Active Extrusion of Potassium in the Yeast *Saccharomyces cerevisiae* Induced by Low Concentrations of Trifluoperazine

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Trifluoperazine (TFP), the antipsychotic drug, induces substantial K^+ efflux, membrane hyperpolarization and inhibition of H^+ -ATPase in the yeast *Saccharomyces cerevisiae*. Investigations on the mechanism of these effects revealed two different processes observed at different incubation conditions. At an acidic pH of 4.5 and an alkaline pH of 7.5, K^+ efflux was accompanied by substantial proton influx which led to intracellular acidification and dissipation of $\Delta\psi$ formed by cation efflux. The results indicated nonspecific changes in membrane permeability. Similar results were also observed when cells were incubated at pH 5.5–6.0 with higher concentrations of TFP (above $75\ \mu M$). On the other hand, low concentrations of TFP (30–50 μM) at pH 5.5–6.0 caused marked membrane hyperpolarization and K^+ efflux unaccompanied by the efflux of other cations and by H^+ influx. Our experiments indicate that under these conditions K^+ efflux was an active process. (1) K^+ efflux proceeded only in the presence of a metabolic substrate and was inhibited by metabolic inhibitors. (2) When 0.3–0.9 mM-KCl was present in the medium at pH 6.0, the concentration of K^+ within the cells (measured at the end of the incubation with TFP) was much lower than the theoretical concentration of K^+ if the distribution of K^+ between medium and cell water was at equilibrium (at zero electrochemical gradient). (3) Valinomycin decreased the net K^+ efflux and decreased the membrane hyperpolarization induced by TFP, probably by increasing the flux of K^+ into the cells along its electrochemical gradient. (4) Conditions which led to active K^+ efflux also led to a marked decrease in cellular ATP level. The results indicate that under a specific set of conditions TFP induces translocation of K^+ against its electrochemical gradient.

INTRODUCTION

Trifluoperazine (TFP), the antipsychotic drug, is known to inhibit calmodulin-dependent regulatory function (Levin & Weiss, 1977; Weiss & Levin, 1978; Weiss *et al.*, 1980), and to induce several calmodulin-independent effects on the cell membranes of higher eukaryotic cells (Seeman, 1972; Luthra, 1982; Im *et al.*, 1984). Our recent investigations on the effects of phenothiazines on yeast, a lower eukaryote, revealed that application of low concentrations of TFP to these cells caused substantial K^+ efflux and membrane hyperpolarization. *In vitro* experiments showed inhibition of the activity of the proton-translocating ATPase by TFP (Eilam, 1983, 1984). The effects of TFP on K^+ efflux and on membrane potential were not observed in the absence of a metabolic substrate, or in the presence of metabolic inhibitors in the medium. This finding could not be accommodated by the simple explanation that TFP induced K^+ efflux by damaging cell membranes, or by some other mechanism which led to a passive K^+ leakage along its electrochemical gradient, and thereby increased $\Delta\psi$ (negative inside).

In the present study we have investigated the mechanism of action of TFP on yeast cells, in an attempt to determine whether TFP-dependent K^+ efflux is an active or a passive process.

Abbreviations: TPP⁺, tetraphenylphosphonium ion; TFP, trifluoperazine.

METHODS

Organism and culture conditions. *Saccharomyces cerevisiae* strain N123 (genotype *MATa/αhis1*) was maintained at 4 °C on YPD-agar slopes and grown at 30 °C in YPD-broth (Bacto yeast extract, 10 g l⁻¹; Bacto peptone, 20 g l⁻¹; glucose 20 g l⁻¹). 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 a cell density of 5×10^7 cells ml⁻¹.

Determination of K⁺, Na⁺ and Mg²⁺ contents. Suspensions of cells (5×10^7 ml⁻¹) were incubated in the indicated medium with continuous shaking. After 30 min, samples (2 ml) were removed and filtered through Sartorius membrane filters (0.45 µm pore size, prewashed with distilled water); the cells on the filters were washed five times with 3 ml distilled water in less than 2 min. After filtration, each filter was immersed in 3 ml distilled water which was then boiled to release the ions from the cells and centrifuged to precipitate the debris. K⁺ and Na⁺ concentrations in the supernatant were determined, after appropriate dilution, using a Perkin-Elmer atomic absorption spectrometer. Mg²⁺ was determined similarly after the addition of LaCl₃ (0.75%) to the supernatant.

