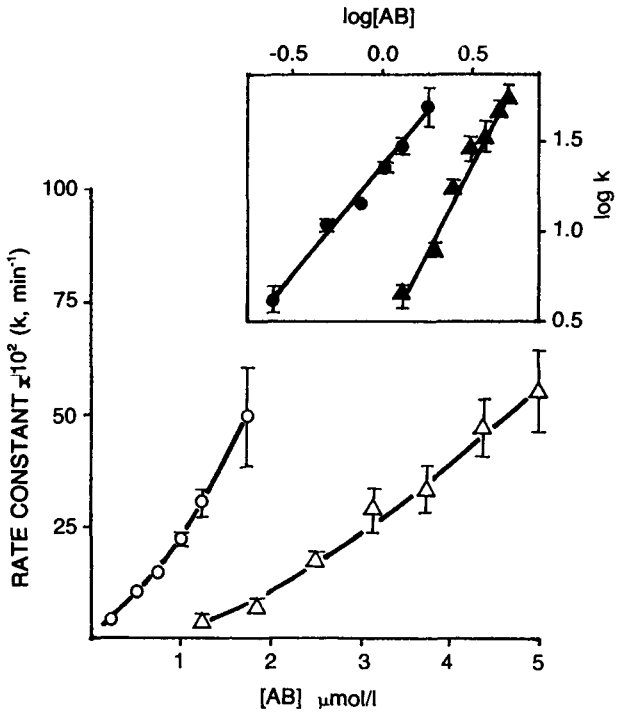

Figure 4. The rate constants of potassium leakage (circles) and thymocyte volume changes (triangles) as a function of amphothericin B concentration. Insert: The same curves in double logarithmic presentation.

recordings (especially for high AB concentrations) were obtained (Fig. 5, right). After AB addition the cells first swelled due to a higher intracellular oncotic pressure. Some time later the cells shrunk, probably due to the efflux of osmotically active contents (ATP, glucose etc.) through amphotericin pores. Since the osmotic equilibrium of a cell is less perturbed by the polyene right after the addition, the initial rate of the processes were used for further analysis.

In order to estimate the effective size of the water pore formed by AB in the thymocyte membrane, we measured the rate of volume changes in the presence of some hydrophilic molecules with different hydrodynamic radii, at 125 mmol/l. Among nonelectrolytes studied only lactose beside sucrose was able to prevent cell swelling. In the presence of other smaller substances the rate was faster than that in the presence of sucrose or lactose but slower than in pure buffer-saline.

To characterize the permeation of small solutes through the pores it is convenient to use the Staverman's reflection coefficient σ (Staverman 1951; Solomon

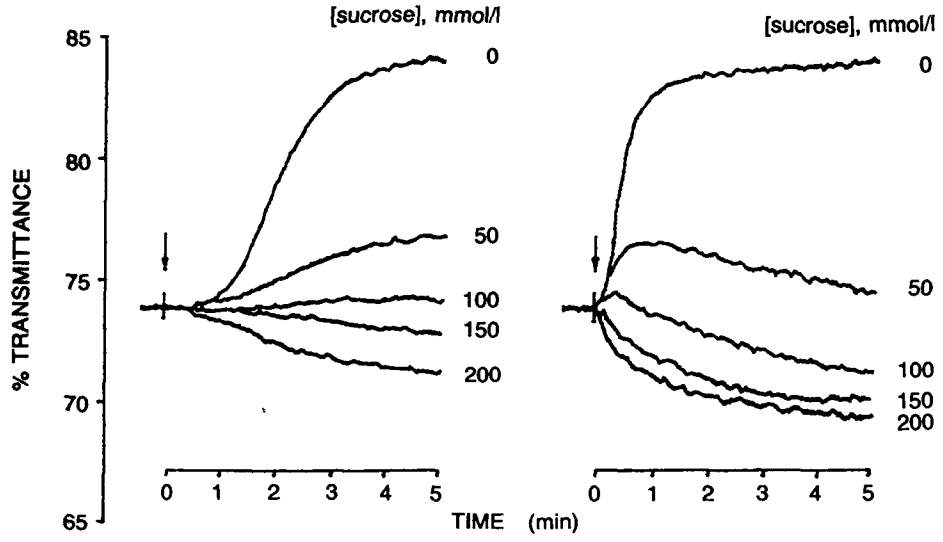

Figure 5. Effect of amphothericin B upon the turbidity of rat thymocyte suspension in standard solutions additionally containing various concentrations of sucrose. The polyene antibiotic was added at time zero (arrow) up to 2.5 μmol/l (left) and 40 μmol/l (right). Typical original recordings are represented. The tracings have been corrected for the decrease in transmittance caused by limited solubility of amphothericin B.

1968) which can be defined as the ratio of the acting operative osmotic pressure to the theoretical van't Hoffe value.

