Recovery of hepatocytes from attack by the pore former Amphotericin B

Adrien BINET* and Jacques BOLARD†‡

*INSERM U.274, Université Paris Sud, 91405 Orsay, and †L.P.C.B. (U.A. CNRS 198), Université Pierre et Marie Curie, 4 Place Jussieu, 75252 Paris Cedex 05, France

Sublytic amounts of the pore former Amphotericin B (AmB) induced transient movements of Na and K ions across the hepatocyte plasma membranes without altering the intracellular free Ca ion concentration. The presence of 1–5 μM -AmB induced leakage of up to 80 % of the intracellular K⁺ within 3 min, followed by Na⁺ entry without loss of cell viability. A repair process occurred after 3–10 min, which restored the initial cationic concentrations. Progressive binding of AmB to the cells could be observed by following the disappearance of the intense excitonic dichroic doublet of free AmB. It was shown that the amount of AmB binding, responsible for the Na⁺ and K⁺ movements, was low (approx. 16 % of total AmB). The recovery process occurred when higher amounts of AmB bound to the cells, and was mediated by Na⁺/K⁺-ATPase. The c.d. spectrum of AmB bound to isolated hepatocyte plasma membranes, indicated that during this step AmB formed a complex with cholesterol, similar to that formed by the binary mixture in water.

INTRODUCTION

It has been observed that cells spontaneously recover from attack by low concentrations of pore formers such as Sendai virus (Bashford *et al.*, 1986) or complement (Morgan *et al.*, 1987 and references therein; Carney *et al.*, 1986; Sims & Wiedmer, 1986). ‘Recycling’ of plasma membranes and shedding of pieces of membranes into the extracellular matrix by vesiculation have been thought to be at the origin of these repairs (see Pasternak, 1986). Repair processes have also been observed with the pore formers polyene antibiotics, particularly Amphotericin B (AmB), and different mammalian cells after removal of the drug (Fisher *et al.*, 1978; Malewicz *et al.*, 1980).

AmB is the drug most widely used for the treatment of systemic fungal disease and has recently received a renewal of interest because it has been shown to be able to stimulate, at sub-lethal doses, different types of cells, particularly immunoreactive cells (see Bolard, 1986). Unfortunately the use of this drug is limited by its toxicity for host cells. In the present work we have studied the recovery of hepatocytes from AmB attack in relation to our belief that this mechanism may be a determining factor in the selectivity of toxicity between fungal and host cells. We have chosen hepatocytes because it was shown (Christiansen *et al.*, 1985) that the highest drug concentration in tissues of patients who had received doses of AmB, were found in the liver (up to 41 % of the total dose), without evidence of drug catabolization.

We have shown that, at sub-lethal doses, AmB induced first the leakage of internal K ions within a few minutes, partially compensated by a Na ion influx and then, always in the presence of the drug, an Na⁺/K⁺-ATPase-mediated process restored the initial internal K⁺ content. We were not able to detect internal Ca ion concentration changes or any other cellular damage during these

events. We used c.d., which has been shown to be the technique most sensitive to the conformational changes of AmB (Bolard *et al.*, 1980), to monitor the binding of the drug to the cells. We obtained indications of the nature of the membrane attack complex, and how its modification with time allowed the repair process to occur.

METHODS

Cell preparation

Hepatocytes were isolated from perfused rat liver by the collagenase method, as described previously (Binet *et al.*, 1985), and maintained in Eagle’s medium containing 116 mM-NaCl, 5.4 mM-KCl, 1.2 mM-CaCl₂, 0.81 mM-MgCl₂, 0.92 mM-NaH₂PO₄, 25 mM-NaHCO₃ and 5.5 mM-glucose, supplemented with 1.5 % gelatin, vitamins and amino acids. The medium was gassed with O₂/CO₂ (19:1, pH 7.4) at 37 °C.

Cell viability was assessed by exclusion of 0.4 % Trypan Blue.

Preparation of plasma membranes was carried out as described by Prpic *et al.* (1984) using Percoll gradient centrifugation.

Cell incubation with AmB

Routinely, cells (3×10^6 cells/ml) were incubated in the isolation medium at 37 °C under continuous gassing and shaking and AmB was added after 20 min of preincubation. Two modes of mixing were used. First, AmB was added from a concentrated solution (5 mg/ml) in dimethyl sulphoxide (final concentration of DMSO not exceeding 0.2 %). Secondly, AmB was diluted from the concentrated DMSO solution in the cell medium, at a concentration 2-fold the final one (DMSO < 0.4 %), and added to an equal volume of a suspension of 6×10^6 cells/ml.

