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Changes in Plasma Membrane Fluidity Lower the Sensitivity of *S. cerevisiae* to Killer Toxin K1

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ABSTRACT. The possible correlation between plasma membrane fluidity changes induced by modified cultivation conditions and cell sensitivity to the killer toxin K1 of *Saccharomyces cerevisiae* were investigated. Cells grown under standard conditions exhibited high toxin sensitivity. Both a membrane fluidity drop and fluidity rise brought about markedly reduced sensitivity to the toxin. These results do not fit the hypothesis of physiological relevance of direct toxin–lipid interaction, suggesting that the essential event in killer toxin action is interaction with membrane protein(s) that can be negatively influenced by any changes of membrane fluidity.

Abbreviations
DPH – 1,6-diphenyl-1,3,5-hexatriene; PMF – plasma membrane fluidity; SSFA – steady-state fluorescence anisotropy

K1 killer toxin is a small extracellular protein secreted by killer strains of *Saccharomyces cerevisiae*, which can kill susceptible cells of other *S. cerevisiae* strains. The lethal effect of K1 toxin involves disruption of the electrochemical potential gradient across the plasma membrane connected with an uncontrolled leakage of K^+ ions, followed by leakage of small molecules from the cell (de la Peña *et al.* 1980, 1981). In susceptible cells, killer toxin K1 interacts first with cell wall receptors containing 1,6-β-D-glucan and is then transferred in a subsequent energy-requiring step to the plasma membrane (for review *see* Magliani *et al.* 1997). There it probably binds first to the plasma membrane receptor Kre1 protein. Its proper function is given by its C-terminus, especially by the GPI anchor located there (Breinig *et al.* 2002). Subsequent events in the action of K1 toxin on sensitive yeast cells remain unclear.

Killer toxin K1 was found to cause both frequent opening and extension of the open state of the endogenous Tok1 transmembrane channel highly selective for K^+ ions, which can lead to an increased leakage of K^+ ions from the cell (Ahmed *et al.* 1999). However, Tok1p is very probably not the main target for killer toxin because the killing effect was found to start by rapid inhibition of net proton efflux, K^+ ions being lost from the cell at later stages (de la Peña *et al.* 1980). Also, the sensitivity of *S. cerevisiae* strains with deleted *TOK1* gene to killer toxin K1 is not markedly decreased (Breinig *et al.* 2002). Moreover, K1 toxin can be incorporated into and form pores in artificial lipid membranes in the absence of any protein (Hianik *et al.* 1984; Martinac *et al.* 1990). It was also shown that killer toxin molecules are able to form *in vitro* oligomers of up to 8 molecules (Bussey 1991). These findings suggest that killer toxin, in addition to interacting with specific protein(s) located in the plasma membrane, might also interact nonspecifically with the membrane lipid matrix, *e.g.*, by forming *de novo* ion channels. Thus, the impact of killer toxin on sensitive yeast cells should be influenced by the composition and physical properties of the plasma membrane, in particular by PMF.

S. cerevisiae is a highly flexible microorganism able to adapt to various cultivation conditions. This ability is often associated with changes in the lipid composition of the plasma membrane and consequently with its fluidity. Changing the relative amount and composition of plasma membrane lipids allows cells to maintain suitable physical membrane properties under unfavorable conditions (Alexandre *et al.* 1996). Data

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on how variations of cultivation conditions might influence cell susceptibility to membrane damaging toxic agents are scarce.

Using survival assays and measurement of steady-state fluorescence anisotropy and time-resolved fluorescence depolarization of the DPH probe, we examined (*1*) whether changes in PMF caused by cultivation of *S. cerevisiae* cells under various conditions can affect the sensitivity of the cells to killer toxin K1 and (*2*) whether there is any unambiguous correlation between fluidity changes and killer toxin sensitivity that would point to killer toxin forming *de novo* pores in the plasma membrane lipid matrix.

