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Efflux of Potassium Ions from Cells and Spheroplasts of *Saccharomyces cerevisiae* Yeast Treated with Silver and Copper Ions

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Abstract—Silver ions induce the efflux of potassium from cells of the yeast *Saccharomyces cerevisiae* but have no such effect on spheroplasts. Copper ions and the natural fungicide 2-O-3-hydroxyhexanoyl-β-D-glucopyranosyl-(1→4)-(6-O-acetyl-β-D-glucopyranosyl-(1→16)-2,15,16-trihydroxyhexadecanoic acid) induce the efflux of potassium ions from both cells and spheroplasts of *S. cerevisiae*. Silver and copper ions inhibit the activity of the plasma membrane H^+-ATPase during the treatment of both cells and spheroplasts. It is supposed that the inability of silver ions to stimulate potassium efflux from spheroplasts results from damage to some components of K^+ transport systems during preparation of spheroplasts.

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Silver and copper ions at relatively high concentrations are toxic for microorganisms and are thus widely used as bactericides and fungicides. The mechanism of their effect on bacterial cells has been studied for a long time [1-9], yet it cannot be considered as fully established. The primary targets of these inorganic cations are thought to be various transport systems of the plasma membrane, including transport systems of phosphate [5, 6] and K^+ [7]. Copper ions are cofactors of some enzymes; therefore, yeast cells possess transport systems that provide their uptake and efflux [1-4]. Special transport systems for Ag^+ are still unknown. In this work, we have compared the effects of Ag^+ and Cu^{2+} and the natural fungicide 2-O-3-hydroxyhexanoyl-β-D-glucopyranosyl-(1→4)-(6-O-acetyl-β-D-glucopyranosyl-(1→16)-2,15,16-trihydroxyhexadecanoic acid) (cellobiose lipid, CL) on K^+ efflux from cells and spheroplasts of the yeast *Saccharomyces cerevisiae*, as well as on the activity of H^+-ATPase, the main enzyme responsible for energization of the plasma membrane in these microorganisms.

Abbreviations: CL) cellobiose lipid 2-O-3-hydroxyhexanoyl-β-D-glucopyranosyl-(1→4)-(6-O-acetyl-β-D-glucopyranosyl-(1→16)-2,15,16-trihydroxyhexadecanoic acid); EKP) electrokinetic potential.

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MATERIALS AND METHODS

The yeast *S. cerevisiae* VKM Y-1173 was cultivated at 29°C in Reader medium [10] with 2% glucose to the logarithmic growth phase. The yeast cells were rinsed with distilled water, centrifuged at 5000*g* for 15 min, and resuspended in water. Then they were used for measuring K^+ efflux and H^+-ATPase activity and for isolation of spheroplasts. Spheroplasts were obtained by treating cells with lyophilized gastric juice of edible snails in 5% citric buffer with 0.8 M mannitol, pH 6.5. After the treatment, they were washed twice with the same buffer.

The efflux of K^+ was measured upon addition of 20 μM $AgNO_3$ and 100 μM $CuSO_4$, as well as 0.4 mM natural fungicide CL produced by the yeast *Pseudozyma fusiformata* VKM Y-2821 (All-Russian Collection of Microorganisms). This compound was obtained from the culture liquid of the *P. fusiformata* as described earlier

[11]. The efflux of K^+ from yeast cells was registered with a K^+-selective glass electrode (Orion, USA). The measurements were performed in a 2.5-ml cell at 25°C under stirring. The medium contained 0.02 M Mes-Tris buffer, pH 7.0 (in the case of Cu^{2+} or Ag^+), or 0.01 M citrate-phosphate buffer, pH 4.0 (in the case of Ag^+ or CL). In the experiments with spheroplasts, buffer solutions contained 0.8 M mannitol. The experiments with cells were performed both in the presence and absence of this osmotic stabilizer. The final concentration of cells or spheroplasts was $(6.0\text{-}6.5)\cdot10^8$ per ml.

The charge on the surface of cells and spheroplasts was estimated by their electrophoretic mobility using a Parmoquant-2 microscope (Carl Zeiss, Germany) at 20°C. The electrokinetic potential (EKP) was calculated using the Smolukhovsky equation without taking into account the effects of surface polarization. Before measurement, cells or spheroplasts ($5\cdot10^6$ per ml) were suspended in 0.01 M citrate-phosphate buffer, pH 4.0, with 0.8 M mannitol and incubated for 15 min at 20°C. The mean EKP values and the mean square deviations were determined for 25 cells in each sample, and EKP histograms for cells and spheroplasts were plotted. The EKP values of both populations in mannitol were corrected for the viscosity of water at 20°C.

