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EFFECTS OF PHENOTHIAZINES ON INHIBITION OF PLASMA MEMBRANE ATPase AND HYPERPOLARIZATION OF CELL MEMBRANES IN THE YEAST *SACCHAROMYCES CEREVISIAE*

YAEL EILAM

Department of Bacteriology, The Hebrew University-Hadassah Medical School, Jerusalem (Israel)

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The transmembranal potential, in *Saccharomyces cerevisiae*, has been calculated from the distribution ratio of the lipophilic cation tetraphenylphosphonium (TPP^+) between the intracellular and extracellular water. Trifluoperazine at concentrations of 10 to 50 μM, caused a substantial increase in the membrane potential (negative inside). This increase was observed only in the presence of a metabolic substrate and was eliminated by the addition of the protonophores 2,4-dinitrophenol and sodium azide, removal of glucose, replacement of glucose by the nonmetabolizable analog 3-*O*-methyl glucose, or by the addition of 100 mM KCl. An increase in $^{45}CaCl_2$ accumulation from solutions of low concentrations (1 μM) was observed under all conditions where membrane potential was increased. Proton ejection activity was monitored by measurements of the rates of the decrease in the pH of unbuffered cell suspensions in the presence of glucose. Trifluoperazine inhibited the changes in medium pH; this inhibition was not the result of an increase in the permeability of cell membranes to protons since in the absence of glucose, trifluoperazine did not cause a change in the rate of pH change generated by proton influx. The activity of plasma membrane ATPase was measured in crude membrane preparations in the presence of sodium azide to inhibit mitochondrial ATPase. Trifluoperazine strongly inhibited the activity of the plasma membrane ATPase. The effect of phenothiazines on transport and on membrane potential reported in this study and in the previous one (Eilam, Y. (1983) Biochim. Biophys. Acta 733, 242–248) were observed only in the presence of a metabolic substrate. The possibility that energy is required for the uptake of phenothiazines into the cells was eliminated by results showing energy-independent uptake of [^{3}H]chlorpromazine. The results strongly suggest that phenothiazines activate energy-dependent K^+-extrusion pumps, which lead to increased membrane potential. Increased influx of calcium seems to be energized by membrane potential, and therefore stimulated under all conditions where membrane potential is increased. The analog which does not bind to calmodulin, trifluoperazine sulfoxide, had no effect on the cells, but the involvement of calmodulin in the processes altered by trifluoperazine cannot as yet, be determined.

Introduction

Phenothiazines, the tranquilizers and antipsychotic drugs, have recently gained renewed interest due to the finding that these drugs bind to calmodulin in the presence of Ca^{2+} and inhibit its regulatory functions [1–3]. In our previous work on *Saccharomyces cerevisiae*, we have found that low concentrations of trifluoperazine and related compounds induced stimulation of the initial rate of Ca^{2+} influx by 10–20-fold, and a substantial efflux of cellular K^+ [4]. Using low concentrations

Abbreviations: $\Delta\psi$, the membrane potential; TPP^+, tetraphenylphosphonium ion; Mes, 4-morpholineethanesulfonic acid; DDA^+, dibenzyldimethylammonium; $TPMP^+$, triphenylmethylphosphonium.

of trifluoperazine, the effects were observed only in the presence of a metabolic substrate. These results indicated that the stimulation of Ca^{2+} and K^+ fluxes were the result of enhanced active, carrier-mediated transport, and not to increased passive ion leak.

In the present work, we continue to investigate the mechanism of the effect of trifluoperazine on yeast cells. It has been found that trifluoperazine inhibits plasma membrane ATPase and activates electrogenic energy-dependent extrusion of K^+, which leads to increased transmembranal potential (interior negative). It is postulated that the activated mechanism consists of electrogenic K^+ pumps. Stimulation of Ca^{2+} influx seems to be the result of the increase in membrane potential.

Recently, calmodulin has been demonstrated in the yeast *S. cerevisiae* [5], but its function in yeasts has not yet been discovered. It is, as yet, impossible to determine whether the reported effects are mediated via inhibition of some calmodulin-dependent regulatory function or alternatively, to calmodulin-independent direct effect of phenothiazines on cellular or membranal component(s).

Methods

Organism and culture conditions. Saccharomyces cerevisiae, strain 123 (genotype MAT a/α his 1) was maintained at 4°C on 1.5% w/v/ agar containing 1% yeast extract, 2% glucose and 2% peptone. Prior to the experiments, cells were incubated in medium containing Bacto yeast extract, (10 g/l) Bacto peptone (20 g/l) and glucose (20 g/l). The cells were grown overnight in shaking flasks at 30°C. Cells were collected from the overnight culture by centrifugation, washed three times by resuspension in distilled water, and finally resuspended in the indicated medium, at cell density of $5 \cdot 10^7$ cells/ml.

