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NYSTATIN EFFECTS ON CELLULAR CALCIUM IN *SACCHAROMYCES CEREVISIAE*

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The primary effects of nystatin, a polyene antibiotic, on the yeast *Saccharomyces cerevisiae* were investigated. Though K^+ leakage was observed shortly after the addition of nystatin, Ca^{2+} leakage was delayed 2–3 h after its application and it occurred only at an acidic pH and in the absence of K^+, Na^+ or Mg^{2+} from the medium. However, within 4 min after application nystatin induced a passive influx of Ca^{2+} into the cells even at a concentration of 1 μM in the medium. These results led to the conclusion that the primary membranal lesion induced by nystatin is not restricted to monovalent cations but is also manifested by increased permeability to Ca^{2+}. The delayed leakage of Ca^{2+} is explained by the assumption that the bulk of cellular calcium is sequestered so that the concentration of free Ca^{2+} in the cytoplasm is very low. The sequestered calcium may be liberated 2–3 h after the addition of nystatin as a consequence of secondary damage to the cells such as intracellular acidification and loss of cations.

Introduction

Polyene antibiotics are important antifungal agents which interact with membranal sterols and cause damage to the cell membranes [1,2]. The specificity of the interactions and the extent of the damage depend on the size of the polyene [3,4], the lipid composition of the membrane, the type of sterol involved [5] and on an array of environmental factors [6]. The immediate effects of polyene on sensitive cells are leakage of intracellular K^+ [7,8] and increased permeability to protons [9]. The late effects are manifested by leakage of Mg^{2+} [10], phosphate ions [11] and carboxylic acids. As a consequence, protein and RNA syntheses decrease and glycolysis is inhibited [10].

The natue of the primary lesion in plasma membranes, with respect to its selectivity to monovalent cations was examined in the present work by investigating the effect of nystatin on the membrane permeability to calcium.

Methods

Organism and culture conditions

Saccharomyces cerevisiae strain 124 (genotype MAP a, his 1) was maintained at 4°C on 1.5% w/v agar containing 1% yeast extract, 2% glucose and 2% peptone. Prior to the experiments, cells were inoculated into medium I containing Bacto yeast extract (10 g/l) Bacto peptone (20 g/l) and glucose (20 g/l). For ^{45}Ca efflux experiments $^{45}CaCl_2$(1 μCi/ml) was added to medium I. The yeast cells were grown overnight with shaking (200 rev./min) at 30°C.

Determination of the cellular content of cations

Cells were grown overnight in medium I and were collected by centrifugation, washed by resuspension in distilled water, and finally resuspended in the indicated medium at a concentration of $5 \cdot 10^7$ cells/ml. The suspensions were incubated at 30°C with continuous shaking during the indicated times. After incubation, 2 ml samples of cell

suspensions were filtered through Sartorius membrane filters, prewashed with distilled water. The cells were washed four times with 5 ml distilled water each. The washing procedure was carried out in less than 2 min. Blank filters, which were similarly washed, yielded less than 5% of the amounts of K^+ and Mg^{2+} found in the samples. These values were substracted from the sample values.

After filtration, each filter was immersed in 3 ml distilled water, boiled to release the cations from the cells, and centrifuged to precipitate the debris. K^+ and Mg^{2+} were determined, after appropriate dilution, using a Perkin-Elmer Atomic Absorption Spectrometer.

Transport studies:

(a) Efflux. The yeast cells grown in the presence of $^{45}Ca^{2+}$ were collected by centrifugation and washed by resuspension in distilled water. The efflux was initiated by suspending the yeast cells at a final concentration of $5 \cdot 10^7$ cells/ml, in media containing 20 mM Hepes buffer, pH 5.2, or Hepes-Tris buffer (pH 7.5) and the indicated cations, substrate, ionophore or nystatin. The media were equilibrated for 15 min at 30°C with continuous shaking before the addition of the yeast cells, and shaken at 30°C throughout the experiment. Samples of 1 ml of cell suspensions were taken immediately after the addition of the yeasts and at the indicated times until the end of the experiment. The samples were rapidly filtered through Sartorious membrane filters (0.45 μM pore size), which had been prewashed with 20 mM $MgCl_2$. The cells on the filter were quickly washed three times with 20 ml 20 mM $MgCl_2$. It was previously reported [12] and confirmed in the yeast *S. cerevisiae* (unpublished data) that under these conditions the amount of ^{45}Ca absorbed to the cells when incubated at 2°C is very small. The filters were dried and the radioactivity determined in toluene-containing scintillation fluid. The results are expressed as percent of the initial radioactivity present in the cells at each sampling time.