MATERIAL AND METHODS

Yeast strains, media and growth. The sensitive laboratory strain *S. cerevisiae* GRF18 (MATα *his3-11,15 leu2-3,112*) and distillery strain *S. cerevisiae* LH 60 (diploid, prototroph) were purchased from the *Collection of Yeasts of the Department of Genetics and Microbiology* (DMUP; *Faculty of Science, Charles University*, Prague). Construction of K1 killer toxin producing strain X3 was described by Vondrejs *et al.* (1983). Sensitive *S. cerevisiae* strains were cultivated for 14–16 h to the exponential growth phase (cell concentration 5–30/nL, *i.e.* 5×10^6–3×10^7 cells per mL) at standard temperature 28 °C (Flegelová *et al.* 2002) or at 20 or 38 °C; in other experiments the YEPG medium (in g/L: bactopeptone 10, glucose 10, yeast autolysate 5) was supplemented with 4, 6 or 8 % ethanol and the cells were incubated under shaking for at least 6 h at 28 °C in flasks with rubber stoppers to avoid ethanol evaporation. Cells of the distillery strain LH60 were used in experiments with ethanol since they tolerate its higher amounts in the cultivation medium than the laboratory strain GRF18, which does not grow in a medium with 8 % ethanol. LH60 differed only slightly from GRF18 in the sensitivity to killer toxin K1. Cells enriched in oligo-unsaturated linoleic (18:2) or linolenic (18:3) acids were obtained according to Howlet and Avery (1997) and Krasowska *et al.* (2002) by growth in 18:2- or 18:3-supplemented YEPG medium, respectively, with a modification (the nonionic surfactant Igepal was used instead of Tergitol for fatty acid solubilization). After a 14-h cultivation with fatty acids at 28 °C, cells growing exponentially to the above concentration were harvested. Cells of all variants were resuspended in the JG medium (pH 4.7) (Flegelová *et al.* 2002) or 10 mmol/L McIlvaine citrate–phosphate buffer, and their sensitivity to killer toxin K1 and membrane fluidity were measured at 22 °C as described below.

Exposure to killer toxin – survival assays. Cultivation of the killer toxin producing strain X3 proceeded in JG medium (pH 4.7). Crude killer toxin K1 was prepared according to Flegelová *et al.* (2002). The survival assays were performed in the same JG medium as follows: 5×10^6 exponentially growing cells were collected by centrifugation and transferred into JG medium containing crude K1 toxin appropriately diluted by the same medium (the same crude toxin was used for all experiments and its concentration was expressed as percentage of crude toxin solution) or into JG medium without killer toxin (as control) and glucose was added to a final concentration of 2 % to give a volume of 1 mL. Incubation proceeded for 2 h at 22 °C under gentle shaking. Viable cells were counted after plating on YEPG agar followed by 2-d incubation at 28 °C. Number of colonies in samples incubated without killer toxin were considered as 100 %. The experiments were done in triplicate.

Plasma membrane fluidity was determined by measuring the steady-state fluorescence anisotropy (SSFA) of the lipophilic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH), which is considered to reflect the rotational mobility and hence the fluidity of the lipid environment of the probe when incorporated into the plasma membrane of living cells (Lakowicz 1999; Shinitzky and Barenholz 1978). Cells were grown and harvested as described above, washed twice in citrate–phosphate buffer (10 mmol/L, pH 4.7) and then resuspended in the same buffer to $A_{550} = 0.2$. Cell suspension was labeled with DPH; 1 mmol/L stock solution of DPH in acetone was added to the sample to a final concentration of 0.5 μmol/L. SSFA of DPH was measured on a FluoroMax-2 spectrofluorimeter (*Jobin-Yvon* SPEX) equipped with standard polarization accessory. DPH fluorescence was excited by a xenon lamp at 350 nm and the emission was detected at 430 nm. The sample was stirred and the sample temperature was kept constant during the measurement. SSFA was calculated as described by Lakowicz (1999) and its value was corrected for intrinsic fluorescence and light scattering from a nonlabeled sample.

Time-resolved fluorescence depolarization experiments were performed on an apparatus consisting of a laser excitation source and a time-correlated single photon-counting detection system. The signal was corrected for apparatus response function and three polarization components, *i.e.* fluorescence decay curves with emission polarizer oriented in parallel, perpendicularly and at the magic angle (54.7°), were simultaneously fitted to the experimental data (for more details *see* Heřman *et al.* 1994) according to the model of DPH fluorescence in membranes. (The fluorescence intensity of DPH in membranes can be described by

a two-exponential decay. Using a simple approximation for hindered rotor, the time-resolved anisotropy is a function of three parameters – rotational correlation time, fundamental anisotropy R_0 and a limiting value R_∞; the anisotropy decays from R_0 to R_∞ exponentially. The limiting value R_∞ is related to the order parameter describing the DPH equilibrium orientation distribution at times longer than the rotational correlation time and can also be interpreted as resulting from an energy barrier that prevents DPH rotational diffusion beyond a certain angle; Lakowicz 1999.)

