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MEMBRANE EFFECTS OF PHENOTHIAZINES IN YEASTS

I. STIMULATION OF CALCIUM AND POTASSIUM FLUXES

YAEL EILAM

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

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Application of trifluoperazine (10–50 μM) to suspensions of the yeast *Saccharomyces cerevisiae* induces the following effects. (1) A marked increase in the initial rate of $^{45}Ca^{2+}$ influx into the cells, accompanied by an increase in the cellular content of calcium. This stimulation in $^{45}Ca^{2+}$ influx (10–20-fold) is observed only in the presence of a metabolic substrate and is completely inhibited by $LaCl_3$. The dose-reponse curves of the cellular accumulation of $^{45}Ca^{2+}$ are of a bell shape, indicating a biphasic response. The concentration of the drug yielding maximal accumulation depends on the density of the cells in the suspensions. The results indicate that the stimulation of $^{45}Ca^{2+}$ influx is mediated by an energy-dependent carrier-mediated process and not by the increase in the passive membrane permeability to Ca^{2+}. (2) Efflux of K^+ from the cells is induced. Removal of metabolic substrate abolishes the effect at concentrations of up to 35 μM and reduces it at higher concentrations. Addition of high concentrations of cations (K^+, Na^+, Mg^{2+}) to the medium abolishes the stimulation of both K^+ efflux and Ca^{2+} influx. Chloropromazine, thioridazine and chlorprothixen display similar effects, but at higher concentrations. The results are discussed in terms of two possible alternative mechanisms; (1) calmodulin-independent effects of trifluoperazine on cell membranes, or (2) inhibition of some calmodulin-dependent processes by low concentrations of trifluoperazine.

Introduction

Phenothiazines and related compounds have been used for years as tranquilizers and antipsychotic drugs. Recently, it has been found that these drugs bind to calmodulin in the presence of Ca^{2+}, and inhibit its regulatory functions [1–3]. Consequently, phenothiazines have been used in many studies as a probe to demonstrate the role of calmodulin in various cellular processes [4,5]. However, due to the hydrophobic nature of these drugs, various effects unrelated to the inhibition of calmodulin have been reported [11].

Calmodulin, the Ca^{2+} receptor protein, is present in almost every eukaryotic cell examined [6]. Recently, the presence of calmodulin has been demonstrated in the yeast *Saccharomyces cerevisiae* [7]. At this stage, it is impossible to determine whether the observed effects of phenothiazines are mediated via inhibition of calmodulin, or by other mechanisms derived from the interaction of the drugs with cell membranes. However, in this article, we describe some striking effects of phenothiazines on Ca^{2+} and K^+ transmembrane fluxes in yeast.

Methods

Organism and culture conditions

S. cerevisiae strain 124 (genotype MAP a/α

Abbreviation: Mes, 4-morpholineethanesulfonic acid.

his1) was maintained at 4°C on 1.5% w/v agar containing 1% yeast extract, 2% glucose and 2% peptone. Prior to the experiment, cells were inoculated into medium I, comprising Bacto yeast extract (10 g/l), Bacto peptone (20 g/l) and glucose (20 g/l). The yeast was grown overnight with shaking (200 rev./min) at 30°c.

Ca^{2+} influx

Cells grown overnight in medium I were collected by centrifugation, washed three times by resuspension in distilled water and finally resuspended in distilled water at $2 \cdot 10^8$ cells/ml. Cell suspensions and the indicated media were equilibrated for 15 min at 30°C with shaking. The experiments were initiated by the addition of the cells to the indicated media, which also contained $^{45}CaCl_2$ at a concentration of 1 μM $CaCl_2$ (1 μCi/ml). The final cell density was $5 \cdot 10^7$ cells/ml or as indicated. The suspensions were shaken at 30°C throughout the experiments. 1-ml samples were removed at the indicated times and filtered on Sartorius membrane filters (0.45 μm pore size) which had been prewashed with 20 mM $MgCl_2$. The cells on the filters were quickly washed four times with 20 mM $MgCl_2$ (20 ml). Blank filters through which 1 ml medium without cells had been filtered were similarly washed and counts remaining on the filters were subtracted from the results. It was previously reported [8] and later confirmed in the yeast *S. cerevisiae* (Eilam, unpublished results) that after such an Mg^{2+} wash, the amount of $^{45}Ca^{2+}$ adsorbed to the cells when incubated at 2°C is very small. The filters were dried and radioactivity was determined in toluene-containing scintillation fluid.

