Role of Phospholipid Head Groups in Ethanol Tolerance of *Saccharomyces cerevisiae*

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Pre-incubation of cells of *Saccharomyces cerevisiae* with 2 M-ethanol led to decreased rates of L-alanine uptake, H^+ efflux and fermentation rate. However, these responses were modified in yeast cells with altered phospholipid composition. Using L-alanine transport and H^+ efflux as indices of ethanol tolerance, it was observed that cells enriched with phosphatidylserine had greater tolerance to ethanol. This resulted from altered charge of membrane phospholipids rather than changes in membrane fluidity. It is suggested that the anion:zwitterion ratio of phospholipids may be one of the important determinants of ethanol tolerance in *S. cerevisiae*.

INTRODUCTION

It is now established that ethanol accumulation is primarily responsible for inhibiting fermentation. Ethanol at concentrations above 12% is toxic to yeast (Rose, 1980) and consequently growth ceases at about this concentration. The inhibition by ethanol of growth of *Saccharomyces cerevisiae* is preceded by the effects of ethanol on cell viability (Thomas *et al.*, 1978; Beavan *et al.*, 1982), accumulation of various nutrients (Thomas & Rose, 1979; Loureiro-Dias & Peinado, 1982; Leão & van Uden, 1982, 1983, 1984a), H^+ fluxes (Leão & van Uden, 1984b; Cartwright *et al.*, 1986; Juroszek *et al.*, 1986) and on fermentation rate (Casey & Ingledew, 1986). Similar effects of ethanol are also known in other organisms (Fried & Novick, 1973; Ingram & Buttke, 1984; Ingram, 1986).

The plasma membrane is considered to be the prime target of ethanol action in yeast (Ingram & Buttke, 1984). It seems that both the structure and function of the yeast plasma membrane are affected (Ingram & Buttke, 1984; Ingram, 1986). Amongst various membrane components, lipids are the chief targets of ethanol toxicity (Thomas *et al.*, 1978; Thomas & Rose, 1979). Alterations in phospholipid and fatty acyl residue composition in the presence of ethanol is known to be due to an adaptive response to the physiochemical interaction of ethanol with the plasma membrane (Ingram, 1977, 1986). Unsaturated fatty acyl residues and ergosterol are considered to be important determinants of ethanol tolerance in yeast (Thomas *et al.*, 1978; Thomas & Rose, 1979; Beavan *et al.*, 1982). However, the precise role of phospholipid head groups in resistance to ethanol has not been ascertained. Since ethanol can modify the hydration state of polar head groups (Rigomier *et al.*, 1980) and is equally important in interfacial regulation of lipid–protein interactions (Sandermann, 1978), it was of interest to study the effect of ethanol on the yeast plasma membrane after phospholipid head group modification.

Availability of a yeast lipid auxotroph (*chol*) and of hydroxylamine chloride, a specific inhibitor of phosphatidylserine decarboxylase (EC 4.1.1.65), have provided an opportunity to enrich yeast plasma membrane with specific phospholipids (Trivedi *et al.*, 1982, 1983). In the present study, using amino acid transport and H^+ fluxes as indices of membrane function, the role of phospholipids in ethanol tolerance has been investigated.

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

METHODS

Strain and culture conditions. S. cerevisiae α ade5 and its mutant derivative KA101 (*chol*) were maintained on YEPD slants and enriched with different phospholipid bases as described previously (Trivedi *et al.*, 1982, 1983). Cells were harvested and washed three times with sodium citrate buffer (10 mM, pH 4·5) before use for further studies.

Measurement of rate of amino acid uptake. The transport assay procedure was similar to that described previously (Rao *et al.*, 1986; Mishra & Prasad, 1987). The rate of L-alanine uptake was determined from plots of the time-course of uptake up to 195 s using linear regression analysis. To examine the effect of ethanol on amino acid uptake, 2 mM-ethanol was added to cell suspensions. After preincubation at 30 °C for 5 min, the reaction was started by the addition of L-[^{14}C]alanine (37 kBq μmol^{-1}). The final concentration of L-alanine was two to four times higher than its K_T value. At the indicated time intervals samples were removed and immediately diluted in 5 ml ice-cold citrate buffer (10 mM, pH 4·5) containing unlabelled L-alanine at the concentration included in the cell suspension. The diluted suspension was rapidly filtered through 0·45 μm pore diameter, Maxflow filter discs. Filters were then washed twice with the same buffer containing unlabelled L-alanine and the radioactivity retained was counted in a Beckman LS-1801 Beta liquid scintillation counter using a toluene-based scintillation fluid.