(b) Influx. Cells were grown overnight in medium I, collected by centrifugation, washed three times by resuspension in distilled water and resuspended in the indicated media at a concentration of $5 \cdot 10^8$ cells/ml. The cells were preincubated at 30°C for the indicated times. Small aliquots of $CaCl_2$ labeled with $^{45}Ca^{2+}$ were added to the cells to a final concentration of 1 μM $CaCl_2$ (1 μci/ml). Samples were removed after short time intervals, filtered through membrane filters and washed four times with a solution containing 20 mM $MgCl_2$. After drying, the radioactivity of the filters was determined in a toluene containing scintillation fluid. Blank filters, through which 1 ml of medium without cells were filtered, were similarly washed and the counts remaining on the filters were subtracted form the results.

$^{45}CaCl_2$ (20 mCi/mg calcium) was purchased from Amersham International (U.K.), and nystatin was purchased from Sigma.

Results

The effect of nystatin on the efflux of cellular calcium is shown in Fig. 1. In a medium of an acidic pH (pH 5.2) and containing no glucose, only 10% of the cellular calcium was extruded from the cells in the absence of K^+ from the medium, and 20–30% in its presence. At pH 7.5

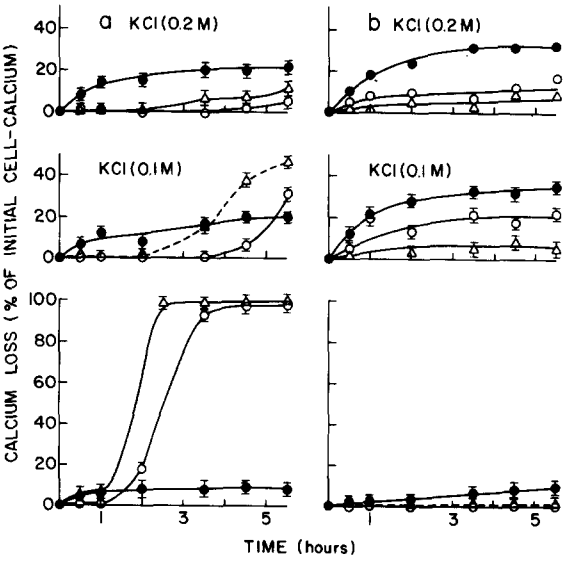

Fig. 1. Effect of nystatin on calcium efflux in the absence of glucose. (a) pH 5.2: the medium contained 20 mM Hepes-Tris, pH 5.2 and KCl when indicated. (b) pH 7.5: the medium contained 20 mM Hepes-Tris, pH 7.5 and KCl when indicated. ●, control; ○, nystatin, 5 μg/ml; △, nystatin, 10 μg/ml. Values represent mean ± S.E. ($n = 4$)

and in the absence of K^+ the efflux of calcium proceeded at a much slower rate, as reported previously [13]. Nystatin showed a dual effect: initially there was a marked inhibition of calcium efflux, while after 2–3 h, at an acidic pH and in the absence of K^+ in the medium, this inhibition was followed by a substantial leakage of cellular calcium. The timing and extent of the leakage depended on the concentration of nystatin in the medium. In the presence of 0.2 M KCl this calcium leakage was completely prevented, whereas 0.1 M KCl reduced and delayed it. At a pH values of 7.5 only the inhibitory effect of nystatin was observed. No calcium leakage followed this inhibition even in the absence of K^+ from the medium.

We have previously reported that in the presence of glucose and K^+ in the medium an exchange between intracellular Ca^{2+} and extracellular K^+ via a Ca/K antiport, took place [13,14]. In Fig. 2 we show the effect of nystatin on cellular calcium in the presence of glucose; the effects of nystatin were similar to those without glucose. At an acidic pH and in the absence of K^+, nystatin induced an initial inhibition of Ca^{2+} efflux followed by a massive Ca^{2+} loss after 2–3 h. In the presence of K^+ however, nystatin markedly inhibited the Ca/K exchange.

These results were most unexpected since data in the literature indicate that nystatin induces leakage of small molecules from the cells. Therefore we examined the leakage of K^+ and Mg^{2+} in the presence of similar concentrations of nystatin. After incubation for 15 min with 10 μg/ml nystatin, most of the cellular K^+ had leaked out of the cells, whereas a decrease in cellular magnesium was observed only after 45 min incubation. Addition of 0.2 M K^+ to the medium prevented the decrease in cellular K^+, and reduced the rate of Mg^{2+} loss both with and without nystatin (Table I).