Determination of the cellular content of K^+ and Ca^{2+}

For the determination of the cellular content of K^+, experiments were carried out as above, except that the media contained 1 μM unlabelled $CaCl_2$, the filters were prewashed with distilled water and the cells on the filters were washed four times with distilled water instead of $MgCl_2$. After filtration, each filter was immersed in 3 ml distilled water and boiled to release the ions from the cells, and the suspensions were centrifuged to precipitate the debris. K^+ was determined using a Perkin-Elmer atomic absorption spectrometer after appropriate dilution.

For the determination of cellular content of Ca^{2+}, experiments were carried out as above, but in larger volumes of media (35 ml).

The cells, suspended at $5 \cdot 10^7$ cells/ml, were collected by centrifugation and washed once with $MgCl_2$ (20 mM) at 2°C and twice with distilled water at 2°C, by resuspension and centrifugation. The pellets were suspended in 0.75% $LaCl_3$ and boiled for 10 min to release the Ca^{2+}. The suspensions were then centrifuged to precipitate the debris and Ca^{2+} contents were determined in the supernatant using a Perkin-Elmer atomic absorption spectrometer. Standard solutions of $CaCl_2$ used for calibration also contained 0.75% $LaCl_3$.

Trifluoperazine, chlorpromazine and antimycin A were obtained from Sigma; chlorprothixene and thioridazine from Taro Pharmaceutic Industry, Haifa, Israel; $^{45}CaCl_2$ (20 mCi/mg calcium) from Amersham International, U.K.

The values in the figures represent mean ± S.E. ($n = 4$).

Results

Trifluoperazine-induced $^{45}Ca^{2+}$ influx

Low concentrations of trifluoperazine caused marked stimulation of $^{45}Ca^{2+}$ influx into yeast cells (Fig. 1). The stimulation was observed within 2 min of the addition of the drug (Table I), and the effect did not increase after preincubation of the cells with trifluoperazine (Table II). Omission of glucose from the medium and addition of antimycin A, a respiratory inhibitor, completely abolished the stimulation of $^{45}Ca^{2+}$ influx by trifluoperazine (Table II). The initial rates of Ca^{2+} influx were markedly increased when the concentration of trifluoperazine in the medium was raised from 0 to 100 μM. Similar results were observed using suspensions at different cell densities (Table I). However, at the lower cell density ($2 \cdot 10^7$ cells/ml) concentrations of trifluoperazine above 20 μM induced efflux of the accumulated $^{45}Ca^{2+}$ (Fig. 2). The balance between the two processes, i.e., the increased initial rate of Ca^{2+} influx and the efflux of the accumulated $^{45}Ca^{2+}$, led to a bell-shaped appearance of the curves showing the amount of $^{45}Ca^{2+}$ in the cells after 20

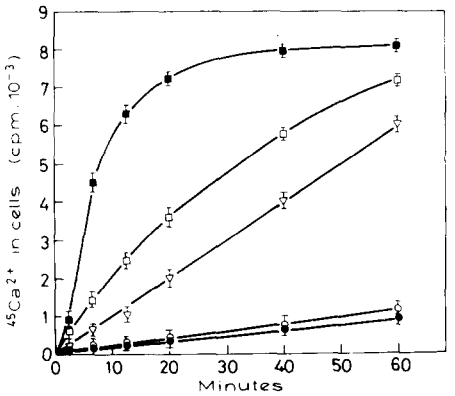

Fig. 1. The time-course of $^{45}Ca^{2+}$ influx in the presence of different concentrations of trifluoperazine. Cells were incubated at a density of $6 \cdot 10^7$ cells/ml in media containing 20 mM Mes-Tris buffer (pH 6.0), 100 mM glucose, 1 μM $^{45}CaCl_2$, (1 μCi/ml), and the following concentrations of trifluoperazine (μM): zero (●); 1 (○); 10 (▽); 20 (□); and 50 (■). Values represent mean ± S.E. of four measurements.