Measurements of tetraphenylphosphonium ion (TPP⁺) uptake. Suspensions of cells (5×10^7 ml⁻¹) in the indicated medium containing [³H]TPP⁺ [1 µM; 0.05 µCi ml⁻¹ (1.85 kBq ml⁻¹)] were incubated at 30 °C with continuous shaking. After 30 min, 2 ml ice cold MgCl₂ (20 mM) was added to 2 ml cell suspensions, which were immediately filtered through glass fibre filters (Sartorius); the filters were immediately washed four times with 2 ml portions of ice cold MgCl₂ solution (20 mM). The Mg²⁺ wash diminished the adsorption of TPP⁺ to cell walls. The filters were dried, and the radioactivity was determined after the addition of toluene-based scintillation fluid, using a liquid scintillation counter.

The amounts of TPP⁺ bound to the cells were determined (for the calculations of the correction coefficients) by two methods. (1) Cells were aerated in MES/Tris buffer, pH 6.0 (20 mM) at 30 °C for 3 h. [³H]TPP⁺ [1 µM; 0.05 µCi ml⁻¹ (1.85 kBq ml⁻¹)] was added to these cells together with antimycin (15 µM), deoxyglucose (15 mM) and 2,4-DNP (1 mM). TPP⁺ uptake was determined as above after 1, 2 and 4 h. Equilibrium distribution of TPP⁺ was attained after 1 h in our strain. Therefore, a 1 h incubation period was routinely used in determinations of TPP⁺ binding. After this period, the cell suspensions were filtered, the filters were dried, and the radioactivity of the de-energized cells (TPP⁺ binding) was determined. (2) Cells were incubated for 1 h in medium containing MES/Tris, pH 6.0 (20 mM), glucose (80 mM), and [³H]TPP⁺ [1 µM; 0.05 µCi ml⁻¹ (1.85 kBq ml⁻¹)]. The cells were filtered as described above and washed four times with MgCl₂ (20 mM). The filters with the cells were each immersed in a flask containing 3 ml distilled water, and the cells were resuspended by a vortex shaker. Methanol (4 ml) was added to each flask and the cell suspensions were incubated for 5 min at room temperature to release the unbound TPP⁺. The cells were collected on glass fibre filters, and washed once with 60% (v/v) methanol and four times with water. The filters were dried and the radioactivity was determined. Both methods yielded similar correction coefficients as calculated according to Boxman *et al.* (1984). The extracellular concentrations of TPP⁺ were determined by incubating the yeast cells under the conditions of the experiment, precipitating the cells by centrifugation, and measuring the radioactivity in the supernatant.

Determination of the water volume of the cells. The water volumes were determined according to Rottenberg (1979). Cells were suspended (2×10^9 cells ml⁻¹) in medium containing MES/Tris pH 6.0 (10 mM), glucose (80 mM), ³H₂O (5 µCi ml⁻¹; 185 kBq ml⁻¹) and [¹⁴C]sorbitol (0.5 µCi ml⁻¹; 18.5 kBq ml⁻¹) and incubated for 10 min at room temperature. Samples (0.6 ml) were removed and centrifuged in a microfuge; 0.1 ml of each supernatant was then transferred to 1 ml HClO₄ (1 M). The rest of the supernatant was removed and 1 ml HClO₄ (1 M) was added to the cell pellets. The cells were resuspended and incubated for 30 min. All samples in HClO₄ were centrifuged again and the radioactivity in the supernatant was determined. The volume of the cells was calculated according to the equation

$$[(^3\text{H}_p/^3\text{H}_s) - (^{14}\text{C}_p/^14\text{C}_s)] \times V = V_i$$

where ³H_p and ¹⁴C_p are the radioactivities in the pellet, ³H_s and ¹⁴C_s are the radioactivities in the supernatant, *V* is the volume of the sample used, and *V_i* is the intracellular volume impermeable to sorbitol.