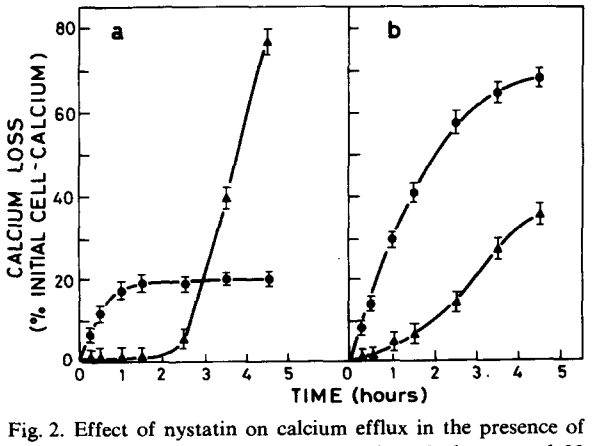

Fig. 2. Effect of nystatin on calcium efflux in the presence of glucose. The medium contained (a) 100 mM glucose and 20 mM Hepes, pH 5.2, or (b) as above with 0.2 M KCl. ●, control; ▲, plus nystatin 10 μg/ml. Values represent mean ± S.E. ($n = 4$)

Further experiments were designed to explain each of the two effect of nystatin.

The late effect of nystatin, calcium leakage from the cells, was inhibited by the presence of K^+ in the medium. In Fig. 3 the effect of other cations on calcium leakage is given. Na^+ shows exactly the same inhibitory effect as K^+, Mg^{2+} had a somewhat lesser effect while Ln^{3+}, which inhibits the energy-dependent calcium extrusion [13], had no effect on calcium leakage.

The initial effect of nystatin, the inhibition of calcium efflux, may be connected with the increased permeability to protons induced by this polyene [9]. Therefore we examined the effect of 2,4-dinitrophenol (DNP), another proton ionophore, on calcium efflux. The results in Fig. 4 show that similarly to nystatin, 2,4-DNP inhibited calcium efflux at pH 5.2 and at pH 7.5, both in the presence and in the absence of K^+, However, no leakage of Ca^{2+} followed the inhibition of efflux even after 5 h of incubation.

Further examination of the results raised the question whether the initial lack of calcium leakage stems from a very low concentration of free calcium in the cytoplasm so that in spite of the increased permeability of the membrane to calcium, no leakage occurs. An alternative explanation is that nystatin does not increase the membrane permeability to Ca^{2+}.

In order to distinguish between these two possibilities we examined the effect of nystatin on calcium influx.

Accumulation of calcium in *S. cerevisiae* is an energy requiring process [12,15,16]. In the absence of glucose only small amounts penetrate into the cells. This influx is probably supported by energy provided from endogenous sources. Glucose stimulates calcium influx 6–7-fold (Table II).

Addition of nystatin to cultures containing no

TABLE I

EFFECT OF NYSTATIN ON THE LEAKAGE OF CELLULAR K^+ AND Mg^{2+} IN THE ABSENCE OF GLUCOSE

Additions to Hepes (20 mM, pH 5.2) medium	K^+ in cells (nmol/mg dry weight) after incubation for (min):			Mg^{2+} in cells (nmol/mg dry weight) after incubation for (min):		
	15	45	105	15	45	105
None	384.2±12.4	380.4±11.8	380.8±12.1	91.2±4.1	88.7±3.4	68.9±2.4
Nystatin (10 μg/ml)	28.5± 1.5	14.5± 0.9	14.5± 1.7	92.5±3.8	64.4±2.1	23.6±1.5
KCl (0.2 M)	395.1±15.2	421.0±17.4	415.5±18.2	89.5±2.9	87.2±4.2	82.8±3.7
KCl (0.2 M) and nystatin (10 μg/ml)	379.8±17.2	384.7±16.5	372.7±12.1	87.1±4.2	59.8±3.8	42.2±2.1

glucose markedly increased the penetration of calcium into the cells. This increase was observed after a lag-period of 4 min, and it took place at both acidic and neutral pH values (Fig. 5). On the other hand, the uptake of calcium in the presence of glucose was inhibited by nystatin (Table II). It appears that this inhibition is the result of the dissipation of ΔpH across the membrane and inhibition of glycolysis by nystatin, thereby reducing the energy supply for Ca^{2+} influx. The presence of K^+ in the medium markedly reduced Ca^{2+} uptake as reported [15,16]. The penetration of calcium in the presence of nystatin was also reduced by K^+ but was still higher than in the K^+-containing controls (Table II).

