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Nystatin changes the properties of transporters for arginine and sugars

An in vitro study

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Abstract

In ergosterol-containing energized yeast plasma membrane vesicles nystatin (5–10 μg/mg total lipid) caused a massive efflux of pre-accumulated arginine while the membrane potential (the principal driving force; −110 mV) decreased by only 10–30 mV. Neither the substrate fluxes nor the membrane potential was influenced by nystatin when the permease was reconstituted in ergosterol-free phospholipid vesicles. The same effect of nystatin was found with the reconstituted sugar transporter from *Chlorella kessleri*. It is suggested that nystatin binding to ergosterol in the vicinity of the permease releases the transport protein from its coupling to energy and converts it to a facilitator.

Key words: Nystatin; Yeast plasma membrane vesicles; Arginine transport

1. Introduction

It is generally accepted that polyene antibiotics such as amphothericin B and nystatin may be used as antifungal agents because they are selectively toxic for fungal cells. This selectivity is thought to originate mainly from the presence of ergosterol in the fungal plasma membranes. These antibiotics, at concentrations that inhibit growth, are considered to damage yeast cell membranes, which results in the leakage of such internal constituents as K^+, Ca^{2+}, Mg^{2+} and PO_4^{3-}; this is thought to be followed by the inhibition of glycolysis and of protein synthesis (for review see [1]).

Recent studies, reviewed by Bolard [2], question the assumption that fungal death results from pore formation induced by the antibiotics; selective K^+ leak being the primary event. The lack of correlation between K^+ leakage and lethality was confirmed with amphothericin B and nystatin in *C. albicans* [3,4] and *S. cerevisiae* [5]. In the latter yeast, the release of Mg^{2+}, of nucleic acid precursors and of amino acids induced by nystatin has been demonstrated (for review see [2]). The conclusion was that under the influence of nystatin the membranes undergo fluidity changes (as opposed to pore formation) which contribute somehow to the release of cell constituents.

We have studied the effect of nystatin using an in vitro system of yeast plasma membrane vesicles. Nystatin seems to affect transport proteins embedded in ergosterol-containing membranes by releasing them from the membrane potential control and converting them to facilitated diffusion mediators.

2. Materials and methods

2.1. Materials

The radiolabelled substances L-[U-^{14}C]arginine (304 mCi/mmol), D-[U-^{14}C]glucose (126 mCi/mmol) and tetra-[^{3}H]phenylphosphonium bromide (33 Ci/mmol) were obtained from Amersham Buchler (Braunschweig).

Cytochrome *c* oxidase was isolated from beef heart mitochondria according to Yu et al. [6].

The lipids L-α-phosphatidylethanolamine (type IX from *E. coli*, approximately 50%) and ergosterol, and miscellaneous chemicals, *n*-octyl-β-D-glucoside, Ph_4PBr, carbonyl cyanide *m*-chlorphenyl hydrazone, horse heart cytochrome *c*, TMPD, nystatin and arginine were from Sigma.

2.2. Yeast strains

In this study a transformed strain *Saccharomyces cerevisiae* RS 453 (CAN1) (ade 2-1, leu2-3,112, his3-11,15, ura52) with a multicopy plasmid pWHY bearing CAN1 gene coding for arginine permease [7] was used. Some supplementary experiments were carried out with a strain *Schizosaccharomyces pombe* transformed by a multicopy plasmid pEVP 11 bearing HUP1 gene coding for hexose uptake protein in *Chlorella kessleri* (*S. pombe* TCY 96) [8].

2.3. Media and growth conditions

The yeast strain *S. cervisiae* RS 453 (CAN 1) was grown on yeast minimal medium containing 0.67% yeast nitrogen base without amino acids, 2% glucose and required bases and amino acids. The strain *S. pombe* TCY 96 was grown on 2% gluconic acid as a carbon source.

