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Ketoconazole and miconazole alter potassium homeostasis in *Saccharomyces cerevisiae*

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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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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].

2.6. $^{86}Rb^+$ uptake

The uptake of $^{86}Rb^+$ was measured by adding 50 mg of cells to 1.0 mL, final volume of 10 mM MES-TEA, pH 6.0, and 25 mM glucose at room temperature (25 °C). After three minutes, $^{86}RbCl$ was added at concentrations of 1, 2, 5, and 10 mM. An aliquot of 100 μL was taken and filtered through a 0.25 μm nitrocellulose filter and washed twice with 5.0 mL of 100 mM KCl. The filters were then dried and placed in scintillation vials and counted in a Beckman scintillation counter.

2.7. Internal pH

Internal pH was measured by the fluorescence changes of pyranine introduced to the cells by electroporation, as described before [24], incubating under the conditions described for each experiment. The cells (25 mg, wet weight) were incubated in 2.0 mL, final volume, of 10 mM MES-TEA, pH 6.0, and 25 mM glucose. The fluorescence changes (460–520 nm) of the dye were measured in a spectrofluorometer (SLM) under continuous magnetic stirring, in a cell compartment thermostated at 30 °C. The internal pH was then calculated from the fluorescence changes with the corrections described before [24].

2.8. Plasma membrane potential

The plasma membrane potential (PMP) difference was initially estimated as described before [25], by following the fluorescence changes of $DiSC_3(3)$. The cells (25 mg) were added to the spectrofluorometer cell containing 10 mM MES-TEA buffer, 10 μM CCCP, 10 μM $BaCl_2$, and 0.25 μM cyanine, in a final volume of 2.0 mL. When indicated, either 25 mM glucose or 10 mM KCl was added. The fluorescence changes (540–590 nm) were followed against time in an SLM spectrofluorometer equipped with a cell holder at 30 °C. In the experiments with miconazole, CCCP was omitted since it produces by itself an increased fluorescence, as stated earlier. In order to obtain a quantitative parameter, in another series of experiments, after incubating the cells, the $DiSC_3(3)$ concentration remaining outside was measured in the supernatant by its fluorescence at the same wavelengths in the presence of 1.0% sodium dodecylsulfate. The internal concentration of cyanine was obtained from this value, considering 0.37 mL g^{-1} wet weight as the internal water volume of the cells [26]. The PMP value was then calculated from the ratio of the internal/external concentrations of cyanine by means of the Nernst equation.

2.9. Inorganic phosphate determination

The efflux of inorganic phosphate was determined in similar experiments to those in which K^+ efflux was measured with the flame photometer, but inorganic phosphate was measured in the supernatant obtained by centrifuging the cells after incubation without or with the antifungals at different concentrations. The method of Fiske and Subbarow was used [27].

2.10. Zeta (ζ) potential

This parameter was obtained from the velocity of cell displacement under an electric field, determined directly under the microscope in a Zeta Meter ZM-75 cell electrophoremeter. The cells (25 mg, wet weight) were mixed with 15.0 mL of 10 mM HOMOPIPES-TEA, pH 4.0, or MES-TEA, pH 6.0, or Bicine-NaOH, pH 8.0, buffer, in the Z-meter electrophoremeter, and after applying 102 V, the migration time for 500 μm was measured, and this was used to calculate the electrophoretic mobility as explained in [18].

2.11. Effects on the viability of the cells

To explore this parameter, after incubating the cells in the presence of variable concentrations of the antifungals, as described to measure H^+ and K^+ fluxes with a selective electrode, a known number of cells were plated on YPD to observe the colony forming units (CFU) as an index of the survival of the cells.

2.12. Oxygen consumption

Oxygen consumption by the cells was measured with a Clark electrode connected to a polarization and measurement device (Yellow Springs) connected to a computer. The medium (3.0 mL, final volume) contained 10 mM MES-TEA buffer, pH 6.0, 25 mM glucose and 25 mg of cells. Different micromolar concentrations of miconazole were already present during the experiment. After 250 s, 10 μM of CCCP was added. The experiments were conducted in a closed chamber, under continuous stirring, at 30 °C.

Reagents were of the highest quality available, obtained from Sigma Chemical Co. (St Louis, MO, USA), except for dichloromethane obtained from Merck, and $DiSC_3(3)$ from Molecular Probes. Octylguanidine was synthesized according to the procedure reported by Phillips and Clarke [28] and recrystallized twice from water.

3. Results

3.1. The uptake of the antifungals

The procedure designed to measure the remaining drugs after incubation with the cells produced reasonable results, except perhaps for the lowest and highest concentrations of ketoconazole, whose uptake was measured only up to 200 μM. As Fig. 1 shows, the uptake was not saturable; it followed a practically linear behavior with the concentration of the drugs, at all concentrations, approximately 75% of the ketoconazole and 90% of the miconazole added were taken up by the cells. Besides, the uptake did not depend on the presence of glucose. In other experiments it was found that the uptake reached similar values when measured by incubating the cells on ice and centrifuging them immediately, or after incubating them at 30 °C for 10 min (not shown).

3.2. The antifungals produce an efflux of K^+ and the entrance of protons

Following the effect of the antifungals with K^+ and H^+ selective electrodes (Figs. 2 and 3), it was found that: a) at concentrations as low as 5.0 μM an efflux of K^+ could be observed with both drugs; b) miconazole was more effective than ketoconazole; c) the effect partially depended on the presence of glucose, more so for miconazole; d) an increase of the pH of the medium was detected with both antifungals starting at 20 to 40 μM, suggesting the entrance of protons to the cells. This apparent influx of H^+ was much lower than the efflux of K^+. Incubation of the cells with various concentrations of each antifungal induced a K^+ efflux to the incubation medium, which increased with concentration and, at around 200 μM, it was close to 100% (see also Fig. 7).

Two triazolic antifungals, fluconazole and itraconazole were tested and they did not produce the efflux of K^+, even when added at 400 μM (not shown). The efflux produced by octylguanidine, an organic cationic molecule previously described to produce an efflux of K^+, was tested. As found before [30], this compound produced some efflux of the cation, but only at concentrations higher than 400 μM (not shown).

3.3. The antifungals do not produce permeabilization of the cells

A possible disruption of the cells was first tested by analyzing the efflux of nucleotides as the material absorbing at 260 nm after

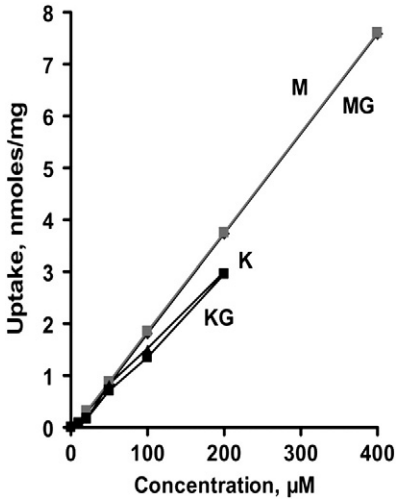

Fig. 1. Uptake of ketoconazole (K) and miconazole (M) by commercial yeast cells (La Azteca S.A., Mexico) at different concentrations in the presence or absence of glucose (G). The cells were incubated for 10 min at 30 °C in the presence of the antimycotic at the indicated concentrations. Aliquots were centrifuged, and the remaining antifungals were measured in the supernatant as described under Material and methods.

extraction of the antifungals with dichloromethane (see Material and methods). Fig. 4A shows that an efflux of this material occurred, but with the highest concentrations of ketoconazole; in the presence of glucose, it reached a maximum of 14% of the total material (measured in the supernatant after boiling and centrifuging the cells). This efflux was also higher with miconazole than with ketoconazole, and partially glucose-dependent, except for the highest concentrations. A small efflux, observed without the antifungals, also required the presence of glucose (it was smaller in the absence of a substrate).