In order to determine whether the influx of calcium in the presence of nystatin may possibly be due to Ca/Ca exchange, calcium efflux was examined in the presence of 1 μM calcium in the medium, after 30 min of incubation with 10 μg/ml nystatin. No calcium efflux was observed and the results were the same as shown in Fig. 1. We

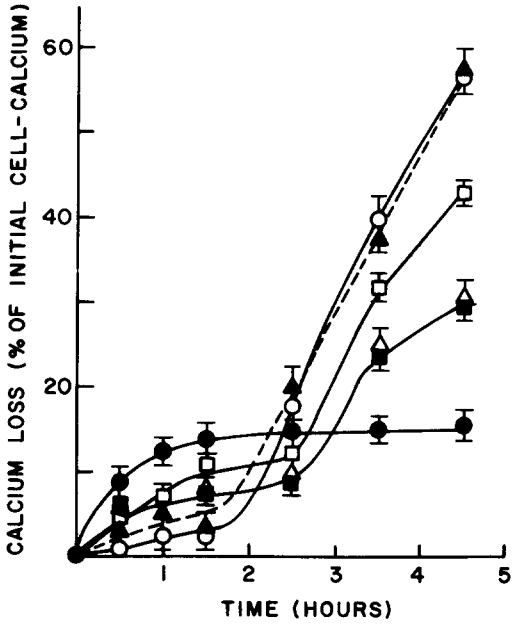

Fig. 3. Effect of cations on calcium leakage in presence of nystatin (10 μg/ml). The medium contained 20 mM Hepes, pH 5.2, and the following: ●, none; ▲, nystatin; ○, nystatin + $LnCl_3$ (0.1 mM); □, nystatin + $MgCl_2$ (2 mM); △, nystatin + NaCl (100 mM); ■, nystatin + KCl (100 mM). Values represent mean ± S.E. ($n = 4$)

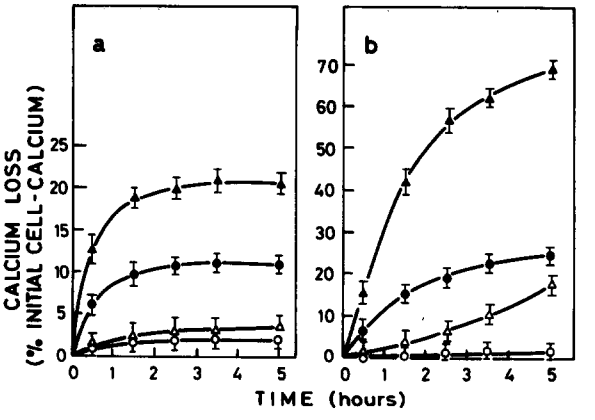

Fig. 4. Effect of 2,4-DNP on calcium efflux. The medium contained 20 mM Hepes, pH 5.2, and the following: (a) ●, none; ▲, glucose (100 M); ○, 2,4-DNP (0.3 mM); △, 2,4-DNP (0.3 mM) + glucose (100 mM). (b) As above in the presence of KCl (0.2 M). Values represent mean ± S.E. ($n = 4$)

TABLE II

THE EFFECT OF NYSTATIN ON CALCIUM UPTAKE

After 30 min incubation in the indicated medium $CaCl_2$ (1 μM) labelled with 1 μCi/ml ^{45}Ca was added for 5 min. The values represent means ± S.E. ($n = 4$).

Medium	Calcium uptake $g \cdot 10^{-18}$/cell per 5 min	
	−glucose	+glucose
pH 5.2		
control (20 mM Hepes)	1.40 ± 0.05	8.25 ± 0.31
+nystatin (1 μg/ml)	1.92 ± 0.03	3.19 ± 0.08
+nystatin (5 μg/ml)	4.01 ± 0.06	4.28 ± 0.01
+nystatin (10 μg/ml)	7.10 ± 0.03	6.75 ± 0.12
+KCl (0.2 M)	0.45 ± 0.002	0.73 ± 0.02
+KCl (0.2 M)	1.69 ± 0.08	0.82 ± 0.03
+nystatin (10 μg/ml)		
pH 7.5		
control (20 mM Hepes-Tris)	1.48 ± 0.05	10.43 ± 0.52
+nystatin (1 μg/ml)	1.90 ± 0.07	3.31 ± 0.11
+nystatin (5 μg/ml)	3.51 ± 0.10	2.72 ± 0.09
+nystatin (10 μg/ml)	5.88 ± 0.13	6.16 ± 0.12
+KCl (0.2 M)	0.59 ± 0.02	1.12 ± 0.03
+KCl (0.2 M)	1.53 ± 0.03	0.96 ± 0.01
+nystatin (10 μg/ml)		

