The Interaction of Amphotericin B Methyl Ester with Protoplasts of *Candida albicans*

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SUMMARY

The interaction of amphotericin B methyl ester (AME) with protoplasts of *Candida albicans* was measured indirectly by following the incorporation of [U-¹⁴C]phenylalanine into the acid-insoluble material. The inhibitory effects of AME at the minimum inhibitory concentration were prevented by the addition of 85 mM-KCl and 45 mM-MgCl₂, as shown by Liras & Lampen (1974) for *Saccharomyces cerevisiae*. In *C. albicans*, pretreatment of the yeast before antibiotic addition was unnecessary. KCl and MgCl₂ did not prevent AME from binding to the protoplast membrane. This interaction was reversed by incubating the protoplasts in the presence of the protecting salts.

INTRODUCTION

The polyene antibiotics interact with sterol-containing membranes of sensitive cells causing leakage of cellular constituents and, ultimately, cell death (Lampen, 1966; Hamilton-Miller, 1974; Kerridge & Russell, 1975). The fungicidal effects of certain members of this group of antibiotics can be prevented by the addition of K⁺ and Mg²⁺ to the growth medium (Marini, Arnow & Lampen, 1961; Liras & Lampen, 1974). It is assumed that the antibiotic interacts with the plasma membrane rendering it permeable to ions, but the presence of K⁺ and Mg²⁺ ions in the suspending medium results in maintenance of the internal concentration required for growth. Polyene antibiotics have been used to affect reversibly the permeability of the plasma membrane of certain animal cells to both ions (Cass & Dalmark, 1973) and DNA (Kumar *et al.*, 1974). In these systems the antibiotic was removed simply by washing the cell suspension with the incubation medium. Amphotericin B, at sub-inhibitory concentrations, has also been used to potentiate the action of rifampicin in strains of yeast normally resistant to this compound (Kobayashi *et al.*, 1972).

The purpose of the studies reported in this paper was to investigate the effect of salts on the interaction of amphotericin B methyl ester (AME) with the plasma membrane of *Candida albicans* and on the reversibility of this interaction. There is considerable phenotypic variation in the sensitivity of *C. albicans* to AME when the organism is grown in batch culture (Gale, 1974; Gale *et al.*, 1975). For this reason protoplasts of *C. albicans* were used rather than intact cells, to eliminate possible interference by the cell wall with antibiotic penetration to the plasma membrane. In other studies on the interaction of polyene antibiotics with *C. albicans*, K⁺ release has been used to monitor the interaction of the antibiotics with the membrane (Gale, 1974), but we could not use this procedure because of the need for high concentrations of K⁺ in the external environment. The incorporation of a radioactively-labelled amino acid into the protein fraction was therefore used as an indirect measure of the functional integrity of the protoplast membrane.

METHODS

Organisms and growth media. *Candida albicans* strain 6406, obtained from the Mycological Reference Laboratory, Public Health Laboratory Service, was used for these studies. The organism was maintained on Difco yeast morphology agar and, after growth at 37 °C, the cultures were stored at 4 °C. For all experiments, the organism was grown at 37 °C in an orbital incubator in the synthetic medium described by Davies (1953). Growth was determined by extinction and dry weight measurements.

Streptomyces violaceus 3196 was obtained from Professor J. R. Villanueva (Department of Microbiology, Salamanca University, Spain).

Protoplast formation. Suspensions of *C. albicans* were harvested during the exponential phase of growth when the culture density was 1 mg dry wt ml⁻¹. The yeast pellet was washed once with 0.1 M-sorbitol, once with 0.8 M-sorbitol and finally resuspended at a density of 10 mg dry wt ml⁻¹ in 0.1 M-NH₄HCO₃ containing 0.8 M-sorbitol. Mercaptoethanol was added to a final concentration of 0.2 M and the suspension was incubated at 37 °C for 5 min. The yeasts were harvested by centrifuging, washed once in 0.05 M-imidazole buffer pH 6.8 containing 0.9 M-sorbitol and resuspended at a density of 10 mg dry wt ml⁻¹ in 0.05 M-imidazole buffer pH 6.8 containing 1.0 M sorbitol and 'strepzyme' [prepared from *Streptomyces violaceus* 3196 as described by Elorza, Munoz-Ruiz & Villanueva (1966)]. After 30 min incubation at 37 °C, bovine serum albumin (BSA) was added to a concentration of approximately 100 µg ml⁻¹ and the incubation was continued until protoplast formation was complete (approx. 90 to 120 min). The 'strepzyme' preparation contained proteolytic enzymes which, although possibly important during the early stages of cell-wall lysis, could subsequently have affected the protoplast membrane proteins. The excess BSA was added during cell-wall removal to minimize this latter effect. The protoplasts were harvested by centrifuging for 10 min at approx. 600 g, washed once with 0.05 M-imidazole buffer pH 6.8 containing 1.0 M-sorbitol and finally resuspended at a density equivalent to 10 mg dry wt yeast ml⁻¹ in the same buffer.