2.4. Isolation of plasma membranes

Plasma membranes were isolated from yeast grown at 29°C on a rotary shaker. At A_{578} = 0.5–0.8 the cells were harvested, washed and broken by shaking with glass beads (diameter 0.45–0.5 mm). Plasma membranes were isolated essentially according to Goffeau and Dufour [9]. During the isolation the mixture of 0.25 M 4-aminobenzamidine dihydrochloride and 0.50 M phenylmethyl sulphonyl floride was re-

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Abbreviations: Ph_4P^+, tetraphenylphosphonium ion; TMPD, *N,N,N,N'*-tetramethyl-*p*-phenylenediamine; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; cytochrome *c* oxidase, ferrocytochrome-*c*:oxidoreductase (EC 1.9.3.1).

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peatedly added in the amounts of 1/100 of the actual isolation solution volume. The isolated plasma membranes, suspended in 50 mM potassium phosphate, pH 6.3, 20% glycerol and 1 mM EDTA, were rapidly frozen in liquid nitrogen and stored at −80°C.

2.5. Preparation of liposomes

E. coli phospholipid liposomes were prepared as described in Opekarová et al. [7]. For preparation of ergosterol containing liposomes, the chloroform solutions of ergosterol and *E. coli* phospholipid were mixed at a desirable ratio and the subsequent procedure was as above.

2.6. Reconstitution of cytochrome c oxidase into proteoliposomes

The procedure used was as described previously [7] with the exception that all the buffers used were of pH 6.3 and 1 nmol of cytochrome *c* oxidase was added per 20 mg of total lipids in 1 ml of buffer.

2.7. Fusion of proteoliposomes with yeast plasma membranes

The proteoliposomes were mixed with yeast plasma membranes at a ratio of 20:1 (lipid/protein) in a final volume of 100–200 μl. The suspension was suplemented with 1 mM $MgSO_4$ and rapidly frozen in liquid nitrogen. Before use the fused membranes were slowly thawed at room temperature and briefly sonicated with a probe-type sonifier (Cole-Palmer Instruments Co.).

2.8. Internal volume

Internal volume of the vesicles was estimated as described previously [7] with the exception that radioactive sucrose was enclosed into the vesicles instead of arginine. Use of the noncharged sucrose for this purpose excluded the unspecific binding observed with arginine probabely due to different lipid composition of the vesicles.

2.9. Uptake assays

The uptake experiments were done at room temperature in an open magnetically stirred vessel. The vesicles were diluted with 50 mM potassium phosphate, pH 6.3, containing 1 mM $MgSO_4$ to a final concentration of 0.05–0.07 mg protein/ml. The energization was started by addition of 22 mM ascorbate, 16 μM cytochrome *c* and 180 μM TMPD. At zero time, radioactively labelled substance was added. Aliquots of 30–50 μl were withdrawn at intervals and diluted with 100 mM LiCl. The suspension was filtered through 0.22-μm pore-size cellulose acetate filters (Schleicher and Schuell) and washed with 2.0 ml of LiCl. The radioactivity was determined by scintillation counting.

2.10. Determination of transmembrane electrical potential ΔΨ

The membrane potential (inside negative) was determined from distribution of tetra-[^{3}H]phenylphosphonium cation (Ph_4P^+) [10] according to Nerst-Donnan equation:

$$\Delta E = -RT/nF \times 2.3 \log([Ph_4P^+]_{in}/[Ph_4P^+]_{out})$$

where $-2.3RT/nF$ represents approximately 59 mV at room temperature.

The calculated values of membrane potential are slightly overestimated since a correction for nonspecific $\Delta\vartheta$ dependent binding [11] was not included.

Throughout this study we use membrane potential as a measure of energization of the system since it was the main driving force for arginine accumulation. The other component of protonmotive force, ΔpH, contributed less than 15% (data not shown).

2.11. Protein estimation

Protein was determined by the method of Bradford [12].