TABLE II

THE EFFECT OF PREINCUBATION AND OF GLUCOSE REMOVAL ON THE TRIFLUOPERAZINE-INDUCED $^{45}Ca^{2+}$ INFLUX

Cells were incubated at a density of $5 \cdot 10^7$ cells/ml in media containing 20 mM Mes-Tris (pH 6.0), glucose and trifluoperazine when indicated. Media without glucose contained 20 μM antimycin. Preincubation (60 min at 30°C) was done in media containing glucose or antimycin, and trifluoperazine (when appropriate). Experiments were initiated by the addition of $^{45}CaCl_2$, and terminated after 2 min. Values represent mean ± S.E. ($n = 4$).

Medium glucose (100 mM)	Preincubation	$^{45}Ca^{2+}$ in cells (cpm/2 min)	
		− trifluoperazine	+ trifluoperazine
+	−	872 ± 38	4034 ± 189
−	−	415 ± 21	482 ± 24
+	+	721 ± 31	4108 ± 30
−	+	312 ± 18	308 ± 14

min of incubations with increasing concentrations of trifluoperazine. This bell-shaped response was evident mainly in suspensions of low cell density (Fig. 3).

Since $^{45}Ca^{2+}$ influx measurements may represent $^{45}Ca^{2+}/^{40}Ca^{2+}$ exchange, as well as net Ca^{2+} influx, measurements were made by the atomic absorption spectrometer to determine changes in the content of cell calcium. An increase in cell calcium content was observed after 20 min incubation with 20 and 50 μM trifluoperazine (Fig. 4).

Trifluoperazine-induced K^+ efflux

Low concentrations of trifluoperazine caused a

TABLE I

THE EFFECT OF TRIFLUOPERAZINE ON THE INITIAL RATE OF Ca^{2+} INFLUX

Cells were incubated at the indicated densities in media containing 20 mM Mes-Tris, (pH 6.0), 100 mM glucose, trifluoperazine at the indicated concentrations, and 1 μM $^{45}CaCl$. Incubation was terminated after 2 min. The values were calculated from the specific activity of the medium and the number of counts in the cells and are presented as 10^{-19} mol/min per cell. Calcium uptake was linear with time for at least 5 min, using all indicated concentrations of trifluoperazine. Values represent mean ± S.E. ($n = 4$).

Trifluoperazine (μM)	Cell density (cells/ml)	
	$6 \cdot 10^7$	$2 \cdot 10^7$
0	1.08 ± 0.06	1.02 ± 0.05
1	1.20 ± 0.05	–
10	2.05 ± 0.09	2.53 ± 0.08
20	6.56 ± 0.32	6.75 ± 0.25
50	9.18 ± 0.41	10.75 ± 0.94
100	22.15 ± 0.98	21.50 ± 1.12

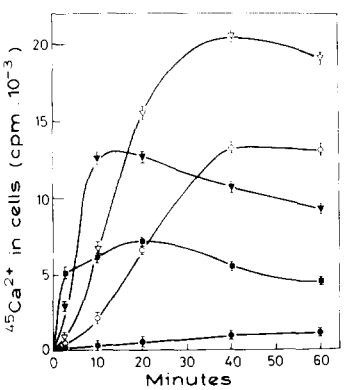

Fig. 2. The effect of low cell density on the time-course of ^{45}Ca influx induced by trifluoperazine. Cells were incubated at a density of $2 \cdot 10^7$ cells/ml in media containing 20 mM Mes-Tris buffer (pH 6), 100 mM glucose, and trifluoperazine at the following concentrations (μM): zero (●); 10 (○); 20 (▽); 50 (▼); 100 (■).

