Biochimica et Biophysica Acta 1808 (2011) 433–445

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Ketoconazole and miconazole alter potassium homeostasis in *Saccharomyces cerevisiae*

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ARTICLE INFO

Article history:
Received 27 July 2010
Received in revised form 24 September 2010
Accepted 29 September 2010
Available online 7 October 2010

Keywords:
Antifungal
Yeast ion transport
Yeast transport
Potassium transport
Ketoconazole
Miconazole

ABSTRACT

The effects of ketoconazole and miconazole uptake on K^+ transport and the internal pH of *Saccharomyces cerevisiae* were studied. The uptake of both drugs was very fast, linear with concentration and not dependent on glucose, indicating entrance by diffusion and concentrating inside. Low (5.0 μM) to intermediate concentrations (40 μM) of both drugs produced a glucose-dependent K^+ efflux; higher ones also produced a small influx of protons, probably through a K^+/H^+ exchanger, resulting in a decrease of the internal pH of the cells and the efflux of material absorbing at 260 nm and phosphate. The cell membrane was not permeabilized. The K^+ efflux with miconazole was dependent directly on the medium pH. This efflux results in an increased membrane potential, responsible for an increased Ca^{2+} uptake and other effects. These effects were not observed with two triazolic antifungals. A decrease of the Zeta (ζ) potential was observed at low concentrations of miconazole. Although the main effect of these antifungals is the inhibition of ergosterol synthesis, K^+ efflux is an important additional effect to be considered in their therapeutic use. Under certain conditions, the use of single mutants of several transporters involved in the movements of K^+ allowed to identify the participation of several antiporters in the efflux of the cation.

1. Introduction

Several imidazolic compounds have been used as antifungals. Their main action has been defined as blockers of sterol biosynthesis [1,2] and that of steroid hormones through the inhibition of P450 cytochromes [3–5]. Miconazole also has an oligomycin-like inhibitory effect on the mitochondrial ATPase [6,7] and produces K^+ efflux, accompanied by an increased Ca^{2+} uptake [8]. Ketoconazole has been identified as an inhibitor of the Ca^{2+}-activated K^+ current in the pituitary gland [9], in erythrocytes [10,11], and in a Ca^{2+}/H^+ exchange in the vacuole of *Trypanosoma bruceii* [12], indicating its interaction with cation transport systems. Resistance genes for these compounds have been described [13,14].

Although the mode of action of these substances has been extensively studied, as pointed out in [15], only ketoconazole uptake has been studied in *Candida albicans* [16], but no reports were found about miconazole, and few other effects have been studied. This work reports results on the internalization of both drugs, as well as other effects on the yeast *Saccharomyces cerevisiae* as a model fungus. Ketoconazole and miconazole were selected among the most used imidazolic antifungals; two other triazolic compounds, fluconazole and itraconazole, were also studied.

2. Material and methods

2.1. Yeast strains and culture conditions

A *S. cerevisiae* wild type strain was isolated as a single colony from commercial yeast (La Azteca, S.A., Mexico).

FY833 was kindly donated by Dr. M. Ghislain through Dr. Mónica Montero, University of Rio de Janeiro, Brazil [17]. All mutants were constructed in FY833 strain, using the PCR-based deletion: *Δnhx1* and *Δkha1* (this work).

The double mutant *Δkha1–Δnha1* was generated from *Δnha1* described in [18].

Δtrk1, *Δtrk2*, and *Δtrk1–Δtrk2* were reported in [19].

W303-1A [20] and TOW (*Δtok1*) were kindly provided by Drs. Lydie Maresova and Hana Sychrova, Academy of Sciences, Prague, Czech Republic [21]. All strains used are described in Table 1.