3. Results and discussion

The alteration of membrane lipids and/or membrane proteins by the polyene antibiotic nystatin can be studied with advantage using plasma membrane vesicles prepared from purified yeast plasma membranes and exogenously added lipids of different composition. In a recent study on lipid composition of subcellular membranes of *Saccharomyces cerevisiae*, Zinser et al. [13] showed that in purified plasma membranes the ergosterol accounts for about 64% of total lipids. It was not possible to prepare functioning plasma membrane vesicles containing this high amount of ergosterol. Only the vesicle suspensions containing 30% of ergosterol exhibited satisfactory membrane potential formation after energization and consequently accumulation of arginine. The vesicle suspension, however, was quite milky and it was difficult to assess what part of the ergosterol had been incorporated into vesicles and what had remainded in solution. Consequently, the estimated vesicle internal volume was 0.5 μl/mg lipids, which was only about one half of that determined for vesicles prepared from *E. coli* phospholipids (0.9–1.0 μl/mg lipids). For comparative studies vesicles containing 12.3% of ergosterol were found suitable, judging from both the appearance of their solution and their internal volume (1.2 μl/mg lipid).

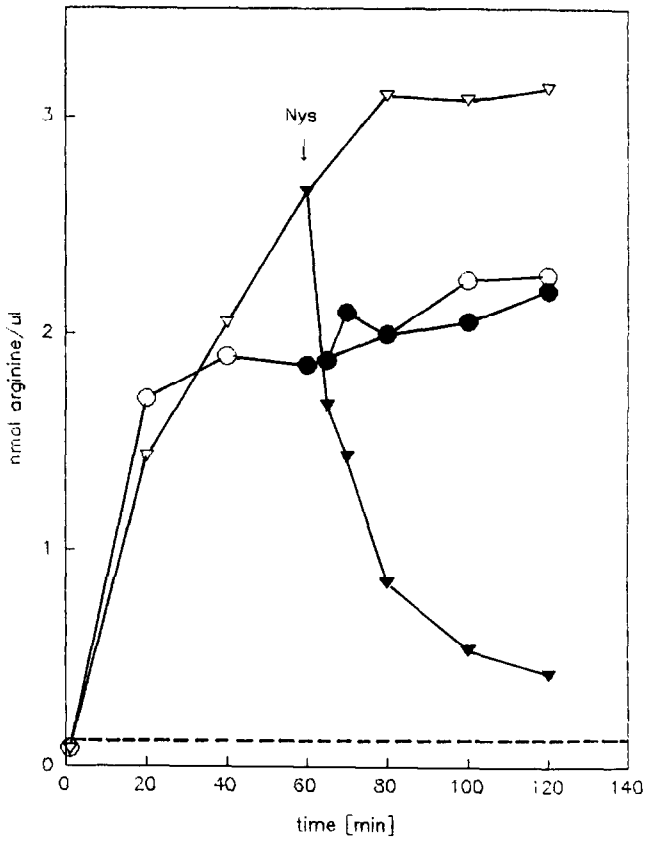

Fig. 1. Uptake of 20 μM arginine in energized yeast plasma membrane vesicles prepared from the membranes of *S. cerevisiae* RS 453 (CAN1) fused with *E. coli* phospholipid proteoliposomes containing cytochrome-*c*-oxidase (○) or with the same proteoliposomes containing in addition 12.3% ergosterol (▽). At arrow nystatin (9.0 μg/mg lipid) was added (●,▼). Dashed line represents calculated equilibrium.

Both ergosterol and pure phospholipid vesicles used in this study exhibited good transporting activity for arginine. The K_m ranged from 10 to 20 μM and V_{max} amounted to 2 nmol/mg protein/min. The arginine accumulation

ratios were much higher than those found previously [7]. Ergosterol containing vesicles accumulated arginine up to 80-fold at 100 μM arginine outside. This is attributed to improvements in membrane isolation procedure used in this study (see section 2).