Determination of the intracellular pH. Intracellular pH values were determined from the distribution of propionic acid between the intracellular water and the medium. Suspensions of cells (5×10^7 ml⁻¹) in the indicated medium were incubated with [¹⁴C]propionic acid [1 µM; 0.1 µCi ml⁻¹ (3.7 kBq ml⁻¹)] for 30 min at 30 °C with continuous shaking. Samples (1 ml) were filtered through Sartorius membrane filters, and the cells on the filters were washed four times with 3 ml distilled water. The filters were dried and the radioactivity determined as described above. The pH was calculated as described by De La Peña *et al.* (1982).

Determination of cellular ATP content. Cells were incubated in the indicated medium (5×10^7 cells ml⁻¹). Samples (0.1 ml) were removed, diluted in 2 ml boiling distilled water and maintained at 100 °C for 5 min. After cooling, 0.2 ml of these solutions were added to 2.8 ml of the reaction mixture in scintillation vials. The reaction mixture contained 1 ml solution A, 1 ml solution B and 0.8 ml H₂O. Solution A contained sodium arsenate (0.1 M), MgSO₄ (40 mM), and was brought to pH 7.4 with H₂SO₄; solution B contained phosphate buffer pH 7.4 (0.4 M)

Fig. 1

Fig. 1. Effect of TFP and pH on K⁺ efflux. Cells were incubated for 30 min at 30 °C in media containing glucose (80 mM), TFP as indicated and MES/Tris (20 mM) pH 4.5 (▲), 5.5 (△), 6.0 (●), 6.5 (○), and 7.5 (■). Values represent means $\pm$ SEM ($n = 4$).

Fig. 2

Fig. 2. Effect of pH on the TFP-induced membrane hyperpolarization. Cells were incubated for 30 min at 30 °C in media containing glucose (80 mM), MES/Tris (20 mM) at the indicated pH, [³H]TPP⁺ (1 μ M, 0.05 μ Ci ml⁻¹) and TFP at the following concentrations: 0 (●), 35 μ M (○), 50 μ M (▲), and 70 μ M (△). Values represent means $\pm$ SEM ($n = 4$).

and MgSO₄ (4 mM). The reaction was initiated by the addition of 75 μ l of a solution of luciferase-luciferin (4 mg ml⁻¹; Sigma), followed by immediate mixing and the placing of the vial into the well of a liquid scintillation counter that had been set for maximum sensitivity with the coincidence circuit off. Each sample was immediately counted for 10 s. Calibration curves were prepared in every experiment by adding different volumes of a solution containing 5×10^{-7} M-ATP dissolved in solution B to the reaction mixture, in which 0.2 ml water replaced the sample volume, and the volume of solution B was adjusted according to the volume of the ATP solution. Valinomycin, TFP (up to 100 μ M) and KCl (0.3–0.9 mM), when added to cell extracts, did not affect the ATP assay.

RESULTS

In order to investigate the mechanism and the specificity of the effect of TFP on yeasts we measured the dependence of TFP-effects on the pH of the medium. TFP caused K⁺ efflux at all pH values between pH 4.5 and pH 7.5, but at the higher pH values the magnitude of K⁺ efflux was somewhat reduced (Fig. 1). On the other hand, membrane hyperpolarization, as determined by TPP⁺-accumulation ratios, was observed only between pH 5.5 and 6.5; at pH 4.5 and 7.5 the increase in the accumulation of TPP⁺ caused by TFP was very small (Fig. 2).

Intracellular pH was calculated from the steady state distribution of [¹⁴C]propionic acid as described previously (Eilam *et al.*, 1984). TFP caused cell acidification at all pH values of the external medium between 4.5 and 7.5, but the magnitude of the decrease in pH_{in} was much greater at pH values of 4.5 and 7.5. When the pH of the medium was 7.5, TFP caused an inversion of Δ pH so that the intracellular pH was more acid than the medium. On the other hand, at pH 6.0 TFP caused only a slight decrease in pH_{in} (Table 1).