The efflux of inorganic phosphate was also measured, and the experiments showed an increased efflux of this anion (Fig. 4B). At the highest concentrations of the drugs, the efflux percentage of this anion reached close to 80% of the total content of the cells, whereas that of K^+ was close to 100%. With ketoconazole, the efflux depended on glucose at all concentrations, whereas with miconazole this dependence was observed only at the lower concentrations.

The efflux of nucleotides and phosphate indicates a permeabilization of the cells. Another way to explore the possible permeabilization of the cells was measuring the activity of glucose-6-phosphate dehydrogenase in intact cells incubated in the presence of various concentrations of the antifungals. If the cells were permeabilized, glucose-6-phosphate and $NADP^+$ should enter the cells, and the corresponding enzyme activity should be detected. In cell-free extracts incubated with glucose-6-phosphate and $NADP^+$, a reduction of the nucleotide could be detected easily as an increase of absorbance at 340 nm in the spectrophotometer. However, by incubating the cells

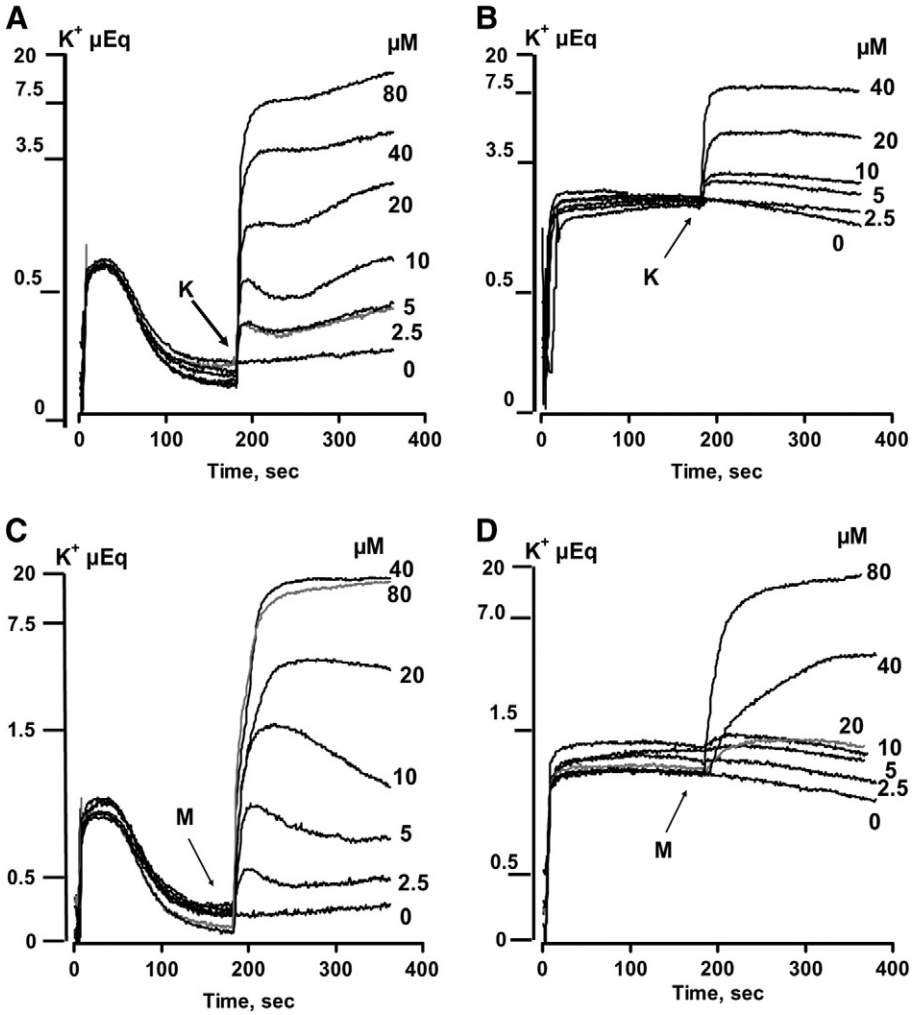

Fig. 2. Efflux of K^+ from yeast produced by ketoconazole (A and B) or miconazole (C and D) in the presence (A and C) or absence of glucose (B and D). Yeast cells were added at the beginning of the tracings. After 3 min, the antifungals were added at the indicated micromolar concentrations. K, ketoconazole; M, miconazole.

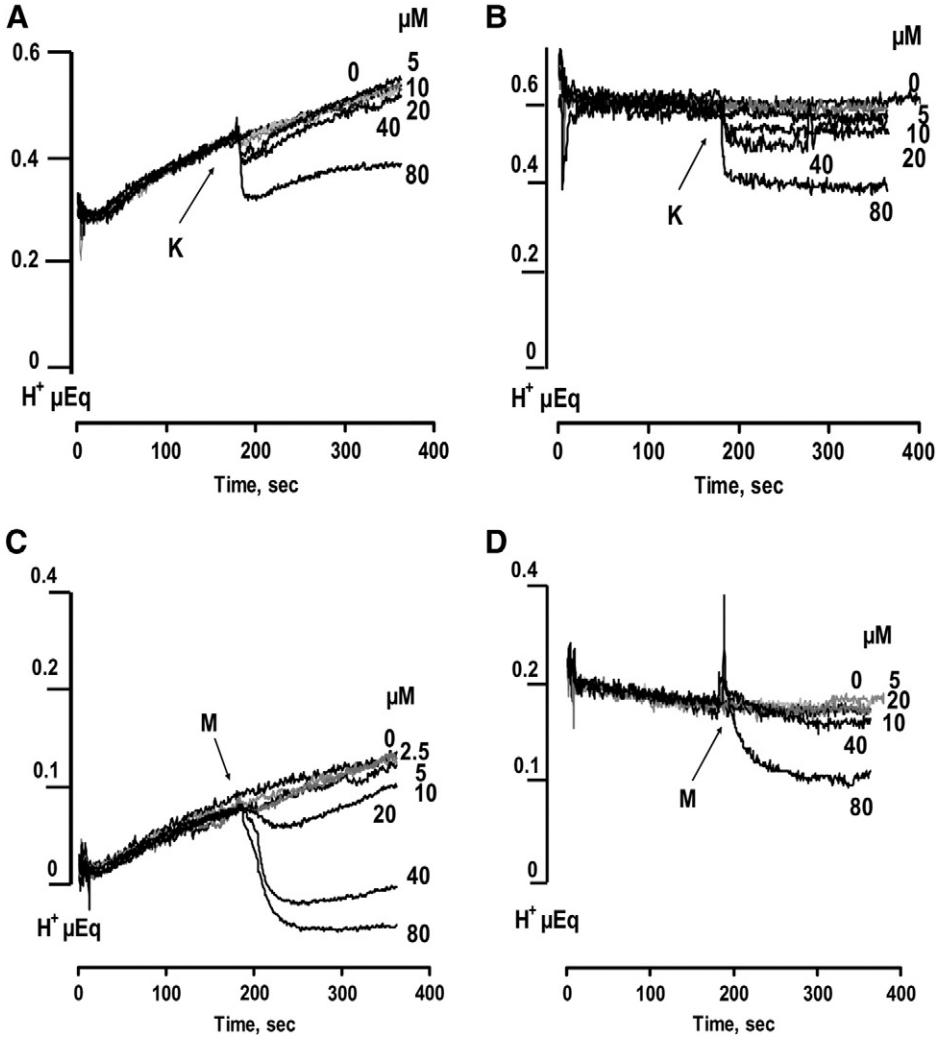

Fig. 3. Effects of ketoconazole (A and B) and miconazole (C and D) in the presence (A and C) or absence of glucose (B and D) on the H^+ concentration of the medium. The experiment was carried out simultaneously to that of Fig. 2, but the changes were followed with a pH selective electrode. K, ketoconazole; M, miconazole.