In Fig. 1 an accumulation of arginine into vesicles prepared from *E. coli* phospholipids and vesicles containing in addition 12.3% of ergosterol is shown. On energization by electron donor system (ascorbate/TMPD/cytochrome *c*) a comparable membrane potential of 100–115 mV was formed in both types of vesicles (Fig. 2). Generally a somewhat higher accumulation of arginine was found in the ergosterol enriched vesicles than in those with phospholipids only. It is evident, however, that ergosterol is not absolutely necessary for reconstitution of arginine transport system. It should be noticed, however, that the endogenous ergosterol of the purified plasma membrane is present in the vesicles; based on the data of Zinser et al. [13] the ergosterol content in the 'phospholipid' vesicles may amount to more than 1%. The vesicles enriched in ergosterol contain approxemately a 10 times higher amount. The transport characteristics in both types of vesicles were similar. The fluxes of arginine in the vesicles, however, differed significantly when nystatin was added (Fig. 1). On addition of nystatin a rapid efflux of accumulated arginine was observed from the vesicles enriched in ergosterol, which was not the case with 'phospholipid' vesicles. The extent of the efflux depended on the nystatin/ergosterol ratio ranging from 95% of accumulated arginine released during 10 min at 70 μg nystatin/mg total lipids to 50% at 7 μg nystatin/mg lipid. For the sake of comparison of the nystatin effect on phospholipid vesicles the amount of nystatin was related to total lipids; the external amount of ergosterol in 'ergosterol vesicles' was the same, 12.3%, throughout the study. To minimize further additional effects and possible unspecific events the lowest amounts of nystatin (5–10 μg/mg total lipid) still causing the marked effects were used.

The observation in Fig. 1 is consistent with the 'sterol hypothesis' according to which the lethal effect of polyene antibiotics on eucaryotic cells is due to formation of polyene–sterol pores [2]. To see whether the arginine efflux proceeds via ergosterol–nystatin pores and to evaluate the overall permeability changes of the system on nystatin addition, the accumulation of tetraphenylphosphonium cation (Ph_4P^+) in the vesicles was followed. This lipophilic cation is accumulated by simple diffusion through lipid bilayer of vesicles in response to the electric potential formed. From its final distribution the membrane potential can be calculated (see section 2). The addition of nystatin did not affect the Ph_4P^+ accumulation in energized phospholipid vesicles (hence, neither the permeability nor cytochrome *c* oxidase activity were affected) while in energized ergosterol containing vesicles nystatin caused a membrane potential drop from

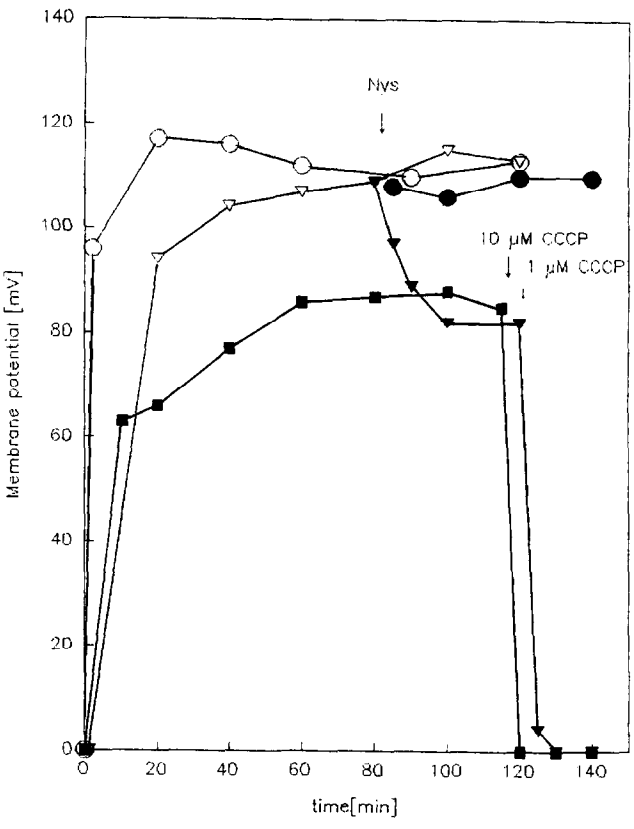

Fig. 2. Membrane potential formation in energized yeast plasma membrane vesicles prepared from the membranes of *S. cerevisiae* RS 453 (CAN1) fused with *E. coli* phospholipid proteoliposomes containing cytochrome-*c*-oxidase (○) or with the same proteoliposomes containing in addition 12.3% ergosterol (▽). At arrow nystatin (9.0 μg/mg lipid) was added (●,▼) or to ergosterol containing vesicles nystatin was added concomitantly with Ph_4P^+ (■). The membrane potential was abbolished by addition of 10 μM or 1 μM CCCP.

about −110 to −82 mV during 40 min. Approximately the same final membrane potential was formed when nystatin was added to the energized vesicles together with the membrane potential indicator (Fig. 2). The observed membrane potential decrease can be explained by an increased permeability for K^+ [14], which, however, most likely is largely compensated for by proton pumping activity of the cytochrome oxidase.