Incorporation of [U-¹⁴C]phenylalanine into protoplasts. Protoplasts, at a density equivalent to 1 mg dry wt yeast ml⁻¹ were incubated statically at 37 °C in a medium containing: imidazole, 0.05 M; succinic acid, 0.05 M; sorbitol, 1.0 M; Difco Casamino acids, 220 µg ml⁻¹; L-cysteine-HCl, L-tryptophan and L-phenylalanine, each at 11 µg ml⁻¹; [U-¹⁴C] phenylalanine (specific activity 495 mCi mmol⁻¹), 0.1 µCi ml⁻¹; other additions as required. Samples (1 ml) were withdrawn at intervals and pipetted into 1 ml 10% (w/v) trichloroacetic acid (TCA). After 15 min at 90 °C followed by 15 min at 0 °C, the precipitated protoplasts were harvested by filtration through glass-fibre filters, and washed with cold 5% (w/v) TCA and 1% (v/v) acetic acid. The filters were then transferred to small glass vials, dried at 105 °C, 1 ml scintillant [0.4% (w/v) 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophene (BBOT) in toluene] was added and the radioactivity was determined using a Packard model 3375 scintillation spectrometer.

Incubation of the protoplasts in the presence of glucose resulted in lysis during the course of the experiment, and succinate was used as the energy source for amino-acid uptake studies. The rate of phenylalanine incorporation into the acid-insoluble fraction was reduced at imidazole concentrations above 0.05 M, and was stimulated by 85 mM-KCl and 45 mM-MgCl₂ (Table 1).

Materials. [U-¹⁴C]Phenylalanine was obtained from The Radiochemical Centre, Amersham, Buckinghamshire. AME and filipin were a gift from Dr C. Schaffner, Department of Microbiology, Rutgers University, U.S.A. Trichodermin was a gift from Dr W. O.

Table 1. The effect of incubation conditions on the incorporation of [U-¹⁴C]phenylalanine into protoplasts of *Candida albicans*

Protoplasts of *Candida albicans* were incubated at 37 °C in the complete incorporation medium modified either by changing the concentration of imidazole or by adding salts, and the incorporation of [U-¹⁴C]phenylalanine into the hot-TCA-insoluble material was measured.

Imidazole concn (M)	Additions	Relative rate of incorporation
0.05	—	100
0.1	—	98
0.2	—	31
0.05	85 mM-KCl	170
0.05	45 mM-MgCl ₂	116
0.05	85 mM-KCl+45 mM-MgCl ₂	136

Fig. 1. The effects of AME on the growth of *C. albicans* and on its incorporation of [U-¹⁴C]phenylalanine. (a) A culture of *C. albicans* was incubated aerobically in synthetic yeast medium at 37 °C and growth was determined spectrophotometrically. AME (0.1 µg ml⁻¹), 85 mM-KCl and 45 mM-MgCl₂ were added as required when the culture was in the exponential phase of growth (at the time arrowed).

(b) A culture of *C. albicans* was incubated in the complete incorporation medium at 37 °C. AME (0.1 µg ml⁻¹), 85 mM-KCl and 45 mM-MgCl₂ were added as required after 10 min incubation, and the radioactivity of the hot 5% (w/v) TCA-insoluble material was determined.

○, Control; △, AME added; ●, AME, KCl and MgCl₂ added.

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RESULTS

Incorporation of [U-¹⁴C]phenylalanine into intact cells

The protective effects of 85 mM-KCl and 45 mM-MgCl₂ observed by Liras & Lampen (1974) for *S. cerevisiae* were also found with *C. albicans*. But in contrast to their findings, we were unable to demonstrate a requirement for pre-incubating the yeast in the presence of the salts before addition of AME to give complete protection (Fig. 1).