Measurements of TPP^+ uptake. Cell suspensions, $1 \cdot 10^8$ cells in 2 ml medium containing glucose (100 mM), Mes-Tris buffer, pH 6.0 (20 mM) and [^{3}H]TPP^+ (1 μM, 0.05 μCi/ml) were incubated at 30°C with continuous shaking. At indicated times, 2 ml of ice-cold $MgCl_2$ (20 mM) were added to the cell suspensions, the suspensions were immediately filtered through glass fiber filters (Sartorious) and the filters were washed four times with 2 ml portions of ice-cold $MgCl_2$ solutions (20 mM). The washing required no more than 1 min. The Mg^{2+}-wash prevented the adsorption of TPP^+ to cell walls as shown by Barts et al. [6] for DDA uptake. The filtered cells were dried, toluene-based scintillation fluid was added and the radioactivity in the filters was determined using a liquid scintillation counter.

Determination of K^+ content. Cell suspensions ($5 \cdot 10^7$ cells/ml) were incubated at 30°C with continuous shaking. At the indicated times, 1 ml samples were removed and filtered onto Sartorious membrane filters (0.45 μm pore size; prewashed with distilled water) and the cells on the filters were washed four times with 3 ml distilled water in less than 2 min. After filtration, each filter was immersed in 3 ml distilled water, boiled to release the ions from the cells and centrifuged to precipitate the debris. K^+ was determined in the supernatant after appropriate dilution, using a Perkin-Elmer atomic absorption spectrometer.

Measurements of Ca^{2+} influx. Cells were suspended at a density of $5 \cdot 10^7$ cells/ml in the indicated medium which also contained $CaCl_2$ (1 μM) labeled with $^{45}CaCl_2$ (0.5 μCi/ml). The suspensions were incubated at 30°C with continuous shaking for the indicated times. 1 ml samples were removed and filtered through Sartorious membrane filters (0.45 μm pore size, prewashed with 20 mM $MgCl_2$). The cells on the filters were quickly washed three times with 20 mM $MgCl_2$. It was previously reported [5,7] that such Mg^{2+}-wash prevents the adsorption of Ca^{2+} to cell walls. The filters were dried out and the radioactivity on the filters was determined in toluene-based scintillation fluid. Blank filters, through which 1 ml medium without cells were filtered, were similarly washed and the counts remaining on the filters were subtracted from the results.

Measurements of proton fluxes. Solutions of glucose (10 mM) and the indicated concentration of trifluoperazine were brought to pH 6 by addition of small aliquots of dilute solutions of NaOH or HCl, as required. Portions of yeast suspensions ($1 \cdot 10^8$ cells/ml) were similarly adjusted to the required pH value immediately before the initiation of the measurement. An expanded scale was selected on the pH-meter (Radiometer, Copenhagen, Denmark, with error limited ± 0.005 pH units)

and the electrode was immersed in a vial containing 1 ml of the indicated medium adjusted to the required pH value. One ml of cell suspension was added forcefully to the medium (to obtain an adequate mixing) and the pH values of the cell suspension were immediately recorded upon the addition of the cells and every 15 s up to 2 min.

All media and suspensions were maintained at 25°C throughout the experiment.Every experiment was repeated at least four times.

Determination of intracellular water volume. Intracellular water volume was determined as described by De la Peña et al. [8] with slight modifications. Cells were suspended at $1 \cdot 10^9$ cells/ml in medium containing Mes-Tris buffer, pH 6.0 (10 mM), glucose (100 mM), ^3H_2O (1 μCi/ml) and ^{14}[C]methoxyinsulin (50 μg/ml, 0.5 μCi/ml). The suspensions were incubated at 30°C for 30 min and then centrifuged. Radioactivity was determined (using toluene-Triton scintillation fluid) in the supernatant and in the pellet after overnight incubation in 1% sodium dodecyl sulfate. The intracellular water volume was calculated from the H^3 and ^{14}C counts according to De la Peña et al. [8]. 10^9 cells yielded 24 μl intracellular water volume.

Assay of the plasma membrane ATPase. Washed yeast cells were suspended at a density of $5 \cdot 10^9$ cells/ml in medium containing 25 mM Mes-Tris, pH 7.0, 0.4 M sucrose, 0.2 mM EDTA and 2 mM mercaptoethanol. After the addition of 10 g glass beads (0.5 mm) to 10 ml of yeast suspension, the yeast cells were homogenized in Braun cell homogenizer. The homogenate was separated from the glass beads and the ATPase activity of the homogenate was determined as follows: The reaction was started by the addition of 100 μl of the homogenate to 1 ml of the reaction-medium containing 5 mM $MgCl_2$, 5 mM ATP, 10 mM NaN_3, 0.5 mM EDTA, 12.5 mM Mes-Tris pH 6.0 and trifluoperazine, as indicated. The reaction was stopped by the addition of 0.2 ml of trichloroacetic acid 100% (w/v). Inorganic phosphate was measured as described by Pullman and Penefsky [11]. The reaction rates were linear with time up to 15 min.