It is evident that no membrane potential could be built up in the vesicles if pores causing the arginine leak were formed. It is assumed therefore that the efflux observed in the presence of nystatin does take place via the arginine transporter. The membrane potential of −80 mV would still be able to drive and maintain approximately a 20-fold arginine accumulation, which is not observed, however; thus obviously the transporter is not coupled to energy anymore; it acts like a facilitator. Passive fluxes of a charged hydrophilic molecule like arginine into and out of vesicles are very small. Virtually no arginine uptake into nonenergized vesicles has been observed (not shown).

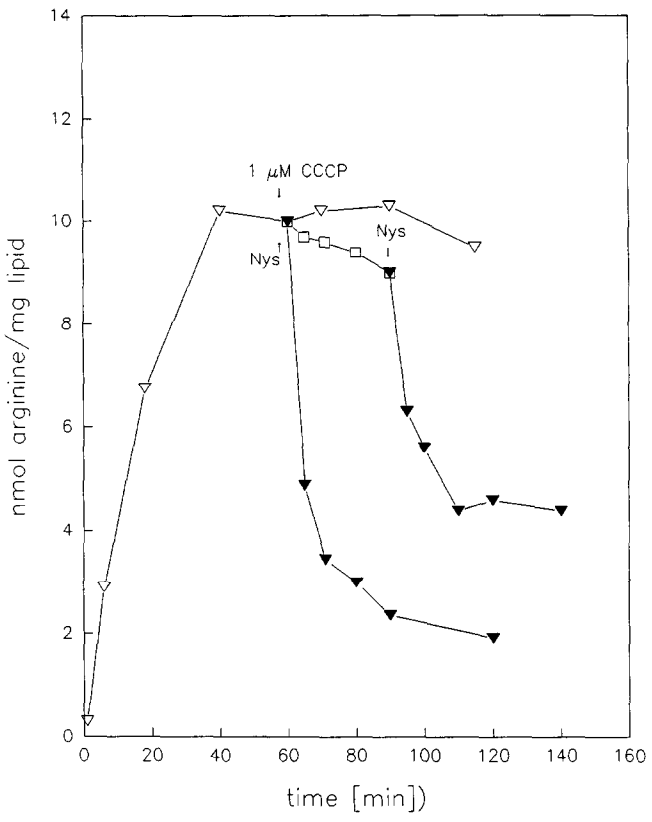

Fig. 3. Uptake of 100 μM arginine in energized yeast plasma membrane vesicles prepared from the membranes of *Saccharomyces cerevisiae* RS 453 (CAN1) fused with *E. coli* phospholipid proteoliposomes containing cytochrome-*c*-oxidase and 12.3% ergosterol (▽). At arrow 1 μM CCCP (□) or nystatin at concentrations of 9.4 and 6.3 μg/mg lipid, resp. (▼) were added.

When membrane potential was rapidly and completely abolished by 10 μM CCCP a considerably slower arginine leak occured as compared to the nystatin induced efflux (not shown), possibly in part reflecting the unidirectional nature of amino acid transport in yeast (for review see [15]). The slow release of arginine from the vesicles after addition of 10 μM CCCP is in contrast to what we observed in our previous study [7]. This discrepancy can be explained by a different uncoupler/lipid ratio employed in this and the previous study, where 10 times more lipids were used for the same uncoupler concentration. When 1 μM CCCP was used for complete uncoupling of the system (Fig. 2) the accumulated arginine was retained in the vesicles for at least 30 min and was released immediately after addition of nystatin (Fig. 3). The high uncoupler/lipid ratio probably causes another effect on the vesicles in addition to uncoupling the protonmotive force and this will be subjected to further studies. The vesicle preparations used throughout this study were able to accumulate high internal concentration of arginine (up to 8 mM) which might make them more fragile. In fact, in some experiments after prolonged arginine accumulation we observed the spontaneous release of the arginine from the vesicles.