Oligonucleotides used to construct deletion cassettes are listed in Table 2. PCR-generated *kha1::HIS3*, *nhx1::kanMX*, disruption cassettes were obtained using the oligonucleotide pairs F1/R1 KHA1, F1/R1 NHX1, respectively, and plasmids pFA6a-TRP1, pFA6a-kanMX6, and pFA6a-His3MX6 as templates according to Longtine et al. [22]. These deletion cassettes contain the specified auxotrophy markers (*TRP1* or *HIS3*) or the kanamycin-resistance marker flanked by *ca.* 40 bp of

Abbreviations: Bicine-TEA, *N,N*-Bis(2-hydroxyethyl)glycine, adjusted to pH 8.0 with triethanolamine; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CFU, colony forming units; CH_2Cl_2, dichloromethane; $DiSC_3(3)$, 3,3′-dipropylthiacarbocyanine or cyanine; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; HOMOPIPES-TEA, Homopiperazine-N,N′-bis-2-(ethane sulfonic acid) adjusted to pH 4.0 with triethanolamine; K, ketoconazole; M, miconazole; MES-TEA, morpholino ethanesulfonic acid adjusted to pH 6.0 with triethanolamine; PMP, plasma membrane potential; TEA, triethanolamine

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doi:10.1016/j.bbamem.2010.09.025

Table 1
Mutants used in this work.

Name	Genotype	Reference
FY833	MATa-*his3-Δ200*, *ura3-52*, *leu2-Δ1*, *lys2-Δ202*, *trp1-Δ63*, *GAL*$^{2+}$	Masuda et al. [17]
Δnha1	MATa-*Δnha1::TRP1*	Peña et al. [18]
Δtrk1	MATa-*Δtrk1::KAN*	Peña et al. [18]
Δtrk2	MATa-*Δtrk2::HIS3*	Peña et al. [18]
Δtrk1–Δtrk2	MATa-*Δtrk1::TRP1-Δtrk2::KAN*	Michel et al. [19]
W303-1A	MATa-*ade2-Δ1*, *can1-Δ100*, *his3-Δ11,15*, *leu2-Δ3,112*, *trp1-Δ1 ura 3-Δ1::URA3*	Wallis et al. [20]
TOW (*Δtok1*)	MATa-*tok1Δ::kanMX*	Maresova et al. [21]
Δkha1	MATa-*Δkha1::HIS3*	This work
Δkha1–Δnha1	MATa-*Δkha1::HIS3-Δnha1::TRP1*	This work
Δnhx1	MATa-*Δnhx1::kanMX*	This work

homology to upstream 5′ and downstream 3′ regions of the target gene on each side, respectively. For *KHA1* and *KHA1–NHA1* deletions, yeast transformants were selected on synthetic defined media lacking the appropriate amino acid; for *NHX1* deletion, transformants were selected in YPD media (1% yeast extract, 2% peptone, 2% glucose) supplemented with G418 (300 μg mL^{-1}). Yeast transformation was performed using the lithium acetate method according to Gietz [23].

Deletions were confirmed by PCR, using external oligonucleotides for each gene, or an oligonucleotide located 5′ to the ORF of the gene and an oligonucleotide located within the kanamycin or the corresponding amino acid coding region.

La Azteca yeast was grown in 500 mL of YPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose). After 24 h of growth, the cells (around 8 g) were collected by centrifugation, washed with distilled water, and aerated in 250 mL of water for 16 to 18 h in an orbital shaker at 30 °C. After starvation, cells were collected by centrifugation, washed with water and suspended in water at a ratio of 0.5 g per mL, and maintained on ice until use, during the same day. FY833 and its mutants were grown as described for La Azteca, in YPD medium, but supplemented with their amino acid requirements. For *Δtrk1*, *Δtrk2*, and *Δtrk1–Δtrk2* the medium was also supplemented with 100 mM KCl and fasted in the absence of the salt. W303-1A and TOW (*Δtok1*) were grown in YPD, but the medium was supplemented with adenine (80 mg L^{-1}) and uracil (30 mg L^{-1}).

In experiments at different pH values, the cells (50 mg, wet weight) were incubated in 10 mM MES-TEA, pH 6.0, or 10 mM bicine-TEA, pH 8.0, or 10 mM HOMOPIPES-TEA, pH 4.0, 25 mM glucose, and the indicated concentrations of ketoconazole or miconazole, in a final volume of 4.0 mL. Glucose or other additions are as indicated in individual experiments.