Abbreviations used: AmB, Amphotericin B; DMSO, dimethyl sulphoxide.

‡ To whom correspondence should be addressed.

Total cell cation determination

Aliquots were withdrawn from the cell incubation mixture and immediately diluted 15-fold in a cold medium containing 140 mM-choline chloride/10 mM-Hepes, pH 7.4, and centrifuged 1 min at 200 *g* and 0 °C. Pellets were washed in the same medium and the final pellet was suspended in water.

Aliquots were used for protein determination by the Folin-Lowry method, or precipitated in HClO₄ (0.5 M) for analysis of internal K_i and Na_i content by atomic absorption spectrophotometry.

For cell Ca determination, the same procedure was used, except that 2 mM-EGTA was added to the cold washing medium.

Internal free Ca determination

Cells were loaded with quin2/AM as described by Berthon *et al.* (1984). In brief, hepatocytes were loaded with 50 μ M quin2/AM in complete Eagle's medium for 2.5 min at 37 °C, washed and resuspended in a saline medium. Fluorescence (excitation wavelength 342 nm; emission wavelength 492 nm) was followed in a spectrofluorimeter (Jobin-Yvon JY3) and corrected for autofluorescence.

C.d. measurements

The binding of AmB to cells was monitored by c.d. on a Jobin-Yvon Mark V dichrograph (Jobin-Yvon, Longjumeau, France) equipped with a thermostat. C.d. has been demonstrated to be more useful than electronic absorption for monitoring AmB interactions (see Bolard, 1986). The recording of AmB c.d. spectra in the presence of isolated plasma membranes was done in conditions similar to those used for studying the interaction of AmB and erythrocyte ghosts (Szponarski & Bolard, 1987).

RESULTS

Effects of AmB on hepatocyte K⁺ and Na⁺ movement

Effects of the addition of AmB on the K_i⁺/Na_i⁺ ratio as a function of time are shown in Fig. 1. At concentrations ranging from 0.5 to 5 μ M AmB induced a transient effect. First of all, addition of AmB to a suspension of hepatocytes induced a rapid efflux of K⁺ from the cells, whereas Na⁺ accumulated; changes which led to a rapid inversion of the K_i⁺/Na_i⁺ ratio. The maximum effect was reached within 3 min and could be prolonged for several minutes, depending on the applied AmB concentration. The initial permeabilization phase was followed by a slower repair phase during which the cells recovered their normal K⁺ and Na⁺ contents. At the highest AmB concentration (> 10 μ M), this repair phase failed to occur (results not shown) and cell death began to be observed.

The initial phase of the AmB effect is detailed in Fig. 2. Hepatocytes were incubated in the presence of 5 μ M-AmB (mixing was performed by the second method described in the Methods section) and aliquots were withdrawn at short intervals after the addition of AmB. After 10 s, 70% of the cell K⁺ had already leaked, whereas notably less Na⁺ (30%) had entered the cell ($P < 0.05$ by the Student's *t* test). This massive K⁺ permeabilization seems to occur immediately after AmB addition and no apparent latency phase could be observed during the first few seconds (results not shown).

Fig. 1. Effect of AmB on K_i⁺/Na_i⁺ ratio as a function of time

Isolated rat liver cells were incubated as described in the Methods section. AmB (5 mM in DMSO) or solvent were added after 20 min of preincubation. Portions were withdrawn at the indicated times and assayed for Na⁺ and K⁺ determination as described in the Methods section. ○, Control; ▼, 1 μ M-AmB; ●, 2 μ M-AmB; ■, 5 μ M-AmB. Results of a typical experiment are shown.

Fig. 2. Rapid effect of AmB on hepatocyte Na_i⁺ and K_i⁺ content

Hepatocytes were preincubated for 20 min at 6×10^6 cells/ml. At zero time, an equal volume of 10 μ M-AmB in Eagle's medium was added to the cells (final concn. 3×10^6 cells/ml, 5 μ M-AmB), and portions were withdrawn at 10, 25, 40, 60, 90 and 180 s. Results are expressed as the percentage of the maximal K⁺ efflux (248 ± 17 nmol/mg of protein) (○) or of the maximal Na⁺ influx (119 ± 4 nmol/mg of protein) (●). Results are means $\pm$ S.E.M. for three experiments.

Furthermore, when the maximum effect was reached at 3 min, we observed that 250 nmol of K⁺/mg of protein had leaked, whereas 120 nmol of Na⁺/mg of protein had entered. This indicates that the AmB-induced permeabilization of isolated hepatocytes is more specific for K⁺ than for Na⁺.