Inhibition constant $K_{i(EtOH)}$ values for L-alanine uptake were derived from reciprocal plots of velocities of L-alanine uptake against increasing concentrations of ethanol (0·5 to 2·0 M) at two fixed substrate concentrations (Dixon, 1953).

Measurement of rates of proton flux. Glucose-stimulated proton efflux was measured by the addition of glucose (100 mM) to a suspension (100 mg wet wt ml^{-1}) of cells enriched with different phospholipids. A constant temperature of 30 °C was maintained by circulating water through a water-jacketted chamber. The cell suspension in the chamber was constantly stirred. Changes in pH of the suspension were recorded by using a REC 80 Servograph with REA 105 pH/mV unit (Radiometer). The effect of ethanol on glucose-induced H$^+$ efflux was measured by supplementing the cell suspension with ethanol after adjusting its pH value to 4·0 prior to the addition of glucose (100 mM). Proton flux was then followed over a 5 min period (Cartwright *et al.*, 1986). The pH range was fixed in order to avoid denaturation of the cellular components of energized organisms (Juroszek *et al.*, 1986).

Fluorescence measurements. A 2 mM solution of 1,6 diphenyl-1,3,5-hexatriene (DPH) was prepared in tetrahydrofuran and 100 μl was added to 50 ml of rapidly stirred potassium phosphate buffer (10 mM, pH 6·8). Excess tetrahydrofuran was removed by flushing with nitrogen. Spheroplasts of normal as well as of phospholipid-enriched cells were prepared as described previously (Jayakumar *et al.*, 1981). These spheroplasts were washed with phosphate buffer (20 mM, pH 6·0) containing 10 mM-MgSO$_4$ and 0·6 M-sorbitol and incubated with 2 μM-DPH for 60 min at 30 °C. Fluorescence polarization was measured by excitation with monochromatic light at 360 nm which was vertically polarized, and emission intensity was detected at 426 nm through an analyser oriented either parallel or perpendicular to the direction of polarized excitation light. The degree of fluorescence polarization (p) and anisotropy (r) were calculated according to Haggerty *et al.* (1976).

Measurement of fermentation rates. The ability of S. cerevisiae to ferment glucose was determined with a Gilson single-valve differential respirometer. Cells (100 mg wet wt ml^{-1}) were suspended in citrate buffer (50 mM; pH 4·5). Glucose (300 mM) placed in the side arm of the Warburg flasks was mixed with the cell suspension after equilibration for 10 min at 30 °C. Prior to the addition of glucose, flasks were shaken (95 oscillations min^{-1}) and the system was continuously flushed with nitrogen gas passed through a trap containing pyrogallol solution. The supply of nitrogen gas was cut off before addition of glucose solution to the buffered cell suspension. Evolution of CO$_2$ was then followed over a period of 5 min (Cartwright *et al.*, 1986).

Chemicals. Ethanolamine, choline chloride, hydroxylamine, DPH, tetrahydrofuran, L-alanine and standard phospholipids were purchased from Sigma. L-[^{14}C]Alanine was purchased from BARC, India. All other chemicals were of analytical grade.

RESULTS AND DISCUSSION

Phospholipid head group modification in S. cerevisiae

The S. cerevisiae auxotrophic mutant *chol* uses the CDP choline pathway (Atkinson *et al.*, 1980) for the synthesis of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and requires phospholipid bases (choline or ethanolamine) for its growth. We have earlier shown that supplementation of this mutant with choline (1 mM) or ethanolamine (1 mM) resulted in the enrichment of PC (18%) or PE (32%), respectively (Trivedi *et al.*, 1982). The addition of 20 mM-hydroxylamine to exponentially growing cells led to a 3·5-fold increase in phosphatidylserine (PS) content because hydroxylamine blocks PS decarboxylase and prevents further conversion of PS to other phospholipids (Trivedi *et al.*, 1983). The phospholipid composition of the mutant and of other strains has been published previously (Trivedi *et al.*, 1982, 1983).