in the absence or presence of ketoconazole or miconazole at concentrations up to 400 μM, no reduction of the nucleotide was observed (not shown). It was verified that none of the antifungals inhibited the enzyme in cell-free extracts.

3.4. The antifungals do not inhibit the uptake of Rb^+

Since after the initial efflux of K^+ detected with the electrode, particularly above 10 μM of the antifungals, no net reuptake of the

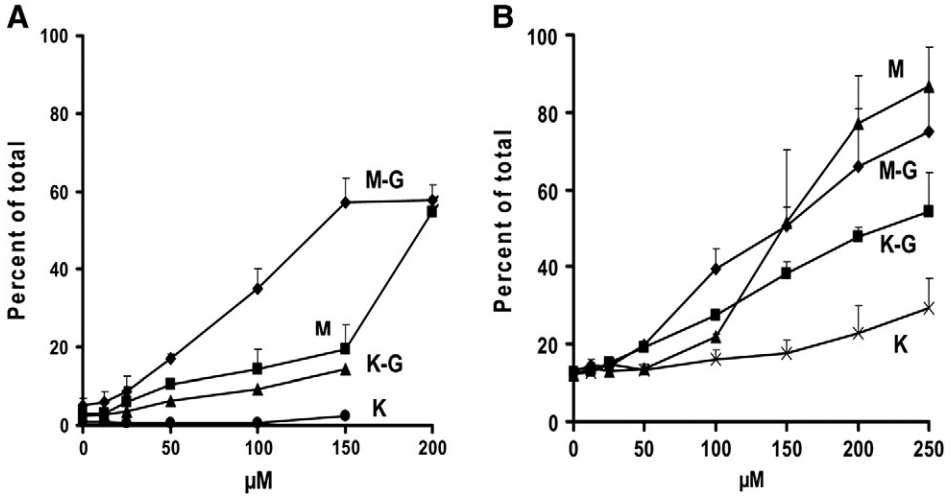

Fig. 4. Efflux from yeast of material absorbing at 260 nm (A) or phosphate (B) produced by ketoconazole (K) or miconazole (M). Incubations were made in the presence (M − G or K − G) or absence (M or K) of glucose. The material absorbing at 260 nm and the phosphate were measured in the supernatant as described under Material and methods.

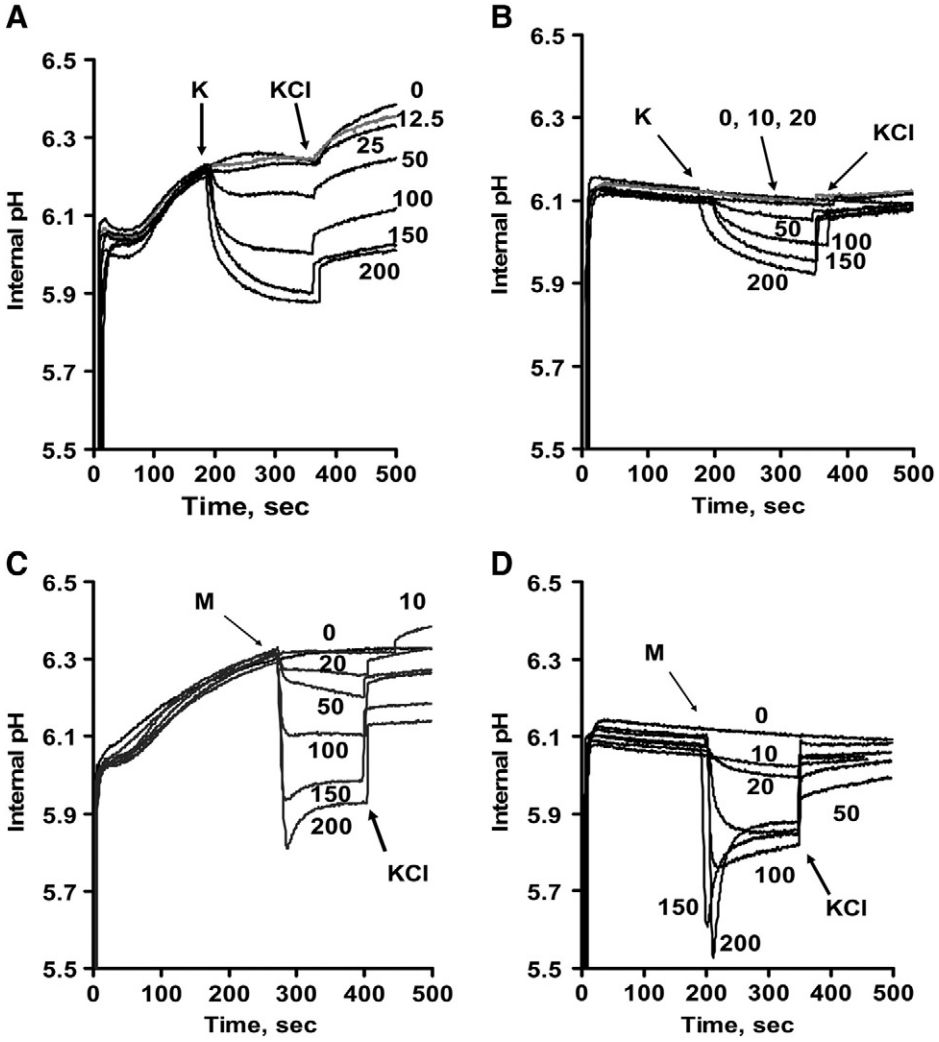

Fig. 5. Effects of ketoconazole (A and B), or miconazole (C and D) on the internal pH of yeast cells measured by the fluorescence changes of pyranine, in the presence (A and C) or absence (B and D) of glucose. The internal pH was measured by following the changes of fluorescence at 460–520 nm, as described under Material and methods. Numbers next to the tracings indicate the micromolar concentrations of ketoconazole (K) or miconazole (M) added where indicated, as well as the addition of 25 mM KCl.

cation was observed, and we tested whether this was due to inhibition of the uptake of the cation by the drugs. The uptake of $^{86}Rb^+$ was measured at concentrations of 1, 2, 5, and 10 mM of $^{86}RbCl$, against 0, 5, 10, 20, and 40 μM of each antifungal. No inhibition of the uptake was observed (not shown).