RESULTS

Cells cultured in ethanol-supplemented media. In *S. cerevisiae* LH60 increasing ethanol levels brought about an increase of SSFA (Fig. 1 *above*) reflecting an ethanol-induced decrease of yeast PMF. To eliminate possible measurement artifacts, we performed time-resolved fluorescence depolarization experiments, which offer more detailed information about the fluorescence signal changes of DPH incorporated in biological membranes. Fig. 2 illustrates the adaptive membrane-fluidity drop in cells cultured in the presence of ethanol as opposed to the short-term fluidizing effect of ethanol added to the measuring buffer containing the harvested cells. The fluidizing effects of 4 or 8 % ethanol added to the measuring buffer completely offset the membrane fluidity drop caused in the cells by cultivation in the presence of the same concentration of ethanol so that the R_∞ values of samples with the same ethanol concentration in cultivation medium and measuring buffer were almost equal. Addition of 4 or 8 % ethanol to ethanol-unadapted cells in the measuring cuvette, which obviously represents a considerable shock for the cells, brought about large variations in measured values that prevented us from evaluating the exact R_∞ value.

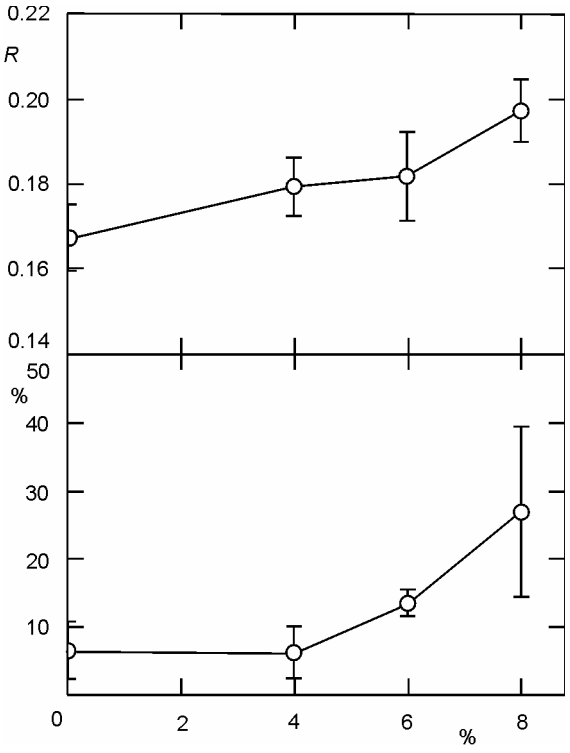

Fig. 1. Steady-state fluorescence anisotropy (*R*; *above*) and cell survival after killer toxin treatment (%; *below*) of *S. cerevisiae* LH60 precultivated in YEPG medium supplemented with ethanol (0–8 %); percentage of living cells determined as colonies growing on YEPG agar after a 2-h incubation with 1 % crude K1 killer toxin (100 % corresponds to samples incubated without K1); means ± SD ($n \geq 5$ for fluorescence data, $n = 3$ for survival data).

Simultaneously performed survival tests showed that the presence of ethanol in the cultivation medium induced concentration-dependent lowering of cell sensitivity to the fungicidal action of K1 killer toxin (Fig. 1 *below*). The presence of maximum ethanol concentration (8 %) in the cultivation medium, which brought about an 18 % increase in SSFA (from about 0.167 to 0.197), increased the survival of the yeast cells about 4-fold (from about 6 % for cells cultivated without any ethanol to 26 %).