Fig. 2. The effect of AME in the presence of KCl and MgCl_2 on $[\text{U-}^{14}\text{C}]$ phenylalanine incorporation into protoplasts of *C. albicans*. Protoplasts were incubated in the incorporation medium; AME ($0.1 \mu\text{g ml}^{-1}$), 85 mM-KCl and 45 mM- MgCl_2 were added as required and the uptake of radioactivity into the hot 5% (w/v) TCA-insoluble material was determined. ○, Control, KCl and MgCl_2 present throughout; ●, AME present throughout; ●, AME present from time 0, KCl and MgCl_2 added after 2 min; △, AME present from time 0, KCl and MgCl_2 added after 5 min.

*Incorporation of $[\text{U-}^{14}\text{C}]$ phenylalanine into protoplasts of *C. albicans**

The incorporation of radioactivity from $[\text{U-}^{14}\text{C}]$ phenylalanine into the acid-insoluble residue by protoplast preparations provided a satisfactory, albeit indirect, measure of the integrity of the plasma membrane. Incorporation did not occur in lysed preparations, and it was completely inhibited by trichodermin ($10 \mu\text{g ml}^{-1}$), an inhibitor of protein synthesis (Cundliffe, Cannon & Davies, 1974), and by AME ($0.1 \mu\text{g ml}^{-1}$).

The inhibitory effect of AME, but not trichodermin, on the uptake of phenylalanine by intact protoplasts was completely reversed when 85 mM-KCl and 45 mM- MgCl_2 were added to the incubation medium. Pre-incubation of the protoplasts with KCl and MgCl_2 was not essential for this protection and, as in the case of intact cells, addition of the salts after AME was effective, and phenylalanine uptake occurred at almost the same rate as in the control (Fig. 2).

The protective effects of KCl and MgCl_2 were not observed when AME was added at concentrations in excess of the minimum inhibitory concentration (m.i.c.). At concentrations of $1.0 \mu\text{g ml}^{-1}$ and above, AME completely inhibited phenylalanine uptake both in the presence and absence of KCl and MgCl_2 . As with *S. cerevisiae*, neither salt alone protected the protoplasts against the inhibitory effects of AME.

The association of AME with protoplast membranes in the presence of K^+ and Mg^{2+} ions

Since we were unable to measure directly the binding of AME to protoplast membranes, we used an indirect approach to study the effects of salts on the interaction of AME with the plasma membrane and on the reversibility of this interaction. The protective effect of salts

Fig. 3

Fig. 4

Fig. 3. The effect of pre-incubation with AME on the synthetic ability of protoplasts of *C. albicans*. Protoplasts were incubated in the incorporation medium supplemented with 85 mM-KCl, 45 mM-MgCl₂ and AME (0.1 $\mu\text{g ml}^{-1}$) as required. After 15 min incubation at 37 °C, they were harvested by centrifuging, resuspended in the incorporation medium in the presence or absence of protecting salts, and the incorporation of [U-¹⁴C]phenylalanine into the hot 5% (w/v) TCA-insoluble fraction was determined. ○, Control incubation, KCl and MgCl₂ present throughout; △, control incubation, KCl and MgCl₂ present during pre-incubation only; ●, KCl and MgCl₂ present throughout, AME present during first incubation only; ▲, KCl, MgCl₂ and AME present during first incubation only; □, lysed control.

Fig. 4. Recovery of synthetic ability by protoplasts of *C. albicans* after treatment with AME. Protoplasts were incubated in the incorporation medium supplemented with 85 mM-KCl, 45 mM-MgCl₂ and AME (0.1 $\mu\text{g ml}^{-1}$) as required. After 15 min incubation at 37 °C, they were harvested by centrifuging and resuspended in the incorporation medium supplemented with 85 mM-KCl and 45 mM-MgCl₂. After a further 0, 45 and 90 min incubation at 37 °C, samples were withdrawn, protoplasts were harvested by centrifuging and resuspended in the incorporation medium, and the incorporation of [U-¹⁴C]phenylalanine into the hot 5% (w/v) TCA-insoluble fraction was determined. ○, Control, KCl and MgCl₂ present throughout; ●, KCl and MgCl₂ present throughout, AME present during first incubation only; □, KCl, MgCl₂ and AME present during the first incubation only; △, KCl, MgCl₂ and AME present during the first incubation, after 45 min incubation in the incorporation medium supplemented with KCl and MgCl₂, the incorporation of phenylalanine by protoplasts was followed in the absence of protecting salts; ▲, KCl, MgCl₂ and AME present during the first incubation, after 90 min incubation in the incorporation medium supplemented with KCl and MgCl₂, the incorporation of phenylalanine by protoplasts was followed in the absence of protecting salts.