3.5. The antifungals produce a K^+/H^+ exchange

The findings shown in Figs. 2 and 3 point out a possible efflux of K^+ through two mechanisms; the main one, at low concentrations of the antifungals, is the extrusion of the cation from the cells without any change of the medium pH. However, at higher concentrations (40 μM or higher), although at a much lower degree, the addition of the antifungals produced an influx of protons, indicating a possible K^+/H^+ exchange. Hence, we decided to follow the changes produced by both drugs on the internal pH of the cells. In the absence of the drugs, glucose addition resulted in an internal cellular alkalinization that can be further increased by the addition of K^+, because the cation abates the plasma membrane potential difference (PMP) [24], which in turn accelerates the H^+-ATPase of the plasma membrane [31]. At intermediate (50 μM) or higher, but not at low concentrations, both drugs produced a decrease of the internal pH (Fig. 5A and C). The response of the internal pH to further addition of K^+ was slightly decreased; at the higher concentrations, this alkalinization presented two phases, one of them very fast, and a second one much slower than that observed in the control cells or those to which the drugs were added at low concentrations. Since the efflux of K^+ was glucose-dependent [29,30], similar experiments were carried out measuring the effect of different concentrations of the drugs on the changes of the internal pH in the absence of substrate (Fig. 5B and D). The presence of the drugs produced a smaller internal acidification, starting at around 50 μM, but similarly to K^+ efflux, this was more clearly observed at higher concentrations. Besides, further addition of K^+ produced a similar biphasic alkalinization, rapid at first, as observed in the presence of glucose. Miconazole, at equivalent concentrations (Fig. 5C and D) was more active, both in the presence and absence of glucose, and the internal acidification of the cells was much faster. Ketoconazole at the higher concentrations, in the absence of glucose (Fig. 5B), also produced a decrease of the internal pH, which could be almost reversed by further addition of KCl.

3.6. The efflux of K^+ is electrogenic

Since the drugs at low concentrations produced an efflux of K^+, apparently not in an exchange for H^+, it was tested whether the phenomenon was electrogenic. For that purpose, the PMP difference was estimated by following the fluorescence changes of the cyanine $DiSC_3(3)$ [25]. It had been reported that miconazole induces a

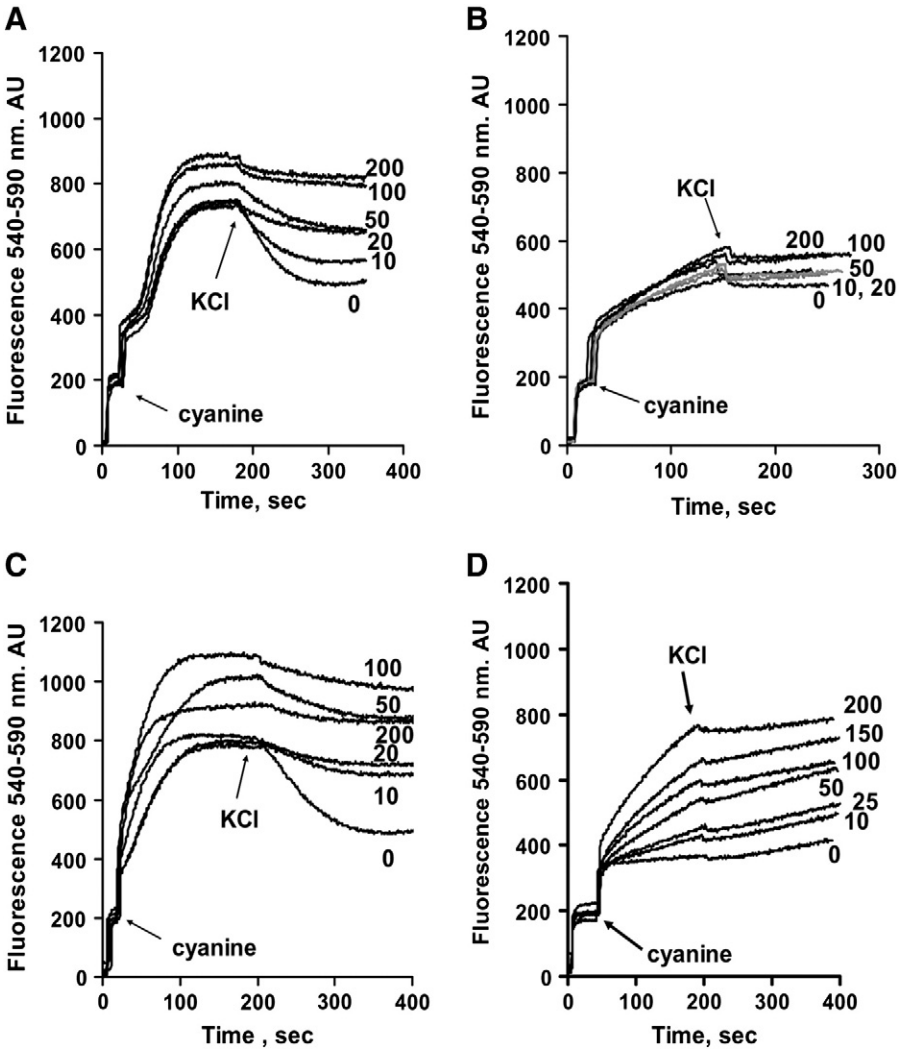

Fig. 6. Changes in the potential difference of the plasma membrane produced by ketoconazole (A and B) or miconazole (C and D), in the presence (A and C) or absence of glucose (B and D). The changes were estimated by following the fluorescence changes of the cyanine $DiSC(3)_3$. Where indicated, 50 mM KCl was added under all experimental conditions. Numbers next to the tracings indicate the micromolar concentration of ketoconazole or miconazole already present in the incubation.

decrease of this parameter in yeast [8]; however, we found that in the presence of glucose, ketoconazole (Fig. 6 A) and miconazole (Fig. 6C) produced: a) an increase in both the rate of increase and maximal values of fluorescence, indicating a faster installation and higher PMP, dependent on the concentration; b) in the absence of glucose (Fig. 6B and D), the effects of both compounds were much smaller; c) in either the presence or absence of glucose, miconazole was more active than ketoconazole at comparable concentrations; d) when K^+ was added, it produced the expected decrease of the PMP [25], which was smaller in the presence of the drugs, as compared with the control tracing.

Besides, as another indicator of the PMP, the effect of ketoconazole and miconazole on the net uptake of $DiSC_3(3)$ was measured (see 3.10.3).

Furthermore, as another indicator of the increased PMP of the cells by the drugs, we measured the uptake of $^{45}Ca^{2+}$, and found that both drugs at 100 μM produced a remarkably increased uptake of about five fold (not shown), in agreement with the report of Eilam et al. [8].

3.7. The K^+ efflux depends on the pH of the medium

At the higher concentrations of the antifungals, results agreed with a stimulation of a K^+/H^+ exchange. Because of this, another experiment was carried out in which the efflux of K^+ was determined as a function of pH. In this experiment the cells were incubated with or without glucose for 10 min at pH 4.0, 6.0, or 8.0; then, the cells were centrifuged and K^+ was measured in the supernatants with a flame photometer. Fig. 7 shows that the efflux produced by 100 μM miconazole increased as the pH was lowered, in either the absence or presence of glucose. With ketoconazole, at the same concentration, dependence of the K^+ efflux on the pH was different, being more or less similar at pH 6.0 and 8.0, but much smaller at pH 4.0.