2.2. Ketoconazole uptake and efflux of nucleotides

Ketoconazole uptake was determined from the concentration of the drug remaining in the supernatant after incubation and centrifugation of the cells, and this supernatant was also used to measure the efflux of nucleotides (material absorbing at 260 nm); the latter as an index of a possible membrane disruption by the drug. After incubation for 10 min at 30 °C and centrifugation of the cells, 40 μL of 1 N NaOH was added to a 2.0 mL aliquot of the supernatant; this addition was found to increase the extraction yields of ketoconazole from the supernatants with dichloromethane (CH_2Cl_2), of which 3.0 mL was found sufficient to extract the drug from the aqueous phase. The extraction was achieved by stirring in a vortex and centrifuging. The absorbance of the upper aqueous layer was determined at 260 nm to measure nucleotide efflux. The absorbance of the remaining lower layer of CH_2Cl_2 was measured at 288 nm and compared with a standard curve of ketoconazole prepared by adding the compound at different concentrations to 2.0 mL of 10 mM MES-TEA buffer, pH 6.0, plus 20 mM NaOH and extracting with CH_2Cl_2 in the same way as the samples. Extraction was necessary because, although ketoconazole shows an absorbance peak at 288 nm, it also shows a strong absorbance at 260 nm.

2.3. Miconazole uptake

Miconazole uptake was measured after incubation of the cells in a similar way, but after centrifugation, 2.0 mL of the supernatant or an adequate dilution in 10 mM MES-TEA buffer, pH 6.0, was mixed with 25 μM pentachlorophenol and 0.5 μM $DiSC_3(3)$. Fluorescence was then measured at 540–590 nm, and the concentrations in the supernatants were calculated by comparing against a standard curve of miconazole prepared in the same way. This method was developed from the fortuitous finding that miconazole, in the presence of FCCP or pentachlorophenol, in micromolar concentrations, produced a linearly increased fluorescence of $DiSC_3(3)$ at 540–590 nm, which was adequate to measure miconazole concentrations of up to 25 μM.

2.4. Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase was measured by incubating 25 mg of cells in the presence of 5 μM $NADP^+$ plus 5 μM glucose-6-phosphate and following the reduction of the nucleotide by its absorbance at 340 nm in the spectrophotometer. The assays were also carried out in the same way with cell-free extracts prepared by breaking the cells with glass beads for 1 min in a vortex and cooling for 1 min, repeating the procedure 10 times, and then centrifuging at 1500 *g* in the SS34 rotor of a refrigerated Sorvall centrifuge during 10 min. Approximately 2 mg of protein was used for the assay.

2.5. Efflux of K^+ and proton pumping

The efflux of K^+ was measured by incubating for 10 min, at 30 °C, 50 mg of cells, wet weight, in a final volume of 4.0 mL of the indicated buffer (10 mM), 25 mM glucose, plus the drugs at the concentrations indicated under each experiment. After incubation, the cells were centrifuged, and the supernatant used to measure K^+. The original total K^+ content of the cells was measured by boiling in a bath for 20 min a similar suspension of the cells in water and centrifuging. K^+ was measured in the supernatant by flame photometry.

The efflux of K^+ and proton pumping were also measured with pH and K^+ selective electrodes, connected to a pH meter and a computer. The incubation medium contained 125 mg of cells, 2 mM MES-TEA, pH 6.0, and 25 mM glucose in a final volume of 10 mL, and incubation was performed in a constant temperature chamber under continuous stirring at 30 °C. The drugs were added after 3 min at the indicated concentrations. In both cases, reference scales were obtained by the addition of known concentrations of KCl or HCl to the medium in the absence of cells.

Table 2
Gene sequences and oligonucleotides used in this study.

F1KHA1 5′-ATGGCAAACACTGTAGGAGGAATTCTGTCGGGTGTAAATCCGGATCCCCGGGTTAATTAA-3′
R1KHA1 5′-AAATTAAGAACAAAGAAAATTAAAAAATTTCTGCTGATACGAATTCGAGCTCGTTTAAAC-3′
F1NHX1 5′-ATGCTATCCAAGGTATTGCTGAATATAGCTTTCAAGGTGCCGGATCCCCGGGTTAATTAA-3′
R1NHX1 5′-TCTAGTGGTTTTGGGAAGAGAAATCTGCAGGTGATTGCGTGAATTCGAGCTCGTTTAAAC-3′

Underlined sequences correspond to the PCR primers used to amplify the transformation modules according to Longtine et al. [22].