The Mode of Action of Polyene Antibiotics; Induced Entry of Hydrogen Ions as a Consequence of Polyene Action on the Cell Membrane of *Candida albicans*

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SUMMARY

The polyene antibiotic candicidin is a potent antifungal agent acting upon the cell membrane of *Candida albicans*. Destruction of selective permeability by polyenes allows cations to leak into the environment and permits the entry of protons to neutralize the charge so created, causing internal acidification. Quantitative studies on proton entry reveal that K^+ leakage alone is not sufficient to account for the degree of acidification recorded. Extracts of untreated *C. albicans*, when acidified to the same extent, precipitated. Electron micrographs of candicidin treated *C. albicans* cells confirm this precipitation.

INTRODUCTION

Candicidin is a polyene antibiotic important in the treatment of human mycoses particularly vaginitis caused by *Candida albicans*. The binding between polyene antibiotics and membrane sterols, causing membrane damage, is well documented (Lampen, 1966).

Membrane destruction leads to the loss of small ions. It was believed that the main cation lost was potassium which was replaced by protons from the environment resulting in internal acidification (Lampen, 1966). We have examined this hypothesis and investigated the effect of acidification on the cytoplasm.

METHODS

Organism and method of culture. *Candida albicans* NCTC713 was grown on Sabouraud's dextrose agar slopes at 37 °C, for 24 h and then maintained at 4 °C. From these slopes 10^6 organisms were inoculated into 100 ml of Sabouraud's dextrose broth and incubated in shake culture for 18 h at 37 °C. Organisms were harvested by centrifuging and standardized suspensions made as described previously (Hammond, Lambert & Kligler, 1974).

Distilled water. The water used to wash and resuspend the cells was triple glass-distilled, boiled and saturated with nitrogen to ensure that it was CO_2 -free. This water was used for all dilutions.

Polyene antibiotic. Candicidin powder (lot 681–NOF2) was supplied by Pharmax Ltd, Bexley, Kent; it was dissolved in dimethyl sulphoxide (DMSO) and diluted with CO_2 -free water to give a final DMSO concentration of 1 %.

Detection of pH change. Standardized suspension (40 ml), maintained at 25 °C and flushed with a stream of nitrogen, was examined with a pH electrode connected to a Vibret 46A pH meter (E. I. L. Ltd, Chertsey, Surrey).

Preparation of cell-free extract. Washed organisms (10^{10} organisms/ml) were pressed at -25°C under 12 tonnes (X-press, A. B. Biotech, Croydon) by the method of Edebo (1961). Larger fragments and whole organisms (approximately 8 %) were removed by centrifuging at 10000g for 15 min.

Determination of pH of disrupted organisms. Standardized suspension (200 ml) was centrifuged after treatment with $20\text{ }\mu\text{g}$ candididin/ml (a known lethal dose), resuspended in 15 ml distilled water, disrupted by freeze pressing, made up to the original volume and the pH determined.

Effect of changes in pH on extract of Candida albicans. An 18 h culture of *C. albicans* was washed three times with distilled water by centrifuging, resuspended in 25 ml water (1.6×10^8 organisms/ml) and extracted by freeze press as described (see above). The extract, diluted 1 in 4, was placed in a flask with a side arm which allowed extinction measurements to be made with a Cecil 303 spectrophotometer. E_{520} measurements were made after addition of 0.1 ml volumes of 0.1 N-HCl, and pH was also recorded.

Titration curves. Using preparations of disrupted organisms, titration curves were constructed to determine the buffering capacity of the cytoplasm.

Preparation for the electron microscope. *Candida albicans* organisms, treated and untreated, were prepared by a modification of the method of Gale (1963). Washed standardized suspensions of *C. albicans* were treated with $10\text{ }\mu\text{g}$ candididin/ml (sub-lethal) or $20\text{ }\mu\text{g}/\text{ml}$ (lethal) for 2 h. The pellet produced after centrifuging (20000g for 30 min) was fixed and stained with 1.5 % potassium permanganate for 15 min at 0°C . After dehydration by an alcohol series the pellet was embedded in Araldite and sections were cut on a L.K.B. ultratome and placed on Formvar-coated grids. An A.E.I. E.M.6B electron microscope was used.