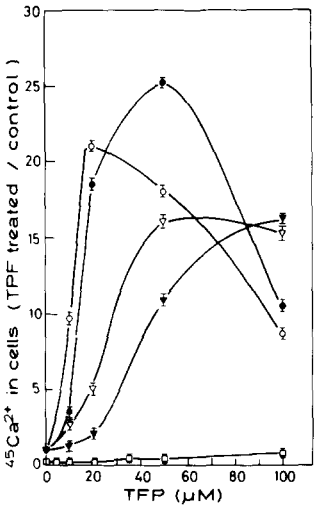

Fig. 3. The effect of cell density on the trifluoperazine-induced $^{45}Ca^{2+}$ accumulation. Cells were incubated at densities of: $2 \cdot 10^7$ (○); $4 \cdot 10^7$ (●); $6 \cdot 10^7$ (▽); and $8 \cdot 10^7$ (▼) cells/ml in media containing 20 mM Mes-Tris buffer (pH 6.0), 100 mM glucose; 1 μM $^{45}CaCl_2$ (1 μCi/ml) and the indicated concentration of trifluoperazine (TFP). At cell density of $6 \cdot 10^7$ cells/ml, cells were also incubated in medium without glucose and with 20 μM antimycin A (□). Incubation was terminated after 20 min.

rapid efflux of K^+ from yeast cells. 41% of all K^+ was lost after 20 min incubation with 35 μM trifluoperazine and 67% was lost when the concentration of trifluoperazine was raised to 50 μM (Fig. 5). Omission of glucose and addition of antimycin A to the medium reduced the effect of trifluoperazine; 35 μM trifluoperazine exerted no effect and 50 μM trifluoperazine caused a 15% decrease in cell K^+ (Fig. 5). Suspensions at cell

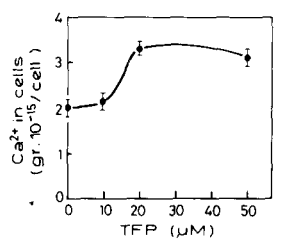

Fig. 4. The effect of trifluoperazine on the cellular content of calcium. Cells were incubated at a density of $5 \cdot 10^7$ cells/ml in media containing 20 mM Mes-Tris (pH 6.0), 100 mM glucose, 1 μM $CaCl_2$, and the indicated concentration of trifluoperazine. Ca^{2+} content was determined as described in Methods.

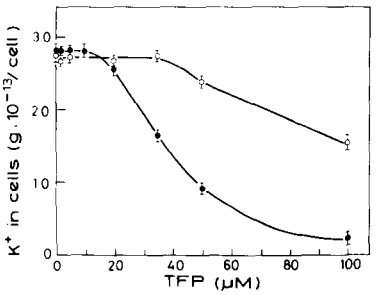

Fig. 5. Dose-response curves for the effect of trifluoperazine (TFP) on K^+ efflux. Cells were incubated at a density if $6 \cdot 10^7$ cells/ml in media containing 20 mM Mes-Tris buffer (pH 6), 1 μM $CaCl_2$, the indicated concentrations of trifluoperazine, and 100 mM glucose (●), or 20 μM antimycin A (○). Incubation was terminated after 20 min.

densities $(2\text{–}6) \cdot 10^7$ cell/ml displayed similar dose-response curves, whereas at $8 \cdot 10^7$ cells/ml, lower sensitivities to trifluoperazine were observed (not shown).

Effect of medium composition on trifluoperazine-induced ion fluxes

Addition of K^+ to the incubation medium markedly reduced the effect of trifluoperazine on both Ca^{2+} influx and K^+ efflux (Fig. 6). A similar reduction in the induced ion fluxes was observed when the concentration of Ca^{2+} in the medium was raised to 10^{-3} M (Fig. 7) or when 100 mM