In our case, the reflection coefficient could be evaluated as follows. It appears reasonable to assume that the rate of thymocytes volume changes (dV/dt) is proportional to the osmotic pressure difference ($\Delta\Pi_0$) between the internal and the external part of a cell (Kotyk and Janacek 1980):

$$(dV/dt)_0 = L \cdot \Delta\Pi_0 \tag{3}$$

where L is the coefficient of proportionality.

In the presence of nonelectrolyte molecules (i) at concentration C_i in external medium the rate of sweelling will be:

$$(dV/dt)_i = L(\Delta\Pi_0 - \sigma RTC_i) \tag{4}$$

where σ is the Staverman coefficient, R is the gas constant, T is the absolute temperature For impermeable nonelectrolyte $\sigma = 1$. Combining Eqs. (3) and (4) gives for the reflection coefficient:

$$\sigma = 1 - (dV/dt)_i/(dV/dt) \tag{5}$$

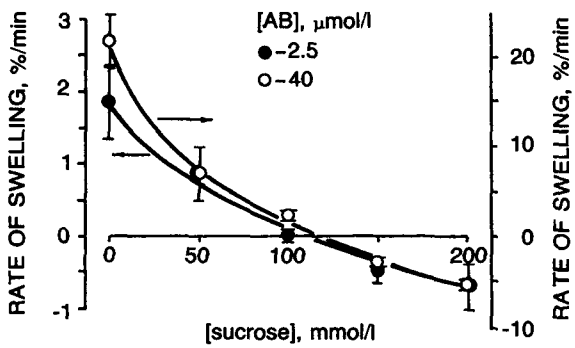

Figure 6. The rate of thymocyte swelling as a function of sucrose concentration. Data from experiments illustrated in Fig. 5.

The values of reflection coefficients for AB-modified cell thymocyte membrane as a function of hydrodynamic radius of nonelectrolytes are shown in Figure 7 (the radii were taken from Sabirov et al. 1993). It can be seen that the smallest molelcules of the nonelectrolytes used (glycerol and ribose) had the smallest reflection coefficients. Increasing the size of the molecules (glucose, inositol etc.) was paralleled by increasing of the values of the reflection coefficients, reaching a constant value (equal to 1) for disacharides. Thus, we can conclude that small molecules – glycerol and ribose – passed through the AB treated cell membrane, but the large molecules of the disacharides did not. The point of intersection of the square fit lines of this dependence with the upper horizontal line (Fig. 7) gives the precise value of the equivalent radius of AB-induced water pore. It was equal to 4.1 ± 0 3 Å independently of the polyene concentration within 2.5–80 μmol/l

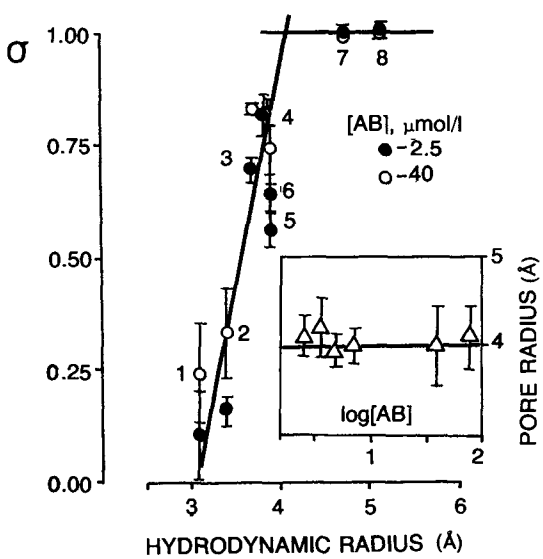

Figure 7. The values of reflection coefficient (σ) for some hydrophylic nonelectrolytes as a function of their hydrodynamic radii. 1–glycerol; 2–ribose; 3–glucose; 4–inositol; 5–sorbitol; 6–tagatose; 7–sucrose; 8–lactose. Insert: Effective pore radius as a function of amphothericin B concentration.

(insert, Fig. 7). This value was close to the values obtained by some investigators for artificial membranes (4 Å, Holz and Finkelstein 1970; ~ 4 Å, De Kruiff et al. 1974) and erythrocyte membranes (2–4.5 Å, Deuticke et al. 1973; 4–4.2 Å El-Soufi et al. 1991). The results of the theoretical modeling of the AB pore structure (4 Å by Khutorsky et al. 1988) also agree with this value of the pore radius.

In conclusion,

i) the size of the AB-induced water pore, and correspondingly the principle of its molecular organization in the cell membrane of thymocytes, is such as established in earlier experiments on erythrocytes and planar lipid bilayer systems;
ii) the lower limit of effective AB-concentrations able to increase both K^+ leakage and cell swelling is close to concentrations reported to enhance immunity. As an increased ion permeation initiates a cascade of biochemical reactions which lead to immune responses (Ashman 1987), ion channel formation by AB in cell membranes can actually be expected to modulate immune responses.

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Final version accepted March 12, 1993