Materials. Trifluoperazine and chlorpromazine were purchased from Sigma, tetraphenylphosphoniumbromide was purchased from Merck, [^{3}H]tetraphenylphosphoniumbromide and [^{3}H]-chlorpromazine were purchased from Nuclear Research Centre, Negev, Israel. ^{45}Ca was purchased from Amersham International, U.K., and trifluoperazine sulfoxide was a gift from Smith-Kline and French Laboratories.

Results

Determination of the membrane potential

It has recently been established that the lipophilic cation tetraphenylphosphonium$^+$ (TPP^+) may be used, at low concentrations, as a probe for determination of the membrane potential in *Saccharomyces* [8–10]. In our strain of *S. cerevisiae*, at 30°C, pH 6.0, TPP^+ was taken up into metabolizing yeast at an initial rate of $0.4 \cdot 10^{-18}$ mol/cell per min and the steady state was obtained after approx. 25 min. Addition of 2,4-dinitrophenol (1 mM) caused a rapid release of 85% of the radioactivity (Fig. 1). Substantial reduction in TPP^+ uptake was also observed under the following conditions: removal of glucose (with or without the addition of antimycin A), replacement of glucose by non-metabolized analogue 3-*O*-methyl glucose, and the addition of NaN_3. Addition of 100 mM KCl or 1 mM $CaCl_2$ caused a reduction in TPP^+ accumulation by 60% and 55%, respectively (Table I). Thus, conditions known to depolarize membranes caused a decrease in the accumulation of TPP^+.

It has previously been reported that the uptake of the lipophilic cations DDA^+ and $TPMP^+$ is mediated via the thiamine carrier and therefore, these cations are unsuitable for quantitative determination of membrane potential [6]. On the other hand, it was shown that TPP^+ is not transported by the thiamine carrier [8–10]. These results were also confirmed in our strain of *S. cerevisiae*. It is shown in Table I that the presence of 1000-fold access of thiamine in the medium did not interfere with the uptake of TPP^+. Therefore, the results shown in Fig. 1 and Table I justify the use of TPP^+ as a quantitative probe for measurements of the membrane potential ($\Delta\psi$) in our cells. The values of the membrane potential ($\Delta\psi$) at 30°C, pH 6.0, were calculated according to Eqn. 1, after corrections for the bound TPP^+ which were determined in the presence of 2,4-di-

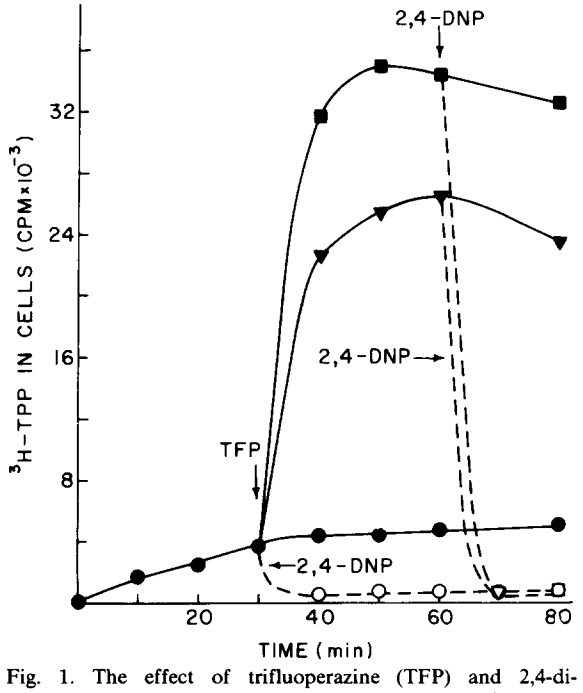

Fig. 1. The effect of trifluoperazine (TFP) and 2,4-dinitrophenol (2,4-DNP) on the time-course of TPP^+ uptake. Cells were incubated in Mes-Tris buffer, pH 6.0 (20 mM), glucose (100 mM) and [^{3}H]TPP^+ (1 μM, 0.05 μCi/ml), Trifluoperazine and 2,4-dinitrophenol were added when indicated. Concentration of trifluoperazine was 50 μM (■), 20 μM (▼) or none (●). 2,4-Dinitrophenol (1 mM) (empty symbols) was added to the control (○) after 30 min of incubation and to the trifluoperazine-treated cells (□, ▽) after 60 min of incubation.