RESULTS

Table 1 shows the changes in pH induced in the standardized suspension by lethal concentrations of candididin, both externally in the supernatant and internally, i.e. changes in the cell sap. The pH of the cell sap of *Candida albicans* grown under the described conditions and determined after freeze pressing was found to be 6.12 ± 0.05 (6 determinations).

Fig. 1 shows the change in the H^+ concentration in the suspension caused by $20\text{ }\mu\text{g}$ candididin/ml over 5 h. From Table 1, change in the pH of the cell sap can be expressed in terms of $\mu\text{equiv.}/\text{mg}$ dry wt (1 ml standardized suspension contains 0.00092 g dry wt/ml):

$$\begin{aligned}\text{pH change in cell sap} &= \text{pH of cell sap after 5 h} - \text{pH cell sap initially} \\ &= \text{pH } 5.20 - \text{pH } 6.12,\end{aligned}$$

$$\begin{aligned}\Delta[\text{H}^+] \text{ in cell sap} &= 6.31\text{ }\mu\text{equiv.}/\text{ml} - 0.75\text{ }\mu\text{equiv.}/\text{ml} \\ &= 5.5\text{ }\mu\text{equiv.}/\text{ml} \\ &= 6.05\text{ }\mu\text{equiv.}/\text{mg dry wt}.\end{aligned}$$

The number of protons leaving the suspension and entering the organisms can also be calculated:

$$\begin{aligned}\text{pH change in the suspension} &= \text{pH of the suspension initially} - \text{pH of the suspension after 5 h} \\ &= \text{pH } 5.6 - \text{pH } 6.5,\end{aligned}$$

$$\begin{aligned}\text{H}^+ \text{ in suspension} &= 2.51\text{ }\mu\text{equiv.}/\text{ml} - 0.24\text{ }\mu\text{equiv.}/\text{ml} \\ &= 2.28\text{ }\mu\text{equiv.}/\text{ml} \\ &= 2.48\text{ }\mu\text{equiv.}/\text{mg dry wt}.\end{aligned}$$

Table 1. *Changes in pH and hydrogen ion concentration of cell sap and a standardized suspension of Candida albicans following treatment by 20 µg candididin/ml*

	Control		Candididin-treated	
	pH	[H ⁺]* (µequiv./ml)	pH	[H ⁺]* (µequiv./ml)
Suspension at t_0	5.12	—	5.60	2.51
Standardized at $t_{15 \text{ min}}$	5.10	—	5.80	1.58
Suspension at $t_{5 \text{ h}}$	5.02	—	6.65	0.224
Cell sap at t_i	6.12	0.75	6.12	0.75
Disrupted at $t_{15 \text{ min}}$	—	—	6.01	0.97
Cell sap at $t_{5 \text{ h}}$	6.12	0.75	5.20	6.31

* Since $\text{pH} = -\log_{10} [\text{H}^+]$ Fig. 1. Loss of extracellular protons from a standard suspension of *Candida albicans* treated with 20 µg candididin/ml.

Similarly it can be calculated that the change in hydrogen ion concentration in the sap was 0.22 µequiv./mg dry wt and in the suspension 1.01 µequiv./mg dry wt at $t_{15 \text{ min}}$.

From titration curves it was determined that the number of protons necessary to cause the pH change recorded in the cell sap was 8.9 µequiv./mg dry wt. From these curves it was also calculated that given the entry of 1.0 µequiv. protons/mg (as at $t_{15 \text{ min}}$) the buffering capacity of the cytoplasm was such that the H⁺ change would appear to be 0.2 µequiv./mg dry wt.

Table 1 shows that treatment with 20 µg candididin/ml causes the organisms of the standard suspension to change in internal pH from 6.12 to 5.20. Fig. 2 demonstrates the change in *E* caused in cell sap by equivalent acidification. There is a threefold increase in extinction which is irreversible.

Fig. 3 shows the normal appearance of *Candida albicans* after staining and fixing. Figs. 4 to 6 reveal the effect of two concentrations of candididin on the morphology of *C. albicans*. Sub-lethal concentrations (10 µg/ml) of candididin cause denser cytoplasm than

Fig. 2. Effect of pH upon turbidity of a cell-free cytoplasmic extract of *Candida albicans*.

normal, but some mitochondria and other organelles are still discernible. Lethal concentrations of candidicin cause complete organelle loss and marked granulation of the cytoplasm. Fig. 6 shows a parting of the cell membrane from the cell wall, a common phenomenon in candidicin-treated *C. albicans* cells.