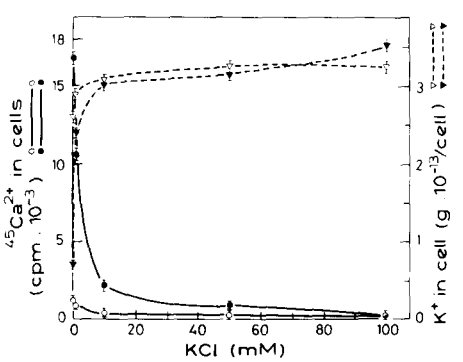

Fig. 6. The effect of trifluoperazine in the presence of K^+. Cells were incubated at a density of $5 \cdot 10^7$ cells/ml in media containing 20 mM Mes-Tris (pH 6.0), 1 μM $CaCl_2$ or $^{45}CaCl_2$, 100 mM glucose. The indicated concentration of KCl and 50 μM trifluoperazine (full symbols) or no trifluoperazine (empty symbols). Incubation was terminated after 20 min. ○, ●, $^{45}Ca^{2+}$ in cells; △, ▲, K^+ in cells.

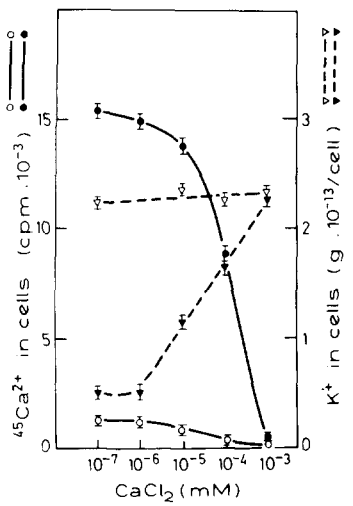

Fig. 7. Effect of trifluoperazine in the presence of different concentrations of $CaCl_2$. Conditions as in Fig. 6; but media contained the indicated concentration of $CaCl_2$ instead of KCl.

NaCl or 1 mM $MgCl_2$ was included in the medium (Table III). $LaCl_3$, an inhibitor of Ca^{2+} influx, completely inhibited the trifluoperazine-induced Ca^{2+} influx, but only partly inhibited the trifluoperazine-induced K^+ efflux (Table III).

Effects of related compounds

Finally, we examined the effect of other phenothiazines on ion fluxes. Chlorpromazine exerted similar effects as trifluoperazine on $^{45}Ca^{2+}$ influx, but smaller effects on K^+ efflux. Chlorprothixen was less effective than trifluoperazine; only at 200 μM did we observe a 5-fold increase in $^{45}Ca^{2+}$ influx but no K^+ efflux. Thioridazine showed effects similar to those of trifluoperazine but at higher concentrations (Fig. 8a–d).

TABLE III

THE EFFECT OF MEDIUM COMPOSITION ON TRIFLUOPERAZINE-INDUCED $^{45}Ca^{2+}$ ACCUMULATION

Cells were incubated for 20 min at a density of $5 \cdot 10^7$ cells/ml, in media containing 20 mM Mes-Tris (pH 6.0), 100 mM glucose 1 μM $CaCl_2$ or $^{45}CaCl_2$, 50 μM trifluoperazine when indicated, and the indicated ions. Values represent mean ± S.E. ($n = 4$).

Medium ions	$^{45}Ca^{2+}$ in cells (cpm/20 min)		K^+ in cells (g/cell) ($\times 10^{13}$)	
	− trifluoperazine	+ trifluoperazine	− trifluoperazine	+ trifluoperazine
Control	910 ± 41	26552 ± 258	2.32 ± 0.15	0.40 ± 0.04
NaCl (10 mM)	514 ± 28	3116 ± 121	2.34 ± 0.18	1.70 ± 0.08
NaCl (100 mM)	235 ± 15	773 ± 32	2.16 ± 0.13	2.28 ± 0.18
$MgCl_2$ (1 mM)	491 ± 22	2794 ± 103	2.44 ± 0.19	1.74 ± 0.10
$LaCl_3$ (0.1 mM)	172 ± 8	240 ± 18	2.39 ± 0.16	1.26 ± 0.08