Cells cultured in media supplemented with oligo-unsaturated fatty acids. Growth of *S. cerevisiae* GRF18 in media supplemented with linoleic (18:2) or linolenic (18:3) acid brought about a substantial (25–30 %) drop in SSFA, *i.e.* a strong increase in PMF, which was more pronounced with linolenic acid (Table I *upper part*). The presence of the non-ionic surfactant Igepal did not influence SSFA. Cells cultivated in media supplemented with these unsaturated fatty acids displayed a reduced sensitivity to killer toxin K1. (The survival assays were performed with 0.1 and 0.5 % concentrations of killer toxin K1 since the higher, 1 % toxin, used in other experiments, brought about a very low cell survival (≈1 %) in all samples, which did not allow us to observe any distinct effects of fatty acids.)

Cells cultured at different temperatures. Cultivation of cells at 20, 28 or 38 °C affected both the SSFA and the sensitivity of the strain GRF18 cells to 1 % killer toxin K1 (Table I *lower part*). A 10 °C increase above the standard value of 28 °C induced a marked increase in SSFA and a dramatic drop in the sensitivity to K1 whereas an 8 °C decrease

below the standard level evoked only a marginal decrease of DPH steady-state fluorescence anisotropy and a similarly marginal drop in the sensitivity to 1 % K1. At low concentrations of K1 (0.5 % and 0.1 %), which were again used in order to increase the differences in survival level, growth at 20 °C brought about an ≈100 % rise in survival despite the meager change in membrane fluidity (Table I *lower part*).

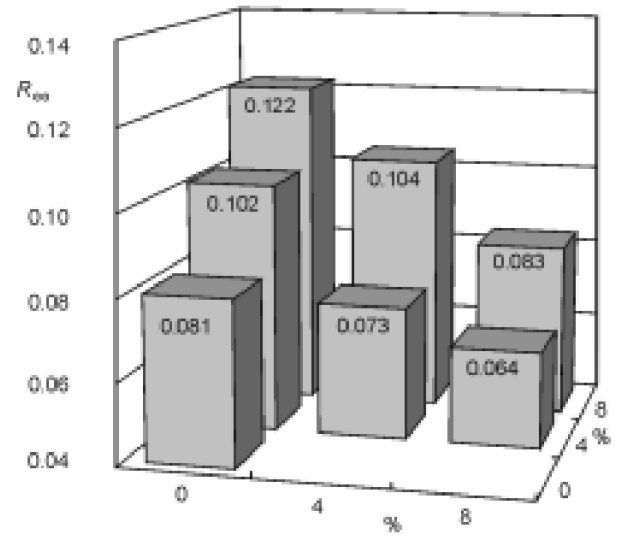

Fig. 2. DPH limiting anisotropy values (R_∞) of *S. cerevisiae* LH60 cells cultivated in media supplemented with ethanol (0, 4, 8 %) and then measured in a buffer with ethanol at concentrations of 0, 4 and 8 %.

Table I. Steady-state fluorescence anisotropy (R) and cell survival (%) after crude killer toxin K1 treatment (0.1, 0.5, 1 %) of *S. cerevisiae* GRF18 precultivated in the presence of linoleic (18:2) or linolenic acid (18:3) (*upper part*) and at 20, 28 and 38 °C (*lower part*)

Sample	R	Cell survival[a]		
		1	0.5	0.1
Control	0.156 ± 0.009	0.85 ± 0.149	5.1	42.6
18:2	0.115 ± 0.007	4.0 ± 0.127	9.7	76.4
18:3	0.102 ± 0.007	1.2 ± 0.092	12.7	59.3
20	0.157 ± 0.005	2.6 ± 0.529	45.6	107
28	0.160 ± 0.005	1.8 ± 0.954	19.8	52.0
38	0.175 ± 0.006	30.3 ± 5.52	–[b]	–[b]

[a]For further details *see Materials and Methods* and Fig. 1. [b]Not determined.

DISCUSSION

The observed effects of different cultivation conditions on PMF were in keeping with the literature data. The plasma membrane of cells cultured at low temperature is more fluid due to an increased content of triacylglycerols and unsaturated fatty acids (Hori *et al.* 1987; Sinigaglia *et al.* 1993). On the other hand, cells cultivated at higher temperature have a decreased amount of unsaturated fatty acids (Sinigaglia *et al.* 1993) and the fluidity of their plasma membrane should therefore be lower. Introduction of exogenous unsaturated fatty acids into the plasma membrane lipid bilayer was also shown to result in increased membrane fluidity (Hazel and Williams 1990).