Table 1. *Effect of ethanol on* L-*alanine uptake in S. cerevisiae*

Cells were grown in the presence of choline, ethanolamine or hydroxylamine as described in Methods. Velocities were calculated from the linear segment of the time-course of L-alanine uptake up to 195 s using linear regression analysis (Mishra & Prasad, 1987). To examine the effect of ethanol on L-alanine uptake, cells were incubated in the presence of ethanol (2 M) for 10 min before the start of transport measurements. Each value is the mean of three experiments.

Strain	Supplement to medium	Velocity of L-alanine uptake [pmol (mg dry wt)$^{-1}$ s^{-1}]		
		Without ethanol	With 2 M-ethanol	Percentage inhibition by 2 M-ethanol
α *ade5*	None	6·5	2·5	61
chol	Choline (1 mM)	5·7	1·3	77
chol	Ethanolamine (1 mM)	2·3	0·8	65
α *ade5*	Hydroxylamine (20 mM)	7·0	5·0	28

Table 2. *Inhibition constants* ($K_{i(EtOH)}$) *for* L-*alanine uptake in S. cerevisiae*

$K_{i(EtOH)}$ values were derived from Dixon plots. Velocity of L-alanine uptake was calculated as described in Methods using linear regression analysis. Values are means of three experiments ± SD values.

Strain	Supplement to medium	Cells enriched with:	Type of inhibition	$10^{-2} \times K_{i(EtOH)}$ (M)
α *ade5*	None	No enrichment	Non-competitive	50·0 ± 2·0
chol	Choline (1 mM)	PC	Non-competitive	12·5 ± 0·6
chol	Ethanolamine (1 mM)	PE	Non-competitive	25·0 ± 1·1
α *ade5*	Hydroxylamine (20 mM)	PS	Non-competitive	75·0 ± 2·5

Effect of ethanol on amino acid uptake in S. cerevisiae

Cells enriched with specific phospholipids were tested for their ability to tolerate ethanol. Amino acid uptake was used as an index of membrane function (Thomas & Rose, 1979; Prasad & Rose, 1986). When wild-type (α *ade5*) and cells enriched with PC, PE or PS were incubated with 2 M-ethanol for 10 min, the rate of L-alanine uptake was reduced in the following order: PC (77%) > PE (65%) > α *ade5* (61%) > PS (28%) (Table 1). The extent of inhibition of L-alanine uptake in the presence of ethanol was minimal in PS-enriched cells. Thus, it is possible that PS enrichment of yeast plasma membrane confers greater tolerance to ethanol in comparison to enrichment with other phospholipids.

To gain further insight into the inhibition by ethanol of L-alanine uptake, the inhibitory constant $K_{i(EtOH)}$ was determined at two different concentrations of L-alanine with increasing concentrations of ethanol (0·5 to 2·0 M). A non-competitive inhibition by ethanol of L-alanine uptake was evident from Dixon plots (not shown), which confirmed earlier observations (Leão & van Uden, 1984a, b; Cartwright et al., 1987). $K_{i(EtOH)}$ values (Table 2) further indicated that the inhibitory effect of ethanol was in the order PC > PE > α *ade5* > PS-enriched cells. Maximum $K_{i(EtOH)}$ value in PS-enriched cells indicated minimal inhibitory effect of ethanol on L-alanine uptake (Table 2).