The rapid permeabilization of the hepatocytes membranes to K⁺ and Na⁺ was followed by a slower recovery phase, dependent on the AmB concentration,

Fig. 3. Effects of AmB on K_i^+/Na_i^+ ratio in the presence of ouabain

Hepatocytes were incubated as indicated in Fig. 1, with (squares) or without (circles) 10^{-3} M-ouabain, $2 \mu\text{M}$ -AmB (from a 2 mM solution in DMSO, closed symbols) or solvent (open symbols) added at 20 min. The incubation was carried out and samples were withdrawn at the indicated times for Na^+ and K^+ analysis. Results of a typical experiment are shown. The low K_i^+/Na_i^+ value at zero time was due to a temperature effect, cells being maintained at 20°C before the incubation which was performed at 37°C (see Berthon *et al.*, 1980).

which led to a return to the normal cellular ionic content. In the presence of 10^{-3} M ouabain, an inhibitor of the Na^+/K^+ pump, which slows down K^+ efflux and Na^+ influx, the permeabilization process still occurred with the same intensity, whereas repair was totally inhibited (Fig. 3). This indicates that the pump is responsible for the return to normal intracellular ionic concentrations, but that AmB does not act by inhibiting or reversing the Na^+/K^+ pump. Furthermore, the recovery phase rate was consistent with normal rates of the Na^+/K^+ pump in hepatocytes (Berthon *et al.*, 1983).

Fig. 4 shows the dose-response curve obtained when the K^+ efflux (or Na^+ accumulation) was measured at 3 min (time of maximal effect as defined above) as a function of the AmB concentrations added. Half-maximal efficiency was obtained with about $2 \mu\text{M}$ -AmB.

Effects of AmB on cell viability and integrity

Surprisingly, during the course of the permeabilization induced by AmB and the recovery, no loss of cell viability was detected (Fig. 5, top). Even when the K_i^+/Na_i^+ ratio had fallen to less than 0.3, no death of cells, as assessed by Trypan Blue accumulation, was observed. Microscopic observation of cells showed that 'blebs' had appeared on the surface of some cells, particularly during

Fig. 4. Dose-response curves for the action of AmB on K^+ efflux and Na^+ influx

Hepatocytes were incubated as in Fig. 1, and various doses of AmB were added from a concentrated solution in DMSO. K_i^+ and Na_i^+ contents were measured after 3 min of incubation, corresponding to the maximal AmB effect on ion movement. Results (means of two experiments) are expressed as the percentage of the initial K_i^+ (○) or Na_i^+ (●) contents.

Fig. 5. Effect of AmB on cell viability and morphology

Isolated rat liver cells were prepared and incubated as indicated in Fig. 1. AmB ($5 \mu\text{M}$) was added at zero time. Portions were withdrawn at the indicated times and counted on a Malassez cell with 0.4% Trypan Blue. The numbers of living cells (excluding Trypan Blue), and living cells with surface blebs, were expressed as percentages of total cells. Results of a typical experiment are shown.

the recovery phase (Fig. 5, bottom). These 'blebs' had completely disappeared when the cells had recovered their normal K^+ and Na^+ contents.

An increase in cellular Ca content is generally believed to precede cell death during various toxic injuries (Nicotera *et al.*, 1986). Accordingly, we looked for possible links between the effect of AmB and any modification in Ca homeostasis. The internal free Ca concentration ($[Ca^{2+}]_i$) was followed using the quin2 method (Berthon *et al.*, 1984). The addition of up to

Fig. 6. C.d. spectra of AmB free or in the presence of purified hepatocyte plasma membranes

The AmB concentration was 5×10^{-6} M. Spectra are of free AmB (---) 1 min after dispersion in culture medium; (-.-) after 40 min incubation at 37 °C in the medium, after 40 min incubation at 27 °C in the presence of plasma membranes at 800 μ g/ml in proteins (—).

2 μ M-AmB did not produce any substantial increase in $[Ca^{2+}]_i$, which stayed under 200 nM (from a level of 160 nM in the non-activated cell) after 5 min, when permeabilization of K and Na was maximal (results not shown). Prolonged incubation (30 min) in the presence of AmB did not increase the $[Ca^{2+}]_i$ either. Higher AmB concentrations would interfere with quin2 fluorescence and were not used. Also, the addition of AmB, at a concentration in the range 0.5–10 μ M, did not affect the total intracellular Ca, as measured by atomic absorption (results not shown). The absence of deleterious effects of AmB was confirmed by the observation that no loss of internal proteins (as measured by extracellular lactate dehydrogenase activity) occurred during the permeabilization of hepatocytes by AmB: 9% of total cell lactate dehydrogenase was found in the extracellular medium after 60 min of incubation in the presence of 5 μ M-AmB, as compared with 5% in the control cells.