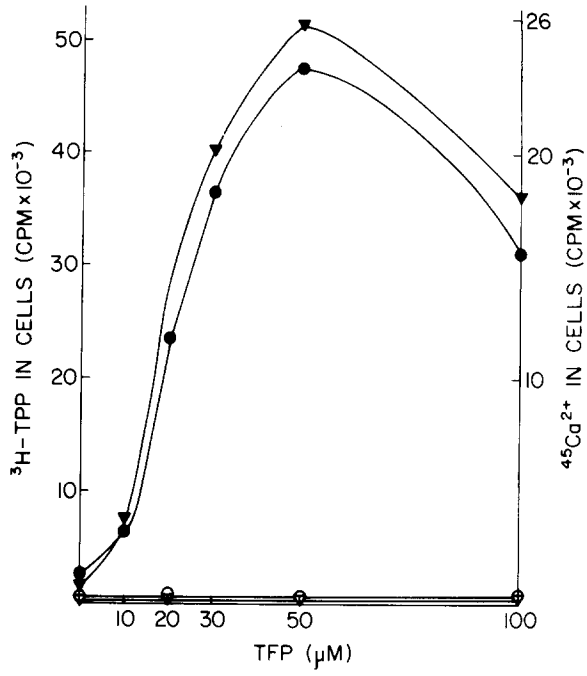

Fig. 2. The effect of different concentrations of trifluoperazine on TPP^+ and $^{45}Ca^{2+}$ uptake. Cells were incubated in Mes-Tris buffer, pH 6.0 (20 mM) with [^{3}H]TPP^+ (1 μM, 0.05 μCi/ml) (●, ○) or $^{45}Ca^{2+}$ (1 μM, 0.5 μCi/ml) (▼, ▽); 100 mM glucose (●, ▼) or antimycin A (○, ▽). Incubation was terminated after 30 min.

nitrophenol.

$$\Delta\psi = -58.8 \log \frac{[TPP]^+ in}{[TPP]^+ out} \quad (1)$$

A value of -100 mV was determined for metabolizing cells; in the absence of glucose, the membrane potential decreased to -60 mV and in the presence of 100 mM KCl, the membrane potential was -56 mV.

The effect of trifluoperazine on membrane potential

Addition of trifluoperazine to the metabolizing cells caused a rapid uptake of TPP^+ into the cells. The initial rate of the uptake was $3.8 \cdot 10^{-18}$ mol/cell per min and the steady-state level of TPP^+ accumulation was up to 8-fold and 20-fold that of the control, using 20 μM and 50 μM trifluoperazine, respectively. Addition of 2,4-dinitrophenol to the trifluoperazine-treated cells at the steady-state level of TPP^+ accumulation caused a rapid efflux of the accumulated TPP^+ (Fig. 1) Membrane potential values were calculated from Eqn. 1. 20 μM trifluoperazine caused an increase in the membrane potential up to -161 mV and 50 μM trifluoperazine up to -178 mV.

The effects of trifluoperazine on membrane potential was dependent on the presence of a metabolic substrate. Reduction in the concentration of glucose in the medium from 80 mM to 10 mM caused a reduction in the accumulation of TPP^+ by 38%. Removal of glucose and the addition of antimycin A abolished the effect of trifluoperazine. The effect was also abolished in the presence of NaN_3, 2,4-dinitrophenol, 100 mM KCl and substantially reduced in the presence of 1 mM $CaCl_2$.

The dose response for the effect of trifluoperazine on the accumulation of TPP^+ was bell-shaped

TABLE I

THE EFFECT OF TRIFLUOPERAZINE ON TPP^+ AND Ca^{2+} UPTAKE AT DIFFERENT MEDIUM COMPOSITIONS

Cells were incubated for 30 min at 30°C in media containing Mes-Tris buffer, pH 6.0 (20 mM), [^{3}H]TPP (1 μM, 0.05 μCi/ml) or $^{45}CaCl_2$ (1 μM, 0.5 μCi/ml) and the indicated substances. Values represent mean ± S.E. ($n = 4$). TFP, trifluoperazine; 2,4-DNP, 2,4-dinitrophenol.

Medium	[^{3}H]TPP^+ uptake (cpm)		$^{45}Ca^{2+}$ uptake (cpm)	
	Control	TFP (50 μM)	Control	TFP (50 μM)
–	455 ± 21	1372 ± 74	521 ± 24	695 ± 31
Glucose (10 mM)	1089 ± 64	14444 ± 105	2473 ± 68	33104 ± 607
Glucose (80 mM)	1137 ± 48	23045 ± 431	3746 ± 72	48288 ± 724
Glucose (80 mM) + antimycin A (20 μM)	1014 ± 38	13604 ± 408	1669 ± 25	21499 ± 76
Antimycin A (20 μM)	405 ± 12	691 ± 22	288 ± 7	516 ± 17
3-*O*-Methyl glucose (10 mM)	546 ± 21	1499 ± 94	652 ± 15	806 ± 27
Glucose (80 mM) + NaN_3 (5 mM)	273 ± 13	242 ± 12	245 ± 10	314 ± 13
Glucose (80 mM) + 2,4-DNP (1 mM)	291 ± 9	357 ± 12	536 ± 15	618 ± 25
Glucose (80 mM) + thiamine (1 mM)	1152 ± 34	24793 ± 528	3525 ± 88	44478 ± 849
Glucose (80 mM) + KCl (100 mM)	442 ± 15	605 ± 18	932 ± 35	1381 ± 51
Glucose (80 mM) + $CaCl_2$ (1 mM)	506 ± 17	1249 ± 29	–	–

in appearance, the maximum being observed at 50 μM trifluoperazine (Fig. 2).