Effect of ethanol on H$^+$ efflux in S. cerevisiae

Earlier studies by Rose and co-workers demonstrated that the addition of glucose (100 mM) to yeast cell suspensions results in a rapid acidification of the external medium (Cartwright et al., 1986), due to net proton efflux caused by Mg^{2+}-dependent proton translocating ATPase (Willsky, 1979; Malpartida & Serrano, 1981). Addition of increasing concentrations of ethanol results in an increase in extracellular pH by decreasing the proton efflux (Cartwright et al., 1986).
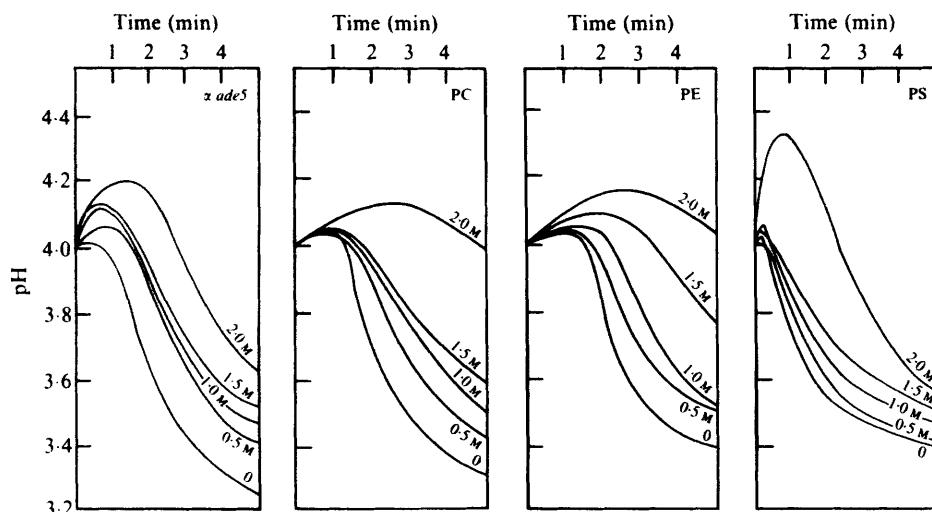
Fig. 1. Effect of ethanol (at the concentration indicated) on the ability of energized *S. cerevisiae* cells to acidify the external medium. The lines are direct tracings of a typical experiment. The initial pH of the cell suspension was adjusted to pH 4·0. Other details were similar to those described in Methods. The four panels of tracings represent experiments done with non-enriched α *ade5* or α *ade5* cells enriched with PC, PE or PS.

Table 3. *Effect of ethanol on fermentative activity of S. cerevisiae*

Cells were suspended in citrate buffer (pH 4·5, 50 mM) and fermentation rates were measured as described in Methods. The percentage inhibition values were calculated from the mean of three sets of observations; the extent of variation was within 5%.

Strain	Cells enriched with:	Percentage inhibition of fermentative activity by 2 M-ethanol
α *ade5*	No enrichment	66
chol	Phosphatidylcholine	69
chol	Phosphatidylethanolamine	57
α *ade5*	Phosphatidylserine	54

Our data indicated that ethanol-induced reversal of H^+ efflux is affected by modification of the phospholipid head group. Rapid acidification of a normal cell suspension was prevented by ethanol in a concentration-dependent manner (Fig. 1). The inhibition of H^+ efflux at various concentrations of ethanol was evident from the increase in extracellular pH. Similar to L-alanine uptake, H^+ efflux was least affected in PS-enriched cells.

From the results of H^+ flux and L-alanine uptake experiments, it became apparent that phospholipid enrichment offers some protection against ethanol toxicity; this protection was greatest in PS-enriched cells. The way in which PS enrichment can affect ethanol toxicity is not clear. There are reports suggesting that an increased ratio of anionic:zwitterionic phospholipids can affect ethanol tolerance (Clark & Beard, 1979; Bohin & Lubochinsky, 1982). It has also been suggested that the phospholipid head group could modify the effect of ethanol on plasma membrane ATPase activity (Stadtlander *et al.*, 1982). Based on H^+-flux measurements, our data do suggest that in PS-enriched cells, H^+-translocating activity of ATPase becomes less sensitive to ethanol as compared to wild-type, PC- or PE-enriched cells (Fig. 1).