DISCUSSION

Candidicin has the typical polyene property (Waksman, Lechevalier & Schaffner, 1965) of binding to membrane sterols, so destroying membrane selective permeability. Lampen (1966) suggested that damage to the membrane allowed leakage of cations, predominantly potassium. The loss of these ions from yeast cells results in a change in internal charge and, if membrane permeability has been altered, one would expect the cations to be replaced (at a pH less than 7.0) by protons from the environment.

This was established, the cytoplasm of *Candida albicans* becoming more acidic (pH 6.12 becomes pH 5.20) concurrent with a loss of protons from the environment. The method of determination of internal cellular pH, by determining the pH of cell sap, gives only a gross picture, but the values obtained agree with those of other authors, determined by other methods (Conway & Downey, 1950).

Lampen (1966) suggested that a direct interchange of protons for lost K^+ ions occurs. Hammond *et al.* (1974), using specific ion electrodes, found that K^+ loss in *C. albicans* treated with candidicin was $0.33 \mu\text{equiv./mg}$ dry wt. Zygmunt (1966), by flame photometry, showed that a different strain of *C. albicans* lost $0.9 \mu\text{equiv./mg}$ dry wt when treated with lethal concentrations of candidicin. Venables & Russell (1972), also by flame photometry, found that the polyene antibiotic nystatin caused leakage of $0.5 \mu\text{equiv./mg}$ dry wt from *Saccharomyces cerevisiae*.

Under the experimental conditions, if there had only been a direct exchange of potassium ions for protons there would have been an almost negligible change in internal pH.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 3. Untreated *Candida albicans* fixed with permanganate.

Fig. 4. *Candida albicans* treated with 10 μg candidicin/ml.

Fig. 5. *Candida albicans* treated with 20 μg candidicin/ml.

Fig. 6. *Candida albicans* treated with 20 μg candidicin/ml. Higher power view than Fig. 5, showing the membrane retracting from the cell wall.

In *Candida albicans* it takes up to 5 h for polyene-induced proton entry to be completed, while potassium efflux is completed in 15 to 20 min (Hammond *et al.* 1974; Venables & Russell, 1972).

If membrane permeability has been altered by polyene action then it seems likely that small anions will diffuse out of the yeast organisms. Scholz, Schmitz, Bucher & Lampen (1959) and Caltrider & Gottlieb (1961) reported phosphate leakage after polyene action. Ghosh & Ghosh (1962) reported a 50 % decrease in cell inorganic phosphate in *Candida albicans*

after nystatin treatment, while Osteaux, Tran-Van-Ky & Biquet (1958) detected leakage of carboxylic acids and phosphate esters. Cirrilo, Harsch & Lampen (1964), using flame-photometry, showed phosphate leakage followed potassium leakage in baker's yeast treated with nystatin. This leakage of negatively charged species would tend to decrease the negative charge resulting from potassium leakage.

After 15 min only 4 % of total proton entry had taken place, while potassium leakage had ceased. There was leakage of 0.33 μ equiv. potassium ions/mg dry wt from *Candida albicans* after 15 min polyene treatment, compared with loss of 1.01 μ equiv. of protons from the suspension. The major part of internal acidification must occur after the efflux of potassium ions is completed. This acidification is equivalent to the entry of 6.31 μ equiv. H^+ /mg dry wt.

Other factors must account for proton entry in addition to potassium leakage. The loss of other cations has been reported and the leakage of these ions would create additional ionic imbalance causing proton entry. Alternatively some protons may be internal in origin. Conway & Downey (1950) reported that the pH of yeast cytoplasm may drop to 5.8 in some organisms at certain stages in the life cycle. The internal production of carboxylic acids could also contribute to internal acidification. It is possible that the slight discrepancy in values obtained for protons leaving the environment and those causing internal acidification is due to the masking effect of leaking carboxylate ions.

The internal acidification caused by polyenes causes precipitation of cytoplasmic components and this was shown by precipitation of cell sap. The pH change induced by lethal concentrations of candididin was sufficient to cause a marked increase in the extinction of cell sap *in vitro*. Venables & Russell (1972), by light microscopy, observed an increase in granularity of yeasts after nystatin treatment. Marked granularity and organelle loss were observed by electron microscopy in cells treated with candididin.

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