The analogue of trifluoperazine, chlorpromazine, exerted similar effects, but at higher concentrations, trifluoperazine-sulfoxide, had no effect on TPP^+ uptake (Table II).

TABLE II

EFFECT OF TRIFLUOPERAZINE SULFOXIDE (TFPS) AND CHLORPROMAZINE (CPZ) ON [^{3}H]TPP^+ AND $^{45}CaCl_2$ UPTAKE

Cells were incubated for 30 min (at 30°C) in media containing Mes-Tris buffer, pH 6.0 (20 mM), [^{3}H]TPP (1 μM, 0.05 μCi/ml) or $^{45}CaCl_2$ (1 μM, 0.5 μCi/ml) glucose (80 mM) when indicated. Values represent mean ± S.E. ($n = 4$).

Medium	Glucose (80 mM)	[^{3}H]TPP^+ uptake (cpm)	$^{45}CaCl_2$ uptake (cpm)
–	+	1094 ± 34	2560 ± 54
TFPS (50 μM)	+	940 ± 21	2568 ± 62
TFPS (100 μM)	+	886 ± 18	1879 ± 49
CPZ (50 mM)	+	16842 ± 542	31892 ± 877
CPZ (100 μM)	+	21477 ± 678	39725 ± 796
CPZ (100 μM)	–	982 ± 31	625 ± 22

The relationships between membrane hyperpolarization, K^+ efflux and $^{45}Ca^{2+}$ uptake

It was reported the trifluoperazine stimulated the uptake of $^{45}Ca^{2+}$ into the cells [4]. The relationship between TPP^+ uptake and $^{45}Ca^{2+}$ uptake are shown in Fig. 2 and Table I.

The dose-response curve for $^{45}Ca^{2+}$ uptake was parallel with the curve of TPP^+ uptake (Fig. 2). All conditions which reduced or abolished the increase in TPP^+ uptake in response to trifluoperazine also reduced Ca^{2+} uptake (Table I).

Finally, we examined the inter-relations between all observed effects of trifluoperazine; K^+ efflux, $^{45}Ca^{2+}$ influx (reported in our previous work [4]) and TPP^+ uptake, as a measure of membrane potential. Cells were preincubated with trifluoperazine and glucose; after 30 min of such preincubation, the cellular K^+ content was reduced by 74%, as compared with that in cells incubated without trifluoperazine, and the membrane potential increased to −180 mV. Cells were then centrifuged and suspended in media without trifluoperazine but with 1 μM Ca^{2+} (labelled with $^{45}Ca^{2+}$) or with [^{3}H]TPP^+. After an additional 30

TABLE III

THE EFFECT OF TRIFLUOPERAZINE-INDUCED HIGH MEMBRANE POTENTIAL ON $^{45}CaCl_2$ UPTAKE AFTER REMOVAL OF THE TRIFLUOPERAZINE

Preincubation media contained Mes-Tris buffer, pH 6.0 (20 mM), glucose (80 mM), [^{3}H]TPP$^+$ (1 μM, 0.05 μCi/ml) and trifluoperazine (TFP) when indicated. After 20 min of preincubation, cells were separated by centrifugation and suspended in the incubation media containing Mes-Tris buffer, pH 6.0 (20 mM), [^{3}H]TPP$^+$ (1 μM, 0.05 μCi/ml) or $^{45}CaCl_2$ (1 μM, 0.05 μCi/ml) or none. Glucose (80 mM), KCl (100 M) and valinomycin (20 μg/ml) were added when indicated. Incubation was terminated after 20 min. Values represent mean ± S.E. ($n = 4$).