C.d. of AmB dispersed in the hepatocyte culture medium (Fig. 6)

In agreement with published data on AmB c.d. spectra in aqueous buffer (Bolard *et al.*, 1980), 5×10^{-6} M-AmB in the hepatocyte culture medium at 37 °C, exhibited an intense dichroic doublet centred at 346 nm with a positive peak at 329 nm ($\Delta\epsilon = +280$), and two negative peaks at 357 nm ($\Delta\epsilon = -170$) and 368 nm ($\Delta\epsilon = -180$). Two additional negative bands were observed at 394 nm

Fig. 7. Decrease in the amount of free AmB in the presence of hepatocytes (Δ) or purified hepatocyte plasma membranes ($\square$, $\blacksquare$) as a function of time

The amount of free AmB was estimated from the magnitude of the dichroic doublet at 329 nm. The AmB concentration was 5×10^{-6} M. The decrease of the dichroic signal of AmB in buffer was also monitored ($\circ$). Hepatocyte concentration of plasma membranes: ($\square$) 80 μ g/ml; ($\blacksquare$) 800 μ g/ml. Temperature, 37 °C.

($\Delta\epsilon = -110$) and 324 nm ($\Delta\epsilon = -60$). After 1 h of incubation at 37 °C the intensities of the bands slightly decreased (13% for the 329 nm peak) and the doublet wavelengths were blue-shifted by 3 nm.

Centrifugation at 150 g , which would be sufficient to sediment the hepatocytes, was without effect on these spectra.

C.d. of AmB in the presence of liver cells or purified plasma membranes

The c.d. of 5×10^{-6} M-AmB is very intense, so the scattering resulting from the addition of 3×10^6 cells/ml was negligible. It was therefore possible to study the interaction of AmB with the hepatocytes by following the c.d. of AmB in the same conditions as those previously used to follow K^+ and Na^+ movements. It appeared that the intense dichroic doublet, characteristic of free AmB, diminished progressively and its amplitude was one-third of its initial value within 1 h (Fig. 7). Centrifugation for 1 min at 150 g was without significant influence: the c.d. spectrum of the supernatant was similar to that of the AmB-containing cell suspension.

It is interesting to note that after 2 or 3 min of incubation, which corresponds to the maximum release of K^+ , only 5% of the AmB was bound to the cells.

The decrease of the AmB c.d. signal indicates the formation of a new dichroic species, characterized by dichroic bands smaller than those corresponding to the free drug. In order to observe the spectrum of this putative bound species, we increased the amount of bound AmB by decreasing the ratio of AmB/hepatocytes. Unfortunately we were unable to detect any new signal

(because of light scattering). Therefore we pursued this study with isolated hepatocyte plasma membranes. Indeed, isolated plasma membranes scatter light much less than whole cells, and the feasibility of detecting the c.d. spectrum of a drug bound to them has already been demonstrated (Schneider *et al.*, 1983; Sato *et al.*, 1986; Szponarski & Bolard, 1987).

We studied the interaction of 5×10^{-6} M-AmB with isolated membranes at two protein concentrations, 0.080 and 0.80 mg/ml. Under these conditions the spectrum of free AmB disappeared (Fig. 7) and was replaced by a new spectrum with a positive peak at 424 nm ($\Delta\epsilon = 11$) and negative peaks at 419 nm ($\Delta\epsilon = -15$) and 383 nm ($\Delta\epsilon = -28$) (Fig. 6). The location of these new dichroic bands, as well as their amplitudes, explain the impossibility to detect them during the previous attempts with whole cells.

DISCUSSION

In this work, we show that AmB strongly permeabilizes hepatocyte plasma membrane to monovalent cations, the major part of the intracellular potassium leaking in a few seconds. The loss of K^+ is only partially compensated for by entry of Na^+ ions. At the same time, no modification of internal free Ca, as well as total calcium content, is detected in the hepatocytes. These results suggest that the permeabilization of the hepatic plasma membrane by AmB is specific for the monovalent cations, and especially for potassium (Fig. 2). No other apparent permeabilization occurred during this process, although the role of anions was not investigated here. The AmB-induced permeabilization of monovalent ions has already been observed for other nucleated mammalian cells (Cybulska *et al.*, 1976; Kotler-Brajtburg *et al.*, 1977; Rozengurt & Mendoza, 1980; Malewicz *et al.*, 1980; Brajtburg *et al.*, 1984) as has its separation from lactate dehydrogenase release (Vertut-Croquin *et al.*, 1986).