For determination of the effects of Ag^+ and Cu^{2+} on H^+-ATPase activity, the cells and spheroplasts were incubated at 30°C for 20 min with 20 μM $AgNO_3$ or $CuSO_4$, centrifuged, and resuspended in 10 ml of 0.01 M Mes-NaOH buffer, pH 6.5, with 10% sorbitol, 0.1 M KCl, and 4 mM $MgSO_4$, followed by addition of 0.5 ml of 1% Triton X-100 and freezing in liquid nitrogen. The experiments with spheroplasts were performed under the same conditions but with 0.8 M mannitol present in all solutions.

Freezing in liquid nitrogen in the presence of 1% Triton X-100 followed by thawing results in an increase in unspecific permeability of the plasma membrane and is useful for the assay of plasma membrane ATPase activity [12]. Before the assay of H^+-ATPase activity, the samples were thawed on ice, centrifuged, and resuspended in 2 ml of the same buffer without Triton X-100. (In case of spheroplasts, the buffer contained 0.8 M mannitol.) For measuring the ATPase activity, 50 μl of cell suspension was added to 0.95 ml of the same buffer containing 5 mM $MgSO_4$ and ATP and incubated for 30 min at 30°C either with or without 100 μM Na_3VO_4. Orthovanadate is a specific inhibitor of P-ATPases including H^+-ATPase of yeast plasma membrane [13, 14], and the level of vanadate-sensitive hydrolysis of ATP was used to determine its activity. After centrifugation at 13,000*g* for 3 min, 100-μl samples were taken from the supernatant and P_i was assayed using malachite green dye as described earlier [12]. The reaction was carried out on microplates for immunoenzyme analysis at 30°C for 10 min on an ELMI Sky Line shaker. Absorbance was measured at 650 nm with an Efos-9305 immunoenzyme plate photometer (Russia).

RESULTS AND DISCUSSION

Silver ions induced fast efflux of K^+ from *S. cerevisiae* cells (Fig. 1a). The total level of intracellular K^+ in the yeast was the same under treatment with 20 μM Ag^+ and under heating at 70°C for 15 min in a water bath. So, Ag^+ provides nearly complete release of K^+ from yeast cells according to the literature data [5, 6]. The effect of Ag^+ was similar at pH 4.0 and 7.0. The efflux of K^+ from cells was independent of the presence or absence of 0.8 M mannitol in the incubation medium. However, during the treatment of spheroplasts with Ag^+, efflux of K^+ was practically not observed (Fig. 1b). The treatment of cells with 20 μM $AgNO_3$ in deionized water also induced K^+ efflux. However, the treatment of spheroplasts with $AgNO_3$ in 0.8 M mannitol was ineffective.

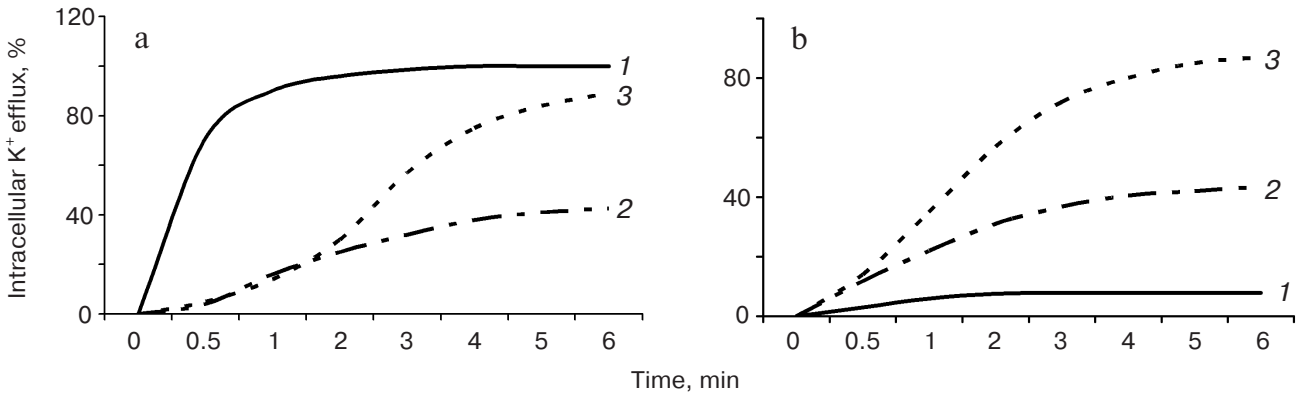

Fig. 1. Efflux of intracellular K^+ from cells (a) and spheroplasts (b) of *S. cerevisiae* during incubation in the presence of 0.8 M mannitol with 20 μM $AgNO_3$ (*1*) (pH 4.0), 100 μM $CuSO_4$ (*2*) (pH 7.0), and 0.4 mM cellobiose lipid of *Ps. fusiformata* (*3*) (pH 4.0).