Preincubation media	TPP$^+$ uptake (cpm)	K$^+$ in cells (g/cell) ($\times 10^{13}$)	Incubation media	TPP$^+$ uptake (cpm)	K$^+$ in cells (g/cell) ($\times 10^{13}$)	^{45}Ca uptake (cpm)
Glucose	1155 ± 54	2.21 ± 0.10	–	1001 ± 28	2.19 ± 0.08	1293 ± 31
			Glucose	1116 ± 34	2.21 ± 0.09	3347 ± 38
			KCL + glucose	420 ± 12	3.29 ± 0.06	647 ± 18
Glucose + TFP (50 μM)	30618 ± 798	0.55 ± 0.04	–	11110 ± 294	0.54 ± 0.1	14239 ± 524
			Glucose	14251 ± 312	0.57 ± 0.1	17674 ± 612
			KCl + glucose	700 ± 28	2.08 ± 0.08	1554 ± 27
			KCl – valinomycin	365 ± 11	2.14 ± 0.09	441 ± 18

min of incubation, the amounts of cellular K$^+$, $^{45}Ca^{2+}$ and [^{3}H]TPP$^+$ were determined. Slow dissipation of the membrane potential was observed in cells incubated with glucose, and somewhat faster in cells incubated without glucose. Cells which were preincubated with trifluoperazine accumulated, in the presence of glucose, 5.2-folds more $^{45}Ca^{2+}$ than the control, and somewhat lower accumulations were observed in the absence of glucose. Addition of 100 mM KCl, in the presence of glucose, caused rapid K$^+$ uptake and a decrease in the membrane potential. A more rapid decrease was observed when K$^+$ was added together with valinomycin.

Effect of trifluoperazine on proton ejection

Increased membrane potential by trifluoperazine may have been mediated by stimulation of the activity of H$^+$-ATPase leading to an increased rate of proton ejection, therefore, the effect of trifluoperazine on proton ejection was investigated. Proton movement was followed in the suspension of yeast cells in unbuffered medium in the presence of glucose by measurements of the medium pH every 15 s. Under our experimental conditions, a linear decrease in medium pH by 0.35 pH units/min was measured during the first 2 min after the addition of the yeast cells to the medium (Fig. 3a). Addition of 100 mM K$^+$ to the medium led to an increased rate of proton extrusion, trifluoperazine decreased the rate of pH changes, both in the presence and absence of KCl (Fig. 3).

A decreased rate of pH change may result from two different processes: (1) decreased activity of

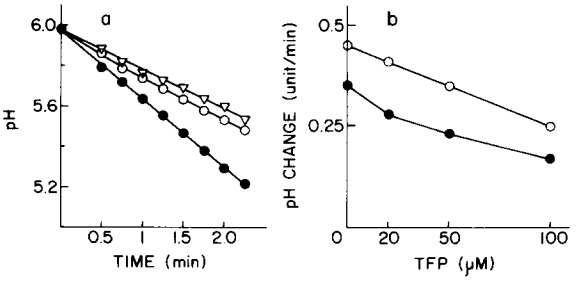

Fig. 3. The effect of trifluoperazine on the rates of proton translocation. Medium containing glucose (10 mM) was adjusted to pH 6.0 by addition of 1–2 μl of 2 μM NaOH, or HCl as required. Measurement was initiated by the addition of 1 ml yeast suspensions ($1 \cdot 10^8$ cells/ml) brought to pH 6.0 as above, to 1 ml medium; pH was recorded immediately after the addition of the cells and every 15 s up to 2 min. (a) The time-course of pH change: control (●); trifluoperazine 50 μM (○) or 100 μM (▽). (b) Rates of pH change. Rates were calculated from pH-change measurements between 0.5 to 1.5 min at different concentrations of trifluoperazine, in absence (●), or presence, of KCl (100 mM) (○).

TABLE IV

EFFECT OF TRIFLUOPERAZINE (TFP) ON PLASMA MEMBRANE ATPase ACTIVITY

The activity of plasma membrane ATPase was measured in aliquots of cell homogenate equal to 0.97 mg yeasts, in the presence of 10 mM NaN_3 (to inhibit mitochondrial ATPase) at pH 6.0. Hydrolysis of ATP was measured between 0 and 10 min of incubation at 30°C. The reaction rate was linear with time up to 15 min of incubation.

TFP (μM)	ATPase activity (nmol/min per mg yeasts)
0	7.1 ± 0.3
20	4.7 ± 0.2
50	2.6 ± 0.11
100	0.5 ± 0.03

the H^+-ATPase in ejecting protons or (2) increased permeability of cell membranes to protons leading to increased passive leak of protons into the cells. To decide between these two possible mechanisms, we measured the rates of pH change in the absence of glucose. Under these conditions, a slow influx of protons was manifested by a slow increase in medium pH. Addition of 50 μM trifluoperazine did not change the rate of this influx (not shown). These results exclude the possibility that trifluoperazine increased the passive permeability of cell membranes to protons.

Effect of trifluoperazine on the activity of plasma membrane ATPase

The results showing inhibition of proton ejection by trifluoperazine led to the suggestion that trifluoperazine inhibits the activity of the plasma membrane ATPase. This hypothesis was examined directly in crude membrane preparation at pH 6.0, in the presence of 10 mM NaN_3 to inhibit mitochondrial ATPase. It is shown in Table IV that trifluoperazine strongly inhibited the activity of the plasma membrane ATPase at concentrations above 20 μM.