The evidence given in this study, i.e. the massive release of accumulated arginine after addition of nystatin to ergosterol vesicles while the overall permeability and membrane potential were not considerably changed, points to a direct interference of this antibiotic with the arginine transporter. The nystatin–carrier interaction can be accomplished only when ergosterol as a primary binding agent for nystatin is present in sufficiently high amounts in the membrane. It can be speculated that an interaction of nystatin with the carrier alternates its properties in that way that it stops sensing the membrane potential (still formed) and is converted to a facilitator.

Efflux of accumulated sugar from *Chlorella kessleri* [16] and of α-amino-isobutyric acid from yeast [17] after addition of nystatin were observed in vivo. Nystatin induced sugar efflux from *Chlorella* cells was interpreted as a consequence of the conversion of the active sugar transporter into a facilitator. This interpretation was based on a countertransport experiment typical for facilitated diffusion. In the vesicle preparation it was not possible, however, to demonstrate countertransport for technical reasons.

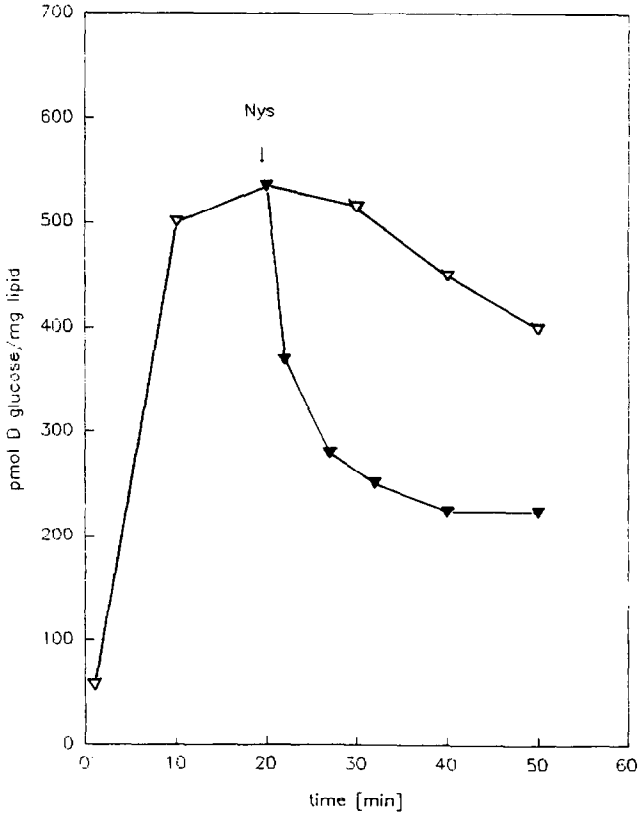

Fig. 4. Uptake of 18.5 μM D-glucose in energized yeast plasma membrane vesicles prepared from membranes of *Schizosaccharomyces pombe* TCY 96 fused with *E. coli* phospholipid proteoliposomes containing cytochrome-*c*-oxidase and 12.3% ergosterol (▽). At arrow nystatin (3.5 μg/mg lipid) was added (▼).

The observations reported in this study for the arginine transporter most likely are more general ones, since the same behaviour after addition of nystatin as described here was found with the hexose/H^+ cotransporter from *Chlorella kessleri* (Fig. 4). This protein, the gene product of the *Chlorella*HUP1 gene, was functionally expressed in *Schizosaccharomyces pombe* and reconstituted in ergosterol containing proteoliposomes. When vesicles not enriched with external ergosterol were used, the addition of nystatin did not lead to an efflux of glucose (data not shown). On the other hand, the fact that the other membrane protein incorporated into the system, the mitochondrial cytochrome-*c*-oxidase, which originally comes from an ergosterol-free membrane, is affected to a small degree only if at all, may suggest that only eucaryotic membrane proteins are sensitive to the antibiotic under study.

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