The time-resolved fluorescence depolarization data show that prolonged exposure to ethanol induces concentration-dependent adaptive fluidity decrease of biomembranes, whereas short exposure brings about an acute membrane-fluidizing effect of about the same magnitude (Fig. 2). Cell adaptation to ethanol is assumed to involve changes in the lipid composition of the yeast cell plasma membrane that serve to maintain an optimum fluidity of the lipid bilayer. Indeed, cultivation in the presence of ethanol resulted in changes of fatty acid synthesis (Beaven *et al.* 1982; Ingram and Butke 1984).

Currently it is still not clear what serves as the killer toxin K1 target: the Tok1p channel (Ahmed *et al.* 1999) or other as yet unidentified plasma membrane proteins (Breinig *et al.* 2002) and whether the formation of *de novo* pores in the membrane lipid matrix (Martinac *et al.* 1990) plays an important role in toxin action. If the latter possibility is physiologically significant, then one would expect a simple correlation between fluidity changes and the sensitivity. However, both a decrease of PMF caused by ethanol or increased cultivation temperature (Fig. 1, Table I *lower part*) and its increase induced in the presence of unsaturated fatty acids during growth or by lower temperature (Table I) are seen to give rise to a decreased sensitivity of the cells to K1. Hence, a certain PMF (SSFA value of ≈0.16 in the yeast strains used) appears to be optimal for interaction of K1 with yeast cells, both higher and lower fluidity caused by different interventions leading to a decrease in killer toxin sensitivity.

PMF is thus seen to play a considerable role in the killer toxin action. The markedly lowered sensitivity to K1 found in yeast cells with modified PMF probably reflects reduced feasibility of killer protein–yeast cell interaction. The mechanism of this effect may involve either a direct interaction of the membrane lipid phase with the killer protein followed by formation of pores or, alternatively, an effect of altered properties of the membrane lipid phase on membrane-associated protein(s) participating in the toxin–yeast cell interaction. Literature data on the action of various membrane-damaging agents lend some support to both mechanisms. Thus increased fluidity of phosphatidylcholine liposome membranes was found to promote the assembly of channel-forming α-toxin of *Staphylococcus aureus* (Tomita *et al.* 1992), and an increased degree of fatty acid unsaturation in *S. cerevisiae*, and consequently increased PMF, was found to enhance the susceptibility to Cd^{2+}-induced plasma membrane permeabilization, which is considered to be the major mechanism of cadmium toxicity in this yeast (Assmann *et al.* 1996; Howlet and Avery 1997). On the other hand, changes in PMF have been reported to have both positive and negative effects on the function of a number of yeast or mammalian membrane proteins such as, *e.g.*, *S. cerevisiae* H^+-ATPase (Alexandre *et al*. 1996) and some membrane receptors (Carmena *et al.* 1991; Ma *et al.* 1994).

Our results favor the latter mechanism, *i.e*. an effect of altered fluidity on membrane-associated protein(s) that participate in the toxin-yeast cell interaction, for the following reasons. Firstly, the pattern of cell survival response to membrane fluidity changes differs from what one would expect for a direct toxin–membrane lipid interaction. As expected, membrane fluidity decrease that should hamper toxin-membrane interaction is accompanied by a rise in cell survival. However, if K1 is incorporated nonspecifically into the lipid membrane matrix to form *de novo* pores, increased membrane fluidity should promote this incorporation and hence enhance the killer toxin sensitivity of cells while, in fact, it brings about again a lowered sensitivity to the toxin. Secondly, there is no clear proportion between the magnitude of PMF changes induced by our treatments and the attendant drop in killer toxin sensitivity. For instance, the relatively small changes in membrane fluidity brought about by growth at both 20 and 38 °C are accompanied by large differences in survival. This lack of a clear correlation indicates that the direct toxin interaction with plasma membrane lipids does not have physiological relevance and the effect of membrane fluidity change on toxin sensitivity is mediated very probably through membrane-associated proteins serving as receptors or primary effectors for K1. The efficiency of their interaction with the toxin can then be negatively influenced by any changes of PMF, *i.e*. by both fluidity increase and decrease. The membrane proteins likely to be involved in this process are Kre1 and/or Tok1 (Ahmed *et al.* 1999; Breinig *et al.* 2002) or other still unidentified targets.

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