Effects of glucose on the uptake of chloropromazine

The results reported in Tables I and II indicate that the effect of trifluoperazine and chlorpromazine on membrane potential is observed only in the presence of glucose. This result may indicate that the process(es) stimulated by trifluoperazine or chlorpromazine is energy-dependent. Alternatively, one may consider the possibility that the uptake of trifluoperazine or chlorpromazine into yeast cells is an energy-dependent process. To examine this possibility, we measured the uptake of [^{3}H]chlorpromazine in presence or absence of glucose. [^{3}H]Chlorpromazine was selected, since radioactive trifluoperazine is not available commercially, and chlorpromazine exerts effects similar to trifluoperazine (Table II and Ref. 4). The amounts of [^{3}H]chlorpromazine in the cells after 15 min of incubation with 50 μM (0.5 μCi/ml) [^{3}H]chlorpromazine were similar in the presence or absence of glucose ($42\,837 \pm 727$ cpm and $42\,147 \pm 652$ cpm, respectively). In the presence of 2,4-dinitrophenol uptake was reduced to $33\,588 \pm 548$ cpm, but the decrease was not large enough to explain the abolishment of trifluoperazine effect on TPP^+ accumulation. It is concluded, therefore, that the uptake of chlorpromazine (and probably also its analog trifluoperazine) is energy-independent. Thus, the glucose requirement for the expression of the effects of chlorpromazine or trifluoperazine may indicate stimulation of energy-requiring processes.

Discussion

It was found in the present work that trifluoperazine exerts the following effects in *S. cerevisiae*: (1) An increase in the membrane potential. (2) An inhibition of proton ejection. (3) An inhibition of the activity of the plasma membrane ATPase.

Increased membrane potential

The use of TPP^+ as a quantitative probe for measurements of membrane potential in our strain of *S. cerevisiae* is justified by the following procedure and results: (1) Corrections for TPP^+ binding was done by subtracting the cellular accumulation of TPP^+ in the presence of 2,4-dinitrophenol, from results obtained in the absence of 2,4-dinitrophenol. Since 2,4-dinitrophenol causes complete dissipation of membrane potential, the intra- and extra-cellular concentrations of TPP^+ in presence of 2,4-dinitrophenol should be equal. Cellular accumulation above this level was used as a measure for TPP^+ binding. Under our experimental conditions, the binding did not account for more

than 25% of the TPP^+ accumulation at the control. (2) Agents leading to dissipation of membrane potential, such as antimycin A and sodium azide [8] almost completely abolished the accumulation of TPP^+, not only in the control, but also in the trifluoperazine-treated cells. (3) The accumulated TPP^+ is immediately released upon addition of 2,4-dinitrophenol to the trifluoperazine-treated and untreated cells. (4) Removal of glucose from the medium or addition of 100 mM KCl or 1 mM $CaCl_2$ decreases TPP^+ accumulation in the control. (5) TPP^+ is not transported via the thiamine carrier. These results confirm the observation of De la Peña et al. [8] and justify the use of TPP^+ as a probe to measure membrane potential. Addition of trifluoperazine or chlorpromazine to the cells caused a quick accumulation of TPP^+. Membrane potential calculated from the ratio TPP^+(in)/TPP^+(out) show an increase from -100 mV in the control up to -178 mV in trifluoperazine-treated cells. This increase is absolutely dependent on the presence of glucose.

In our previous work, it was found that trifluoperazine generate a glucose-dependent efflux of K^+ from the cells [4]. In the literature, efflux of K^+ is shown to occur following ATP-depletion or addition of protonophores such as 2,4-dinitrophenol [12,13]. These conditions lead to a decrease in membrane potential due to shortage of ATP for H^+-ATPase function, or dissipation of $\Delta\psi$ by an influx of protons. Efflux of K^+ under these conditions is a passive process which appears to be caused by the decrease in membrane potential and does not lead to a substantial increase in membrane potential.

The simplest explanation to the increase in membrane potential in the presence of trifluoperazine would postulate the formation of 'passive' K^+ diffusion potential by K^+ leakage. However, this interpretation is incompatible with the results showing the requirement of glucose for K^+ efflux. Since the uptake of phenothiazine drugs into the cells is not affected by the presence or absence of glucose, it is suggested that the efflux of K^+ in the presence of TFP is an active process.

Under normal conditions, membrane potential in yeasts is maintained mainly by H^+-ATPase, which ejects protons from the cells, against their electrochemical gradient [14,15]. Our results show inhibition of both proton ejection and the activity of plasma membrane ATPase by trifluoperazine, therefore, the increase in the membrane potential by trifluoperazine cannot be mediated via this process. I suggest that the increase in membrane potential by trifluoperazine is mediated by activation of an electrogenic, energy-dependent K^+ extrusion pump. The presence of an electrogenic K^+ transport system has been suggested previously in yeasts [16]. Recently, K^+ influx system coupled to ATP hydrolysis was reconstituted in proteoliposomes [17]. The electrogenic K^+ extrusion system activated by phenothiazines may operate via the reversal of the K^+ influx system or via a separate system.