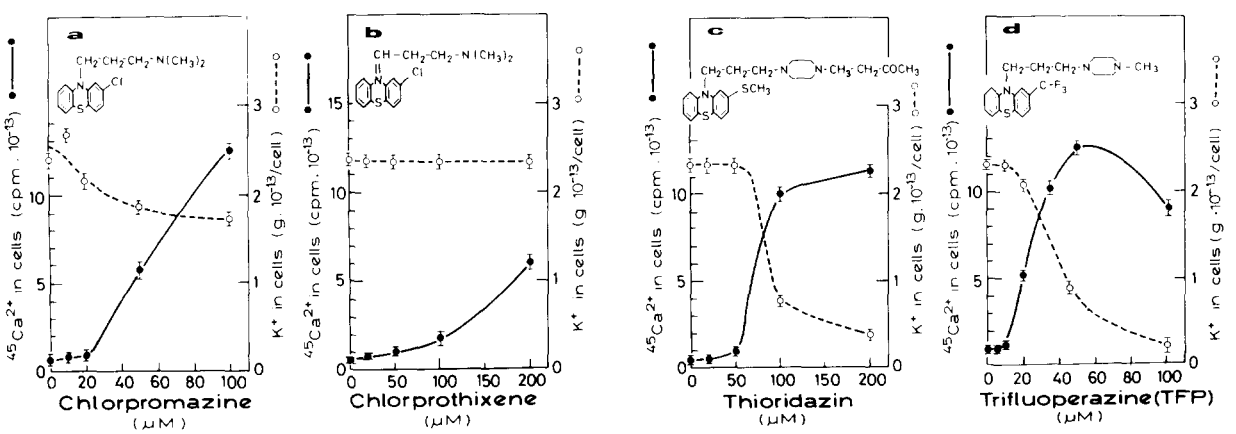

Fig. 8. The effects of different phenothiazines on K^+ efflux and ^{45}Ca influx. Cells were incubated at $5 \cdot 10^7$ cells/ml in media containing 20 mM Mes-Tris (pH 6.0), 100 mM glucose, 1 μM $CaCl_2$ or $^{45}CaCl_2$ and the indicated phenothiazine. Incubation was terminated after 20 min. ●, $^{45}Ca^{2+}$ in cells, ○, K^+ in cells.

Discussion

The present study has demonstrated that trifluoperazine and some related compounds induced a marked increase in K^+ efflux and Ca^{2+} influx in yeast. Two possible mechanisms could account for these results. (a) Trifluoperazine may induce an increase in the passive permeability to K^+ and Ca^{2+} causing leakage of K^+ from the cells and leakage of Ca^{2+} into the cells due to the low concentration of Ca^{2+} within the cytosol. Such effects were observed using nystatin [9]. (b) Alternatively, trifluoperazine may stimulate the carrier-mediated Ca^{2+} influx and possibly also the carrier-mediated K^+ efflux.

The results do not support the first possible mechanism for Ca^{2+} influx for two reasons. (a) Stimulation of Ca^{2+} influx by trifluoperazine requires a metabolic substrate. No stimulation is observed without glucose and in the presence of antimycin A. On the other hand, increase in the passive influx of Ca^{2+}, as induced by nystatin, does not require metabolic energy [9]. It is unlikely that energy is required for the uptake of the drugs into the membranes considering the hydrophobic nature of these drugs, and the energy-independent effects of phenothiazines in mammalian cells [11]. In yeasts, the partial persistence of the drug-effect on K^+ leakage in the absence of glucose indicates that the uptake of the drug is energy-independent. (b) La^{3+} is an inhibitor of the carrier-mediated Ca^{2+} influx [10]. The presence of $LaCl_3$ in the medium does not inhibit the nystatin-induced Ca^+ fluxes [9] but completely inhibits the trifluoperazine-induced Ca^{2+} influx. The possibility that La^{3+} interferes with the interaction of the drug with the membrane is excluded by the results showing the persistence of part of the trifluoperazine effects on K^+ efflux in the presence of La^{3+}.