could result either from preventing the antibiotic from binding, possibly by precipitating the AME, or from the high concentrations of salts maintaining the necessary internal concentrations of K⁺ and Mg²⁺ ions. If the former, phenylalanine incorporation should take place once the excess antibiotic and salts were removed; whereas if the latter were the cause, uptake should cease once the antibiotic and salts were removed if membrane damage had been sustained in the presence of the salts. This was investigated by incubating protoplast suspensions in the presence of AME together with the protecting salts. After 15 min incubation at 37 °C, the protoplasts were harvested by centrifuging and resuspended in the incorporation medium in the presence or absence of the protecting salts, and the synthetic abilities of the protoplasts were measured. The results of such an experiment are shown in Fig. 3. Radioactive phenylalanine was only incorporated when the protecting salts were present in the incubation medium. This would suggest that AME was bound to the membrane in the presence of K⁺ and Mg²⁺, but once these ions together with any free AME were

removed from the suspending medium, leakage of internal constituents occurred, resulting in the loss of synthetic ability by the protoplasts.

The association of amphotericin B with both animal cells (Cass & Dalmark, 1973; Kumar *et al.*, 1974) and mycoplasma cell membrane (D. B. Archer, University of Cambridge, personal communication) is reversible, and so we investigated the possibility that the binding of AME to protoplast membranes was also reversible. A suspension of protoplasts was incubated with AME at its m.i.c. together with 85 mM-KCl and 45 mM-MgCl₂. After 30 min incubation, the protoplasts were harvested and resuspended in the incubation medium lacking [U-¹⁴C]phenylalanine but containing the protecting salts. Samples were removed at intervals, protoplasts harvested and resuspended in the normal incorporation medium and the uptake of phenylalanine into the acid-insoluble residue was measured. Initially the protoplasts were unable to incorporate phenylalanine when resuspended in the absence of KCl and MgCl₂; but there was a progressive recovery of synthetic activity, presumably due to the release of AME from the plasma membrane, and after 90 min the protoplasts had completely regained their synthetic ability (Fig. 4). The addition of trichodermin had no effect on the recovery of synthetic ability under these conditions.

The effect of filipin on the incorporation of phenylalanine into protoplasts of C. albicans

The presence of K⁺ and Mg²⁺ does not protect *S. cerevisiae* against the action of the polyene antibiotic filipin (Liras & Lampen, 1974). We confirmed these findings with protoplasts of *C. albicans*. The incorporation of [U-¹⁴C]phenylalanine into *C. albicans* incubated in the presence of AME, 85 mM-KCl and 45 mM-MgCl₂ was completely inhibited by adding filipin at 10 µg ml⁻¹. Thus, under these conditions, the association of AME with components of the plasma membrane did not prevent the subsequent disruption of the membrane by filipin.

DISCUSSION

It is generally agreed that AME interacts with sterols present in the plasma membrane of sensitive cells, resulting in the formation of pores through which low-molecular-weight constituents of the cell can pass, and that it is this loss of cellular constituents which is ultimately responsible for loss of viability (Hamilton-Miller, 1974). The prevention of the fungicidal action of certain of the polyene antibiotics by addition of potassium and magnesium ions to the suspending medium has been explained by the role of these ions in maintaining the internal concentration within the cell. The finding that this protection did not occur when the polyene antibiotic AME was present at concentrations in excess of the m.i.c. would suggest that the increased damage to the protoplast membrane cannot be compensated for by environmental changes, and emphasizes the importance of studying the effect of an antibiotic over a range of concentrations. Although K⁺ and Mg²⁺ ions protected protoplasts of *C. albicans* against the action of AME, they did not prevent the antibiotic from binding to the plasma membrane impairing membrane function. The binding of AME to the protoplast membrane could be reversed, with recovery of membrane function, provided the protecting salts were present during this period. The fungicidal effects of the polyene antibiotics therefore result from the irreversible damage to the cell caused by leakage of cellular constituents rather than the irreversible binding of the antibiotic to the plasma membrane. It is unlikely that the protective effects of salts and the reversibility of the polyene binding to the yeast plasma membrane are important in the treatment of clinical infections since both require the presence of K⁺ and Mg²⁺ at high concentrations. However the

reversibility of the effects of the polyenes on animal cells in the absence of protecting ions might be important in minimizing the damaging effects of the antibiotic on the host's cells during treatment of systemic fungal infections.

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