3.8. Miconazole reduces the negative Zeta potential of the cells

Amiodarone, another cationic amphipathic molecule, has been shown to partially bind to yeast cells, increasing (to less negative values) the ζ potential [18] and also increasing the PMP of yeast cells [18,32]. Due to the general similar characteristics of the effects of miconazole, experiments were performed to measure this parameter. As shown in Fig. 8, this parameter changed to more negative values as the pH increased. Low concentrations (20 μM) of the antifungal were found to produce also an increase (to less negative values) of the ζ potential. This parameter was found to be dependent on the pH of the medium, being smaller as the pH was increased.

3.9. The effect of the antifungals on growth is reversible

It was decided to look at the effects of the antifungals on growth, after incubating similarly to the experiment of Fig. 4, and spreading a

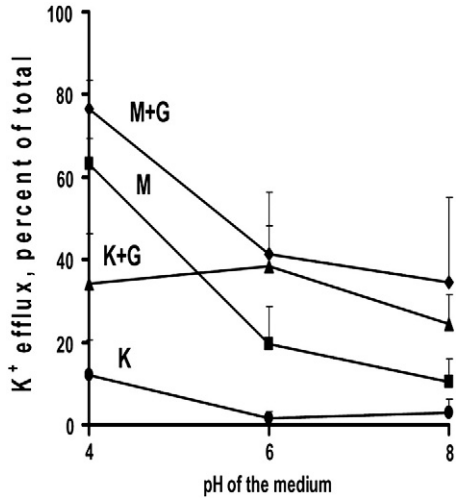

Fig. 7. Efflux of K^+ produced by the addition of 100 μM ketoconazole (K) or miconazole (M) to fasted yeast at different pH values, with (M + G or K + G) or without (K or M) glucose as substrate. Results are expressed as the percentage of internal K^+ concentration leaving the cells after incubation. The mean total concentration of K^+ in the cells was 171 mM. n = 4.

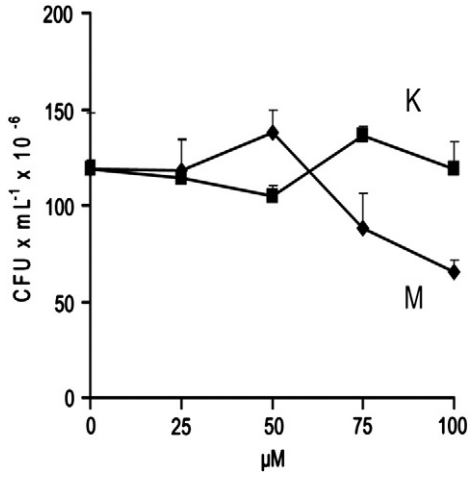

Fig. 9. Colony forming units (CFU) of cells incubated with variable concentrations of ketoconazole (K) or miconazole (M). Cells were incubated as indicated under Material and methods. A suitable dilution was spread with a known number of cells on YPD plates to detect CFU after incubation for 48 h at 30 °C, n = 3.

known amount of cells on YPD plates. This may be important, regarding the possible reversibility of the effects observed after 10 min incubations. Fig. 9 shows that, under these conditions, the number of CFU's was only slightly decreased by miconazole at concentrations much higher than those producing the efflux of K^+. These results were similar with three different wild type strains, FY833, W303-1A, and the commercial strain La Azteca.

3.10. Effects of the antifungals on mutants deficient in cation carriers

Experiments were also carried out in an attempt to define the possible K^+ carriers involved in the effects of the drugs. Particularly important is the Tok1p, identified as an outward-rectifying channel [33]; however, other known transporters were also explored: Nha1p [34], Kha1p [35,36], and Nhx1p [37,38]. Although involved in the uptake of monovalent cations [39,40], the mutant of Trk1p was also studied.

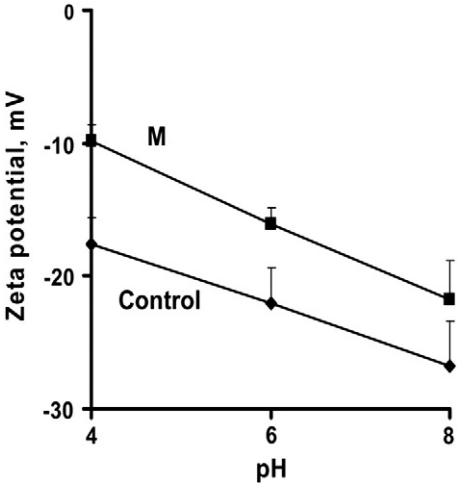

Fig. 8. Changes of the ζ potential produced by miconazole (M) in yeast cells. This parameter was measured through the electrophoretic mobility of the cells in an electric field. From that, the ζ potential (mV) was calculated as described under Material and methods.

3.10.1. Potassium content of starved and non-starved cells

First, it was considered necessary to measure the internal content of the different strains used, both with and without starvation for 18 h, after growing for 24 h (Table 3). In non-starved cells, the K^+ content of the cells was not very different among the strains, except for a tendency of the *Δkha1–Δnha1* and *Δnhx1* mutants to a higher K^+ accumulation, as compared to the parental strain FY833, and both the W303-1A and its *Δtok1* mutant (TOW), as well as the Azteca strain, showed a lower accumulation of the cation. In fasted cells, the *Δtrk1*, but not the *Δtrk2* mutant, was found to contain a lower K^+ concentration, as expected because it was starved in the absence of the cation and due to its inability to reaccumulate it during starvation.

3.10.2. Effects of the azoles on K^+ efflux in the mutants

The effects on the mutants were determined with 50 μM ketoconazole. In non-starved cells, results presented in Table 4, comparing each mutant with its corresponding parental strain can be summarized as follows: Contrary to what was expected, in the non-fasted *Δkha1*, but not in the *Δnha1* mutant, more, not less, efflux was observed. In addition, as expected, a much larger efflux was produced in both *Δtrk1* mutants, but not in the single *Δtrk2* mutant, since the former mutation results in the incapacity to reuptake the extruded monovalent cation.

Experiments were also performed with starved cells, with 50 μM ketoconazole (Table 5). Contrary to what was found in non-starved cells, with these cells, a significantly lower efflux of K^+ was found to be produced in the *Δkha1* and *Δnha1*, and the double mutant, as well as in the *Δnhx1*. As expected, a much higher efflux was also observed in the *Δtrk1* mutants, single or combined with the *Δtrk2* deletion. In contrast to what we expected, the *Δtok1* mutant did not show any difference as compared to its W303-1A parental strain.

3.10.3. Effects on the apparent PMP

As another indicator of the increased PMP produced by the drugs, their effect on the actual uptake of the cyanine $DiSC_3(3)$ was measured. The experiments were performed in the presence of glucose to energize the cells. To avoid cyanine accumulation by the mitochondria, 10 μM CCCP was added, as well as 10 μM $BaCl_2$ to prevent its binding to the surface of the cells. From the ratio of the internal/external concentration of the cyanine and the Nernst equation, a value for the PMP was calculated (see Material and

Table 3
Potassium content of the different wild type and their corresponding mutant strains after growth, or after growth and fasting for 18 h.