It is, therefore, reasonable to conclude that the stimulation of Ca^{2+} influx by trifluoperazine is mediated by an energy-dependent increase in the rate of the carrier-mediated Ca^{2+} influx. This increase $^{45}Ca^{2+}$ influx does not merely represent isotope exchange, but also an increase in the net amout of Ca^{2+} within the cells.

The bell-shaped nature of the dose-response curves, showing the amounts of $^{45}Ca^{2+}$ in the cells after 20 min of incubation, reflects the balance between two effects of drug: (1) an increase in the initial rate of $^{45}Ca^{2+}$ influx; and (2) increase in the efflux of the accumulated $^{45}Ca^{2+}$. The second effect became evident mainly at trifluoperazine concentrations above 20 μM, using suspensions of low cell density. Bell-shaped curves have previously been observed for the dose-responses of the effects of chlorpromazine on membrane stability in erythrocytes and in other membranes [11]. The dependence of the dose-response curves on the cell density is probably due to the effect of the number of the cells on the concentrations of the drug within the membranes.

Interpretation of the drug effects on the stimulation of K^+ efflux is more ambiguous. Removal of metabolic substrate abolished the effect of trifluoperazine at low concentrations (up to 35 μM) and reduced it at higher concentrations. The results may be interpreted in terms of a biphasic response. At low concentrations of trifluoperazine, the results probably represent stimulation of the carrier-mediated, energy-dependent K^+ extrusion, whereas above 35 μM, an increase in the passive membrane permeability to K^+ is also observed.

The reason for the inhibition of the trifluoperazine effects by high concentrations of cations in the medium is also not clear. One possibility is that cations interfere with the drug-membrane interactions, but conclusive evidence is still lacking.

Phenothiazines are recognized as calmodulin inhibitors [3]. In many systems where calmodulin stimulates Ca^{2+} fluxes [12] such as Ca^{2+}-ATPase in erythrocytes [13–15], phenothiazines exert inhibitory effects on Ca^{2+} fluxes. Inhibition of Ca^{2+} influx by phenothiazines is also observed in some secretory cells, although the role of calmodulin in these processes has not yet been determined [16–19]. The present results in yeasts showing stimulation of Ca^{2+} fluxes by trifluoperazine are contrary to the general pattern in higher eukaryotic cells.

Two alternative mechanisms may be suggested for the interpretation of the results:

(A) The observed trifluoperazine effects may be mediated via calmodulin-independent reactions. Recently, several studies have shown calmodulin-independent effects of phenothiazines in mammalian cells, in disruption of the mitochondrial energy production [20], inhibition of calmodulin-

insensitive ($Na^+ + K^+$)- and Mg^{2+}-ATPase activities [21], and probably also in the neuroleptic effects of phenothiazines [22]. Chlorpromazine has been found to induce the following changes in mammalian cell membranes [11]: (a) protection of erythrocytes against osmotic hemolysis [23,24]; (b) expansion of membrane area [11,25]; (c) displacement of membrane-bound Ca^{2+} [26]; (d) decrease in the passive influx of Na^+ in erythrocytes in the presence of Ca^{2+}, and increase in the passive permeability in the absence of Ca^{2+} [27]; (e) shape change in erythrocytes [28]; and (f) phase transition in membranes leading to increased membrane fluidity [29,30]. None of the above effects can adequately explain the increase in Ca^{2+} influx in yeast, except perhaps the increase in the fluidity of the membrane.

(B) Recently, calmodulin has been demonstrated in the yeast *S. cerevisiae* [7]. This finding may raise the possibility that the stimulation of the initial rate of $^{45}Ca^{2+}$ influx and the energy-dependent K^+ efflux are mediated via inhibition of some calmodulin-dependent processes. This would imply that calmodulin may be involved in the regulation of the transmembranal fluxes of K^+ and $^{45}Ca^{2+}$ in *S. cerevisiae*. At concentrations above 35 μM, it is likely that some nonspecific drug effects may, perhaps by damage to the cell membrane, lead to the loss of accumulated $^{45}Ca^{2+}$ and to the energy-independent K^+ efflux. Further work on the mechanism of the effect of phenothiazines in yeasts is now in progress.

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