The trifluoperazine-induced increase in $\Delta\psi$ is inhibited by the presence of high concentrations of cations in the medium. The finding that KCl (100 mM) abolishes the effect of trifluoperazine on $\Delta\psi$ might have been interpreted as being due to increased influx of K^+ into the cells, which neutralizes the increase in $\Delta\psi$. However, 1 mM $CaCl_2$ also diminishes trifluoperazine -effect on $\Delta\psi$, and almost completely abolishes the effect of trifluoperazine on K^+ efflux. If the decrease in trifluoperazine effect on membrane potential was the result of cation-influx, one should have seen the persistence of K^+ efflux and enhanced Ca^{2+} influx in the presence of 1 mM CaCl, but this situation was not observed. On the contrary, it is shown in the previous work [4] that 1 mM $CaCl_2$ abolishes the effect of trifluoperazine both on K^+ efflux and on Ca^{2+} influx. It should be concluded, therefore, that high concentrations of K^+ or Ca^{2+} (and also Na^+ or Mg^{2+} [4]) in the medium prevent the activation of K^+ efflux system by trifluoperazine.

Trifluoperazine effect on Ca^{2+} influx

Trifluoperazine stimulates Ca^{2+} influx from media with low Ca^{2+} concentrations (1 μM) [4]. Ca^{2+} uptake may be stimulated via K^+/Ca^{2+} antiporter found to function during Ca^{2+} efflux in *S. cerevisiae* [18]. A different interpretation may postulate that Ca^{2+} uptake is stimulated as a result of the increase in $\Delta\psi$, which drives the influx of Ca^{2+}. To decide between these two possible mechanisms, we disassociated between the K^+ efflux and Ca^{2+} influx in time. Cells were first allowed to generate $\Delta\psi$ in the absence of Ca^{2+}, then trifluo-

perazine was removed, K^+ efflux ceased, and $^{45}Ca^{2+}$ was added (Table III). Cells preincubated with trifluoperazine, which maintained higher $\Delta\psi$, accumulated higher amounts of Ca^{2+} than cells preincubated without trifluoperazine. These results indicate that the increase influx of Ca^{2+} is mediated by the increased $\Delta\psi$, and not by K^+/Ca^{2+} antiport. Our results show direct correlation between changes in $\Delta\psi$ and Ca^{2+} influx (Fig. 2, Table I) and support the assumption that Ca^{2+} influx is energized by $\Delta\psi$ [7,19,20]. It should be mentioned, however, that in addition to $\Delta\psi$ other factors, such as cell pH and surface potential, have been shown to affect Ca^{2+} uptake in yeasts [20].

Inhibition of proton ejection and the activity of the plasma membrane ATPase

The inhibition of proton extrusion by trifluoperazine seems to be mediated by different mechanisms than the increase in $\Delta\psi$, since the effect on pH-change persists in the presence of 100 mM KCl in the medium. The possibility of increase permeability to protons in the presence of trifluoperazine is excluded by the experiment showing no increase in the permeability to protons by trifluoperazine in the absence of glucose. It may still be argued that the increased $\Delta\psi$ in the presence of glucose drives an increased influx of protons into the cells, thus, the decreased rate of pH change in the presence of trifluoperazine (and glucose) may be the result of increased $\Delta\psi$. Another interpretation, may assume that K^+ efflux activates K^+/H^+ exchange, leading to proton influx. The results in the presence of 100 mM KCl eliminate these possible interpretations, since under these conditions, $\Delta\psi$ is not increased by trifluoperazine and K^+ efflux does not occur, yet rates of pH-change are inhibited. It is concluded, therefore, that trifluoperazine cause inhibition of proton ejection. Direct measurements of the activity of plasma membrane ATPase in crude membrane preparation had shown that trifluoperazine strongly inhibits the activity of this enzyme. It appears, that similarly to diethylstilbesterol and dicyclohexylcarbodiimide [23], trifluoperazine displays inhibition of both proton ejection and ATPase activity.

In mammalian cells, it was reported that trifluoperazine inhibits various ATPase, both calmodulin-dependent, such as Ca^{2+}-ATPase [1,21] and calmodulin-independent, such as Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase [22]. Thus, the inhibition of ATPase activities and functions by phenothiazines seems to be a general phenomenon.

Finally, it should be mentioned that the analog of trifluoperazine, trifluoperazine sulfoxide, which does not bind to calmodulin and therefore, does not inhibit calmodulin-dependent functions, has no effect on our cells. However, the involvement of calmodulin in the mechanisms altered by trifluoperazine in *S. cerevisiae* cannot, as yet, be determined.

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