	Internal K^+, mM; mean ± S.D.	
	Non fasted	Fasted
Azteca	248 ± 14	171 ± 18
FY833	276 ± 4	181 ± 13
Δkha1	265 ± 22	180 ± 7
Δnha1	273 ± 21	179 ± 18
Δkha1- Δnha1	295 ± 20	183 ± 1
Δnhx1	302 ± 19	172 ± 8
Δtrk1	273 ± 11	157 ± 9
Δtrk2	287 ± 20	172 ± 10
W303-1A	248 ± 12	138 ± 8
TOW (*Δtok1*)	249 ± 13	137 ± 3

After growth for 24 h, the cells were collected by centrifugation, washed, and either used immediately or after aeration in water for 18 h. K^+ was measured as indicated in Materials and methods (n = 5).

methods). We found that, in fact, miconazole produced a higher accumulation of $DiSC_3(3)$, and of course, a higher value for the calculated PMP; in order to simplify, only experiments with 50 μM ketoconazole are presented. Results with the different mutants are compared to those with the FY833 and W303-1A used as parental strains of mutants of several transporters involved in the movements of K^+ (Table 6, non-fasted cells; Table 7, fasted cells). In all cases, even in those with a low significance (high p value), due to some dispersion of the data, the addition of the drug was found to produce a significant increase of the uptake of the cyanine, and, consequently, of the calculated PMP; these cases are indicated in the tables by a double asterisk.

3.10.4. Inhibition of respiration by miconazole

Miconazole inhibits respiration of isolated mitochondria, reportedly due to an oligomycin-like effect on the ATPase [6,7], similarly to other organic, cationic, and amphipathic molecules capable of entering the mitochondria and inhibiting respiration [41]. Results in Fig. 10 show that miconazole produced an inhibition of respiration only at concentrations of 150 μM or higher. Considering the effects reported by Portillo and Gancedo [6,7], of an oligomycin-like effect of miconazole, the effect of the uncoupler CCCP, which stimulated respiration, was tested; once respiration was inhibited by the drugs, no reversal of the inhibition was observed with the uncoupler.

Table 4
Efflux of K^+ produced by 50 μM ketoconazole in wild type and mutant strains of *S. cerevisiae*. Non-fasted cells.

Strain	Mean efflux, mM (percent of control ± S.D.)	n	Significance (p)
FY	19.32 (7.0 ± 2.3)	7	
Δkha1	30.74 (11.6 ± 4.5)	13	<0.01
Δnha1	60.95 (23.4 ± 6.8)	9	<0.01
Δkha1- Δnha1	46.9 (15.9 ± 8.5)	3	0.21
Δnhx1	60.1 (19.9 ± 5.4)	8	<0.01
Δtrk1	168 (61.8 ± 16.0)	3	0.026
Δtrk2	38.7 (13.5 ± 4.0)	3	0.090
Δtrk1–Δtrk2	166.8 (61.8 ± 8.2)	3	<0.01
W303-1A	43.6 (17.6 ± 5.3)	3	
TOW (*Δtok1*)	56.5 (22.7 ± 1.5)	3	0.10

After growth for 24 h, the cells were collected by centrifugation, washed, and used within few hours. Incubation was performed either in the presence or absence of 50 μM ketoconazole. After incubation, the cells were centrifuged, and K^+ was measured in the supernatant by flame photometry (n as indicated). The significance level was determined by the Student's t test (value of p). Results are expressed as the efflux of K^+ in mM concentration, followed in parenthesis by the percentage of the total content of each strain ± S.D.

4. Discussion

4.1. The antifungals are taken up by diffusion and binding

The transport of both antifungals is shown to occur by free diffusion, as indicated by the linearity of the uptake curves at different concentrations; this mechanism may also explain why the uptake, differently from other cationic organic molecules [29], did not depend on the presence of glucose. *S. cerevisiae* was used because this yeast has similar drug sensitivity and extrusion properties to *C. albicans*, a common human pathogen [15]. Our results with ketoconazole confirm the uptake mechanism reported for *C. albicans* [16]. It is worthwhile mentioning that at all concentrations the uptake of ketoconazole and miconazole was around 75% and 90%, respectively, showing a large concentration of both drugs by the cells. Moreover, although these experiments were carried out after 10 min at 30 °C incubation, similar ones (not shown) were performed in which the uptake was studied immediately after adding the antifungals, at room temperature or in ice water, and the uptake was similar, implying a physical diffusion phenomenon because of the amphipathic and cationic nature of the molecules, with a further binding to the internal hydrophobic structures of the cell. Binding occurred also at the surface of the cells, as shown by the changes in the ζ potential (Fig. 8). These data are important, since no reports exist about the mechanism of uptake of these drugs in *S. cerevisiae* [15], and the finding of the actual concentration inside the cells should be considered regarding their concentration–effect ratio. Besides, the effects of these antifungals could be observed at concentrations lower than those attained in humans upon the therapeutic administration of the drugs. Ketoconazole has been reported to reach values of 50 μg/mL of serum, *i.e.*, around 100 μM [42]. Interestingly, it has been found recently that lipid rafts are involved in the entrance of miconazole [43]. The usually larger effects with miconazole described later may be a consequence of its higher uptake, as compared to ketoconazole.

4.2. The antifungals produce an efflux of K^+

Although the primary mode of action of ketoconazole and miconazole involves the inhibition of sterol synthesis through its action on P450 cytochromes [1–4], our results extend the findings regarding their effects on cation transport systems [9–12]. Ketoconazole and miconazole produced an efflux of K^+ at low concentrations and, at around 200 μM, they produced almost depletion of the cation from the cells. At low concentrations, this effect depended on the presence of glucose, as it has been found for other agents that produce K^+ efflux in yeast, such as streptomycin [44], ethidium bromide [45], and Tb^{3+} [46]. This partial dependency on glucose as an energy source for the effect, and not of the uptake, may imply that the K^+ efflux system is activated by some intermediary of metabolism or the energy state of the cell. Miconazole is somewhat different in this respect, since its glucose requirement to produce the efflux of K^+ and other changes was less marked. It is possible that both antifungals produce the K^+ efflux by acting on different transporters, or that their effect is similar, but influenced by their structure, aside from the imidazolic moiety, which seems to be the active portion. The lower effect of ketoconazole in the K^+ efflux may be explained because of its structure, and it appears to be less hydrophobic, due to the presence of polar groups in its non-polar tail. Otherwise, their pK's are similar, and the pH dependency of the effects observed do not appear to be due to protonation differences of the molecules, as previously found by Beggs [47]. It is also worth mentioning that the effects of these drugs are fungistatic, at least regarding lethality under our conditions, as shown by the fact that, after the incubation, the number of CFU's did not show a large decrease. After incubation with the drugs, the cells recovered their viability when spread on YPD in their absence. It has to be stressed that the K^+ efflux produced by the fungistatics is

Table 5
Efflux of K^+ produced by 50 μM ketoconazole in wild type and mutant strains of *S. cerevisiae*. Fasted cells.

Strain	Mean efflux, mM (percent of control ± s. d.)	n	Significance (p)
FY	35.2 (19.5 ± 3.9)	6	
Δkha1	11.9 (6.6 ± 3.3)	16	≪0.01
Δnha1	15.2 (8.5 ± 5.0)	9	≪0.01
Δkha1–Δnha1	21.4 (11.7 ± 3.6)	3	≪0.01
Δnhx1	21.1 (12.3 ± 3.3)	9	≪0.01
Δtrk1	103.3 (65.8 ± 2.9)	2	≪0.01
Δtrk2	30.7 (17.9 ± 6.8)	2	0.80
Δtrk1–Δtrk2	118.1 (69.5 ± 5.9)	2	0.03
W303-1A	38.2 (27.7 ± 14.5)	3	
TOW (*Δtok1*)	30.0 (21.9 ± 10.0)	3	0.60

After growth for 24 h, the cells were collected by centrifugation, washed, and used after aeration in water for 18 h (see Materials and methods). Incubation was performed either in the presence or absence of 50 μM ketoconazole. After incubation, the cells were centrifuged, and K^+ was measured in the supernatant by flame photometry (n as indicated). The significance level was determined by the Student's t test (value of p). Results are expressed as the efflux of K^+ in mM concentration, followed in parenthesis by the percentage of the total content of each strain ± S.D.

partially compensated by a reuptake of the cation, as indicated by the results obtained with the *Δtrk1* mutants; in which, being unable to transport it inside, the efflux was found remarkably higher than in other strains or mutants.