It is well known that agents which permeabilize the plasma membrane of mammalian cells, such as ionophores or detergents, induce, with more or less lag time, the death of the cells. This event is generally preceded by an increase in cytosolic Ca which stimulates some intracellular proteases (Nicotera *et al.*, 1986). In this action of AmB on hepatocytes, it is interesting to point out the correlation between the absence of effect on Ca homeostasis and the maintenance of cell viability when an important permeabilization to monovalent cations has occurred. These results indirectly support this generally accepted role for Ca in cell death. At much higher AmB doses (200 μ M) calcium was shown to be a determining factor in the cell death induced by the drug (Schanne *et al.*, 1979).

The other major fact that appears during the action of AmB on hepatocytes is the repair process. This process is characterized by a slow return (within 1 h) to the normal cellular K and Na content. The restoration of the initial internal ion concentrations, while the AmB pores are superseded by the AmB-cholesterol complex, seems to be done by Na^+/K^+ -ATPase. Its inhibition by ouabain is in agreement with this hypothesis and the rate at which it is done corresponds to the normal rate of the pump in the present conditions of ionic cell contents (Berthon *et al.*, 1983).

During the repair process, blebs can be observed on

the surface of the plasma membrane. The significance of these blebs is unknown, and they are probably different from the blebs that have been described elsewhere in cell injuries, and which precede cell death (Lemasters *et al.*, 1987). A tentative explanation may be that these blebs occur to somehow compensate the des-equilibrium of the intracellular osmotic pressure modified by the greater K^+ efflux than Na^+ entry. Furthermore, the small proportion of cells showing these blebs may suggest that their occurrence is relatively transient.

In the present study we have also been able to correlate the different steps of drug action with its binding to the cells and to obtain information on the state of AmB when the cells recover from K^+ loss. First, it is noticeable that K^+ leakage is induced when only a small amount of AmB is bound. Typically only 5 % of the total AmB amount is bound when 80 % K^+ has leaked. For 3×10^6 cells/ml and 5×10^{-6} M-AmB (total), this corresponds to 1 molecule of drug bound to the membrane for every 100 molecules of cholesterol. It is not possible to obtain the c.d. spectrum of this AmB species because it is hidden by the intense c.d. spectrum of free AmB.

Paradoxically, the recovery process occurs when more AmB binds to the cell and is completed when approx. 60 % of the total AmB is bound. At that time the c.d. spectrum observed in the presence of purified hepatocyte plasma membranes is identical to the spectrum obtained in the binary mixtures of AmB-cholesterol in water (Bolard & Chéron, 1982). It is different however from the spectra obtained in the presence of unilamellar vesicles and assigned to the species inducing permeability (Bolard *et al.*, 1980; Bolard & Chéron, 1982; Vertut-Croquin *et al.*, 1983). We think therefore that the progressive recovery from K^+ loss can be explained by the formation of a new type of interaction between AmB and the cell membrane. Initially, specific complexes are formed which induce K^+ leakage, but are superseded by inactive AmB-cholesterol complexes as more AmB binds. These binary complexes could either be expelled in water or endocytosed into the hepatocytes. The last possibility seems to be favoured because it would explain why so much AmB is found in liver and why these high concentrations are not active against fungi when they are present in the liver (Christiansen *et al.*, 1985). Under our experimental conditions, analytical determination of the presence of cholesterol in the aqueous medium would be difficult because of its low concentration ($\sim 10^{-6}$ M) but the presence of cholesterol has been observed in preparations of erythrocytes and higher AmB concentrations (d'Hollander & Chevallier, 1972).

Degradation of the polyene antibiotic was postulated as being one of the possible mechanisms involved in the repair process (Malewicz *et al.*, 1980). That could have been the case in their study involving baby hamster kidneys where the process took 8 h but it cannot be significant in our study where the repair process is taking place in less than 1 h.

Our observations are to be distinguished from those made on the repair process of cells which are transferred to a drug-free medium after the AmB-induced leakage (Brajtburg *et al.*, 1984; Malewicz *et al.*, 1981). In this latter case the small amount of bound AmB is removed from the membranes and released in the drug-free medium; as it is known to happen during washing of the cells (see Bolard, 1986).

Finally it appears that the recovery of hepatocytes

from AmB attack is another example of the processes observed with other pore formers. However AmB-induced K^+ and Na^+ movements we described here are very specific and do not involve Ca ions.

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