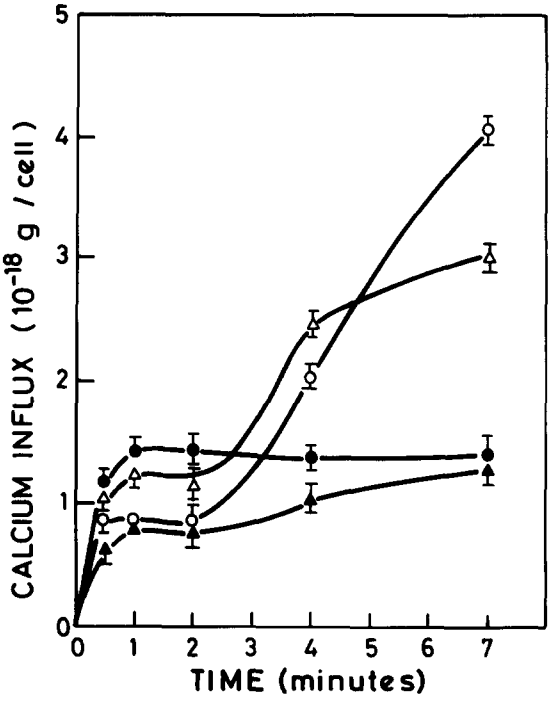

Fig. 5. Effect of nystatin on calcium influx in the absence of glucose. The medium contained: ●, Hepes (20 mM), pH 5.2; or ○ as above with nystatin (10 μg/ml); ▲, Hepes-Tris, (20 mM) pH 7.5; or △, as above with nystatin (10 μg/ml). Values represent mean ± S.E. ($n = 4$)

concluded that nystatin induced an energy-independent net influx of calcium into the cells even when the concentration of calcium in the medium was as low as 1 μM.

Discussion

The immediate effects of polyene antibiotics on yeast are manifested by the leakage of K^+ [7,8] and by an increased permability to protons [9]. The present work shows that the leakage of calcium occurs only at an acidic pH, 2–3 h after leakage of K^+ began. This considerable lag in initiating the calcium leakage indicates that it is probably the result of some secondary damage to the yeast cells. Intracellular acidification or K^+ loss may be the primary causes for the late calcium loss, since addition of K^+ or adjusting the pH of the medium to neutral prevented calcium leakage.

The finding that the immediate effect of nystatin is limited to leakage of K^+ (but not of Ca^{2+}) may be interpreted as indicating that there was no increase in the membrane permeability to calcium. An alternative explanation is that the lack of Ca^{2+} leakage results from the low concentration of free calcium in the cytoplasm and not from the impermeability of the cell membranes to calcium. These two possibilities were examined by measuring calcium influx in the presence of nystatin. The results support the second hypothesis; the inward electrochemical gradient, in the presence of nystatin and 1 μM calcium in the medium, prevented the leakage and induced a passive influx of Ca^{2+} into the cells. The concentration of free calcium in the cytoplasm is therefore bound to be very low.

However, measurements of calcium content by atomic absorption spectrometry and by the isotope equilibrium technique showed that the cells contain a substantial amount of calcium (2.5 nmol/mg dry weight [13]). Thus the bulk of cellular calcium must be sequestered in intracellular compartments such as mitochondria or vacuoles. This compartmentalized calcium is presumably liberated follow-

ing secondary damage to the cells as discussed above.

The initial effect of nystatin was manifested not only by the lack of calcium leakage but also by a marked inhibition of calcium extrusion. This inhibition could be due to two possible causes: (1) leakage of K^+, which presumably increased $\Delta\Psi$ (negative inside); (2) dissipation of ΔpH due to the increased permeability to protons. The first possibility (K^+ leakage) was not supported by the results since in the presence of K^+ in the medium leakage was not observed, yet Ca^{2+} efflux was inhibited. On the other hand, similarly to nystatin, 2,4-DNP, a proton ionophore, inhibited calcium extrusion at pH values of 5.2 and 7.5 both in the presence and in the absence of K^+ in the medium. Therefore, we conclude that the initial inhibition of calcium extrusion by nystatin is likely to be due to the dissipation of ΔpH across the plasma membrane, thus abolishing the energy source for calcium extrusion.

In conclusion, it was shown that the initial membranal lesion induced by nystatin is not restricted to monovalent cations but also involves increased permeability to calcium. The lack of early Ca^{2+} leakage stems from the finding that most of the cellular calcium in yeast is compartmentalized or bound so that the concentration of free calcium in the cytoplasm is very low.

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