4.3. The antifungals do not produce disruption of the cells, but the secondary efflux of anions and the influx of H^+

It appeared possible that the effects were due to the disruption of the cells, as indicated by the efflux of material absorbing at 260 nm and of phosphate in the supernatant obtained after incubating the cells with the drugs and centrifuging. However, although these materials appear in the supernatant, their proportion of the total content of the cells, compared to K^+ efflux, was smaller. Nucleotides, which are the main material absorbing at 260 nm, as well as phosphate, are of anionic nature, and may, at least partly, be accompanying potassium in its efflux from the cell through anionic transporters. It is understandable that the efflux of phosphate was larger than that of nucleotides, because the former is the most abundant anion of the cells, apparently forced to accompany K^+. The efflux of K^+ produced by Tb^{3+} is accompanied by the efflux of phosphate [37]. It is also interesting that even in the absence of the antifungals, a small efflux of material absorbing at 260 nm was observed that depended on the presence of glucose. This finding is not surprising: ketoacids [48] and succinate [49] have been found to be extruded from yeast cells. Moreover, the internal/external

Table 6
Effects of 50 μM ketoconazole on the PMP of the *Saccharomyces cerevisiae* strains and mutants of different K^+ carriers. Non-fasted cells.

	Calculated PMP, mV	p, *vs.* FY	p, *vs.* control*
	Means ± S.D.		
FY	−173.6 ± 4.9		
FY K 50	−197.7 ± 9.1		0.004
*Δkha1**	−184.8 ± 5.9	0.068	
Δkha1 K 50	−199.9 ± 8.6		0.028
Δnha1	−180.6 ± 3.4	0.123	
Δnha1 K 50	−196.8 ± 6.6		0.008
Δkha1–Δnha1	−170.3 ± 4.0	0.031	
Δkha1–Δnha1 K 50	−194.0 ± 4.1		0.002
Δnhx1	−172.2 ± 1.5	0.672	
Δnhx1 K 50	−190.7 ± 2.7		0.002
*Δtrk1**	−183.7 ± 4.4	0.051	
Δtrk1 K 50	−208.3 ± 9.7		0.035
Δtrk2	−166.0 ± 10.5	0.341	
Δtrk2 K 50	−188.8 ± 10.1		0.054
*Δtrk1–Δtrk2**	−194.5 ± 4.9	0.057	
Δtrk1–Δtrk2 K 50	−211.8 ± 7.7		0.236
W303-1A	−176.6 ± 5.8	0.528	
W303-1A K 50	−184.0 ± 4.2		0.144
TOW (*Δtok1*)	−178.3 ± 6.5	0.030	
TOW (*Δtok1*) K 50	−185.5 ± 6.1		0.211

Non-starved cells (50 mg, wet weight) were incubated as indicated under Materials and methods. The supernatant, after the addition of 1.0% sodium dodecyl sulfate, was measured by its fluorescence at 540-590 nm. From the amount in the supernatant and the total added, the internal concentration of the cyanine was obtained. Using the Nernst equation, the PMP was calculated. K 50, in the presence of 50 μM of ketoconazole. Values are given as the means of 6 or 7 experiments ± S.D.

* Experiments in which although significance was not observed by the p value, in all of them, a higher value for the PMP was observed, as compared to the reference strain (control).

Table 7
Effects of 50 μM ketoconazole on the PMP of the *Saccharomyces cerevisiae* strains and mutants of different K^+ carriers. Fasted cells.

Strain	Calculated PMP, mV	p, *vs.* FY	p, *vs.* control*
	Mean ± S.D.		
FY	−181.4 ± 8.0		
FY K 50*	−194.1 ± 10.4		0.106
*Δkha1**	−199.1 ± 11.5	0.130	
Δkha1 K 50	−203.6 ± 7.8		0.290
Δnha1	−183.2 ± 7.6	0.764	
Δnha1 K 50	−198.3 ± 6.3		0.023
Δkha1–Δnha1	−184.3 ± 7.8	0.626	
Δkha1–Δnha1 K 50	−197.7 ± 4.2		0.033
Δnhx1	−183.8 ± 7.1	0.667	
Δnhx1 K 50	−195.2 ± 6.0		0.051
*Δtrk1**	−199.6 ± 8.8	0.023	
Δtrk1 K 50	−208.7 ± 7.5		0.165
Δtrk2	−186.3 ± 8.7	0.890	
Δtrk2 K 50	−197.5 ± 8.8		0.099
*Δtrk1–Δtrk2**	−203.2 ± 8.3	0.075	
Δtrk1–Δtrk2 K 50	−207.3 ± 9.4		0.277
W303-1A	−194.2 ± 6.9	0.053	
W303-1A K 50	−201.0 ± 5.6		0.175
TOW (*Δtok1*)	−192.3 ± 6.5	0.705	
TOW (*Δtok1*) K 50	−205.3 ± 2.6		0.021

The experiment was performed as for Table 4, but cells grown for 24 h and then starved for 16 h were used.

* Experiments in which although significance was not observed by the p value, in all of them, a higher value for the PMP was observed, as compared to the reference strain. K 50, in the presence of 50 μM of ketoconazole. n = 4.

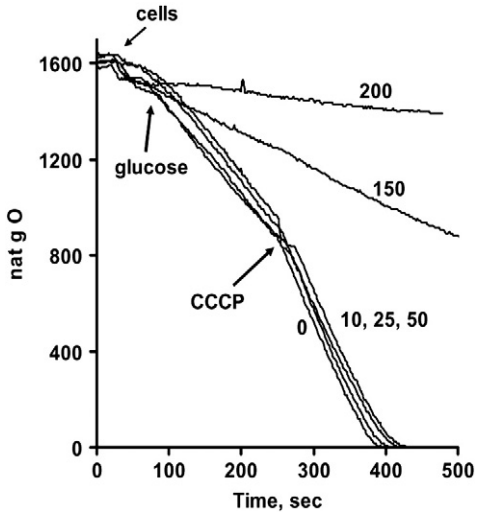

Fig. 10. Oxygen consumption by yeast cells and the effects of miconazole and CCCP. Numbers next to the tracings indicate the micromolar concentrations of miconazole already present during the experiment. Where indicated, 10 μM of CCCP was added.

concentration ratio of bicarbonate depends on the presence of K^+ [50,51]. In addition, the dependence on glucose of the nucleotide efflux in the absence of the antifungals may indicate that the efflux is not due to the disruption of the cells, but to another mechanism, or due to other anions. Against the simple disruption of the cell membrane are the following facts: a) the changes of the internal pH of the cells could not be expected to recover by the addition of K^+ after the drugs; b) in the presence of the antifungals, the PMP was not only not decreased, but rather increased: c) no activity of glucose-6-phosphate dehydrogenase was detected in the presence of the antifungals; d) it was found that both antifungals did not produce any changes in the uptake of $^{86}Rb^+$.