Control experiments were performed with Cu^{2+}, the effect of which on yeast cells is well studied [1-9]. These ions in the concentrations used disturb the barrier function of plasma membrane and induce leakage of K^+, amino acids, and nucleotides [15]. Copper ions, in contrast to Ag^+, induce K^+ efflux from cells (both in the presence and absence of 0.8 M mannitol) and spheroplasts (in the presence of 0.8 M mannitol) with similar efficiency, but only at pH 7.0 (Fig. 1).

The effect of the natural fungicide CL inhibiting cell growth at pH 4.0 [11] on cells and spheroplasts was examined. This compound induces leakage of ATP from yeast cells, acting as a detergent that destroys the plasma membrane [11]. This glycolipid fungicide induces K^+ efflux at 10 times lower concentration than is known for the detergent SDS [16, 17]. The efflux of K^+ from cells and spheroplasts was observed at similar concentrations of this compound (Fig. 1, a and b). In the case of cells, exclusion of mannitiol from the incubation medium did not affect this efflux (not shown). Thus, only Ag^+ was ineffective in the case of spheroplasts.

The data are in agreement with the opinion that Cu^{2+} and CL [11, 15] cause K^+ efflux by destroying the barrier function of the plasma membrane. In this case, the presence of cell wall is not necessary.

Supposing that insensitivity of spheroplasts to Ag^+ may be due to the change in surface charge after the removal of the cell wall, we determined the EKP values of cells and spheroplasts. These values characterize the negative charge on the cell/spheroplast surface. The mean values of EKP were -12.88 ± 0.40 and -7.52 ± 0.76 mV for cells and spheroplasts, respectively. For spheroplasts, increase in heterogeneity of EKP values is typical as compared with cells (Fig. 2). The decrease in EKP of spheroplasts is explained by the loss of negatively charged components such as polyphosphates and polysaccharides on elimination of the cell envelope [18].

The decrease in surface charge may be one of the factors causing the absence of K^+ efflux on the treatment of spheroplasts by Ag^+. So, we compared the effect of Ag^+ and Cu^{2+} on an integral protein of plasma membrane, H^+-ATPase, under treatment of cells and spheroplasts with these ions.

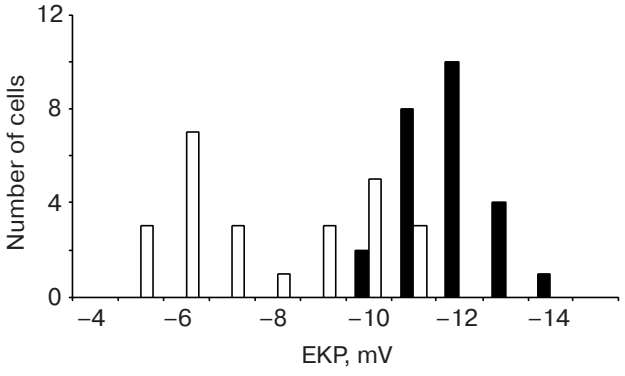

Fig. 2. EKP histograms of cells (black columns) and spheroplasts of *S. cerevisiae* (white columns).

Effect of Ag^+ and Cu^{2+} on vanadate-sensitive ATPase activity *in situ* upon treatment of *S. cerevisiae* cells and spheroplasts

Treatment	*In situ* ATPase activity (%)	
	cells	spheroplasts
Control	100*	100**
20 μM $AgNO_3$	63	57
20 μM $CuSO_4$	63	33

* 60 mU per g wet weight.
** 36 mU per g wet weight of initial cells.

The ATPase activity was assayed *in situ* after cell permeabilization as described in section "Materials and Methods". It turned out that both cations inhibited H^+-ATPase to the same extent (see table). Thus, the changes in the surface charge on preparation of spheroplasts had no effect on the interaction of Ag^+ and H^+-ATPase.

The inability of Ag^+ to induce K^+ efflux from spheroplasts is probably explained by changes in transport systems involved in K^+ homeostasis after elimination of the cell wall. The K^+ homeostasis system of yeast cells involves proteins that provide both uptake and efflux [19, 20]. We suggest that, in contrast to Cu^{2+} or CL, which induce K^+ release through destruction of the barrier function of plasma membrane, Ag^+ affects proteins responsible for K^+ efflux. During preparation of spheroplasts, these components are eliminated and no K^+ efflux is observed. Such effects, therefore, should be taken into consideration when spheroplasts are used as a model for studying the processes of transport across the yeast plasma membrane.

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