Effect of ethanol on fermentative activity of S. cerevisiae

The inhibition of the fermentative ability of an organism in the presence of ethanol is the best indicator of the potential of a given strain to produce ethanol (Casey & Ingledew, 1986). This is
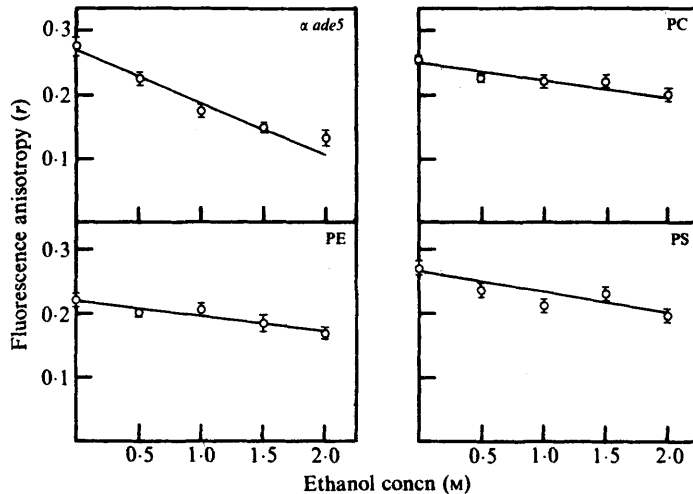
Fig. 2. Effect of ethanol on the anisotropic value (*r*). Spheroplasts were prepared either from non-enriched α *ade5* or α *ade5* cells enriched with PC, PE or PS. Anisotropic measurements were done as described in Methods. The four panels represent the anisotropic values of the different cell types. Each point is the mean of three separate determinations; the vertical bars represent ± SEM.

because the effect of ethanol on fermentation rate is not influenced by the nutritional conditions or the growth status of the cells and its value correlates well with the upper limits of ethanol production (Hayashida & Ohta, 1981).

Among cells enriched with various phospholipids the rate of fermentation was greatest in PS-enriched cells and was comparable to that of wild-type cells (data not shown). The percentage inhibition of fermentation by 2 M-ethanol was least in PS-enriched cells. The fermentative abilities of cells of *S. cerevisiae* with altered phospholipid compositions suggest that those enriched with PS acquire the greatest resistance to ethanol (Table 3).

Effect of ethanol on membrane fluidity in *S. cerevisiae*

Ethanol interacts with and perturbs the organization of lipid bilayers (Paterson *et al.*, 1972; Jain & Wu, 1977); in particular it lowers the transition temperature and increases membrane fluidity (Jain & Wu, 1977). Since addition of ethanol results in a reduction in dielectric strength and thereby leads to altered charge interaction at the phospholipid surface (Fried & Novick, 1973), it was of interest to monitor the effect of ethanol on the fluidity of yeast plasma membrane enriched with different phospholipids. We therefore used DPH, a fluorescent probe, to monitor changes in membrane fluidity (Van-Blitterswijk *et al.*, 1987). As revealed by the anisotropic data, except in PE-enriched cells the alteration in phospholipid head group did not significantly alter the membrane fluidity as compared to wild-type cells. However, increasing concentrations of ethanol led to decreased anisotropy or increased fluidity (Fig. 2). The decrease in anisotropy suggests that the fluidizing effect of ethanol was greater in α *ade5* cells as compared to cells enriched in different phospholipids.

Since the fluidity (anisotropy) of the membrane was not affected by a change in phospholipid head group, the ethanol tolerance of cells enriched in PS must reflect lipid properties other than fluidity (Cartwright *et al.*, 1986). In this regard Jones & Greenfield (1987), using passive permeability of acetic acid as a criterion of membrane fluidity, showed that optimum membrane stability rather than increased membrane fluidity is of greater relevance to ethanol tolerance in yeast.

Apart from the long-term effect of ethanol on the yeast cell membrane, where adaptation results in the alteration of fatty acyl composition (Ingram, 1976), the short-term effects of ethanol on membrane functions are equally important. Our results, based on amino acid uptake, H⁺ efflux and fermentative activity suggest that head group composition rather than fluidity

renders yeast membranes more resistant to ethanol. The role of membrane fluidity, however, cannot be overlooked (Thomas *et al.*, 1978; Thomas & Rose, 1979). Recent results from our laboratory further confirm earlier findings of Rose and co-workers (Thomas *et al.*, 1978; Thomas & Rose, 1979) that the degree of unsaturation is a decisive factor in ethanol tolerance when other membrane components remain unaltered (unpublished observations).

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