Besides the K^+ efflux, the measurement of proton pumping by the cells into the incubation medium showed that immediately after their addition, and starting at around 40 μM, both antifungals produced a pH increase of the medium, indicating an influx of protons, partly compensated by the proton pumping by the plasma membrane H^+-ATPase. This proton influx was absent at the lower concentrations, much smaller than the efflux of K^+, and larger at the intermediate or higher concentrations tested. At the highest concentrations of the drugs, the efflux of K^+ was close to 20 μEq, while the maximum influx of protons with miconazole was around one-hundredth (0.2 μEq). This means that the efflux of K^+ is the primary effect, and only at the higher drug concentrations, at a much lower proportion, an influx of protons is produced. Both drugs may be activating a K^+/H^+ exchange system in the cell. These antiporters have been found widely distributed among living cells [52,53], and, in yeast, three of them have been studied: Nha1p [34], Kha1p [35,36], and Nhx1p [37,38].

This K^+ efflux was more marked in the presence of glucose, and is partly balanced by an uptake, as derived from the fact that, even after the significant internal acidification of the cells observed at the higher concentrations of the drugs, the addition of K^+ produced a rapid alkalinization of the cell interior, followed by another slower but appreciable alkalinization rate (Fig. 5). This assumption is also reinforced by the fact that the efflux was much higher in the *Δtrk1* mutants, lacking the main K^+ influx transporter.

Indicating also the possible role of a K^+/H^+ exchanger are the results of K^+ efflux (Fig. 7), which increased with miconazole as the external pH was lowered. This is also supported by the lower effect of ketoconazole on mutants of all three antiporters. It is however difficult to explain a) why was this difference only observed in starved cells, and b) why was this efflux less sensitive to the external pH with ketoconazole; the behavior of this compound appears to be more complex. Differences were also observed in the rate of internal acidification, which was slower with ketoconazole, as well as in effectiveness, glucose dependence, and effects on the membrane potential. To this respect, the degree of protonation of the drugs could also be involved, as proposed by Beggs [47].

4.4. A net K^+ outward system

Our findings show that the drugs produce a large net efflux of the cation in which there is a role of K^+/H^+ exchangers; their main and probably primary effect is to stimulate a net K^+ efflux system. The first possibility would be a channel, such as those described in yeast [33], resulting in the hyperpolarization of the cells as shown by the increased fluorescence and uptake of $DiSC_3(3)$, as well as the increased uptake of $^{45}Ca^{2+}$ (not shown), also found by Eilam et al. [8]. The efflux of K^+ through such a system would be partially modulated by the energy status of the cell, as indicated by the partial requirement of glucose to produce the efflux of K^+, and that when the substrate was omitted smaller changes in the fluorescence of the cyanine, indicating a lower increase of the PMP, were observed with ketoconazole (Fig. 6B), whereas with miconazole, which showed a less strict glucose requirement to produce the efflux of K^+, a higher increase of the fluorescence signal was detected also in the absence of glucose (Fig. 6D). In the control cells, the addition of K^+ produced a clear decrease of the PMP, but this decrement was slower in the presence of the drugs. Together with the reduced rate of K^+ uptake after the efflux (Fig. 2), this might be an indication that both ketoconazole and miconazole are also inhibitors of the uptake of the cation; however, the measurement of the uptake of $^{86}Rb^+$ failed to show an inhibition at concentrations of up to 40 μM of the antifungals. It has been reported [8] that miconazole produces K^+ efflux from the cells, part of which is concentrated in the vacuole, but in contrast to our results, the authors found a decreased PMP. However, their method to measure the membrane potential implied the incubation of the cells with the membrane potential indicator for 90 min, which may be a method less reliable to measure the changes right after the addition of the drug, particularly because of the profound changes on the ionic composition produced by these drugs. With both methods used by us, a clear increase of the PMP, most probably resulting from the efflux of K^+, was observed.

4.5. Mechanism proposed

Taken together, the results could be visualized as follows, as already proposed for amiodarone [18] and supported by similar findings of Maresova et al. [32]: the drugs appear to bind to the surface of the cell by virtue of both their amphipathic and cationic nature, and by decreasing (turning less negative) the surface charge of the membrane, and decreasing the actual concentration of K^+ available for its transport into the cell, according to a given PMP; if this concentration decreases, an efflux of the cation is possible. Another possibility is that by the same change of the surface charge, the membrane structure becomes disrupted by the interaction of the antifungals with lipid rafts [43] and may contribute to this effect or to the functioning of the cation transporters. As a consequence, both antifungals at low concentrations can stimulate the efflux of K^+, but ketoconazole in a partially energy-dependent way. At higher concentrations, the higher PMP generated at the level of the plasma membrane can stimulate or drive the uptake of H^+, which might imply the participation of a K^+/H^+ exchanger. The increased negative PMP could also drive the efflux of anions out of the cell, as well as the influx of H^+. Results indicate that after producing the K^+ efflux, particularly at the lower or intermediate concentrations of the antifungals, the cells appear to maintain a cycling of K^+, in which a balance towards the efflux is established against an unaltered influx through the K^+ uptake systems.

Probably, the main finding from the experiments with the mutants is that the mutation of Tok1p eliminated the most plausible candidate for a straightforward mechanism; its absence did not result in any difference in the effect of the azoles as compared to the wild type strain; differences were found in neither the K^+ efflux nor the changes produced by the drugs on the PMP. However, although different results were obtained in the non-starved and starved cells, regarding the mutation of the Kha1p and Nha1p, in the starved cells, results indicate the participation of these H^+/K^+ exchangers. From these data, it appears probable that ketoconazole and miconazole may affect the function of these transporters, but since they do not alter the uptake of K^+, as demonstrated by the $^{86}Rb^+$ uptake experiments, a large part of the effects of the drugs may be compensated by the combined action of the H^+-ATPase and the influx of the monovalent cation through Trk1p. This may be supported by the fact that in the mutants of Trk1p, without the main uptake system for monovalent cations, the efflux produced by both imidazolic compounds was remarkably larger.

4.6. The effects on respiration

Respiration was affected by miconazole and ketoconazole, but only at concentrations above 150 μM. This is probably related to the cationic nature of the imidazolic portion of the molecule. Amidine groups, originally found to interact with sodium channels [54], and

other compounds, particularly octylguanidine, were found to inhibit mitochondrial respiration [55]. A similar effect was described for ethidium bromide in mitochondria [41]. This inhibition also means that these antifungals can reach this organelle, although its mechanism would require further studies with isolated mitochondria. In our experiments, the inhibition of respiration was not reversed by CCCP, neither with ketoconazole nor with miconazole, as would be expected from the data of Portillo and Gancedo [6,7]. In any case, the effects on respiration in intact cells were observed only at high concentrations of the antifungals.

4.7. Final remarks

It is important to point out that the efflux of K^+ could be observed with ketoconazole and miconazole, similar antifungals, in agreement with other authors [14], but not with two triazolic antifungals, fluconazole and itraconazole, which may indicate that the active group of the molecule in the interaction with the carriers is the imidazole moiety of the molecule.

Our incapability to fully explain the results may be due to our still incomplete knowledge of the fine mechanisms and possible interactions of the different transporters involved in the homeostasis of monovalent cations in yeast.

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