Table 1. *Effects of TFP on the intracellular pH in yeast cells*

Cells were incubated for 30 min at 30 °C in media containing MES/Tris (20 mM), glucose (80 mM), KCl (0.3 mM), TFP as indicated and [^{14}C]propionic acid (1 μM ; 0.1 $\mu\text{Ci ml}^{-1}$). Values represent means $\pm$ SEM ($n = 4$).

pH of medium	Intracellular pH		ΔpH	
	Control	+ TFP (35 μM)	Control	+ TFP (35 μM)
4.5	6.29 $\pm$ 0.03	5.82 $\pm$ 0.05	1.79 $\pm$ 0.03	1.32 $\pm$ 0.05
6.0	6.72 $\pm$ 0.05	6.66 $\pm$ 0.04	0.72 $\pm$ 0.05	0.66 $\pm$ 0.04
7.5	7.82 $\pm$ 0.08	7.01 $\pm$ 0.06	0.32 $\pm$ 0.08	-0.5 $\pm$ 0.06

The specificity of the TFP effect on K^+ efflux was determined by measuring the intracellular concentrations of Na^+ and Mg^{2+} after incubation with TFP at different pH values. Na^+ and Mg^{2+} efflux were induced by TFP mainly at an acidic pH (4.5 and 5.5). At pH values of the medium of 6.0, 6.5 and 7.5, a small efflux of Na^+ and Mg^{2+} was induced only at a higher concentration range of TFP (75–100 μM) (Fig. 3). These results indicate that at pH 6.0 the efflux of K^+ caused by low concentrations of TFP (30–50 μM) was not due to a non-specific decrease in the barrier functions of the cell membranes.

The following experiments were aimed at determining whether low concentrations of TFP at pH 6.0 caused the translocation of K^+ along or against its electrochemical gradient. Cells were incubated in media containing 0.3 mM-KCl, glucose, MES/Tris buffer and 35 μM -TFP. The concentrations of K^+ within the cells were determined from the measured amounts of K^+ in the cells and the cell water volume. The membrane potential values ($\Delta\psi$) were calculated from the equilibrium accumulation ratios of TPP^+ . From the values of $\Delta\psi$ and K_{out}^+ we calculated K_{in}^+ , the theoretical concentration of K^+ within the cells at equilibrium (zero electrochemical gradient).

$$\Delta\psi = 58.8 \log \text{K}_{\text{in}}^+ / \text{K}_{\text{out}}^+$$

$$\text{Thus } \text{K}_{\text{in}}^+ = \text{K}_{\text{out}}^+ \times 10^{(\Delta\psi/58.8)}$$

K_{out}^+ is the concentration of K^+ in the medium at the end of the experiment, which equals the initial concentration of K^+ (0.3 mM in this experiment) and the concentration of K^+ in the medium derived from K^+ efflux. The results given in Table 2 show that the actual concentration of K^+ within the cell water (K_{in}^+) was higher than K_{in}^+ in the control, and in the presence of TFP at pH 4.5, 6.5, and 7.5. However, at pH 5.5 and 6.0, in the presence of TFP, K_{in}^+ was lower than K_{in}^+ , indicating translocation of K^+ against its electrochemical gradient.

The requirement for metabolic energy for K^+ efflux was estimated by two types of experiments. (a) The cellular ATP level was determined after 30 min incubation with or without TFP at three different pH values of the medium, with 0.3 mM- K^+ in the medium. TFP in the presence of glucose caused a decrease in the level of cellular ATP at all three pH values, but the largest decrease (45%) was at pH 6.0. A much smaller decrease in cellular ATP was induced by TFP in the absence of glucose (Table 3). (b) TFP-induced K^+ efflux was inhibited in the absence of glucose or in the presence of glucose together with the uncoupler 2,4-DNP (Table 4). This inhibition of K^+ efflux was more pronounced at pH 6.0. The same conditions also caused inhibition of TFP-induced ATP-decrease (Table 3).

Previously, it has been reported (Peña, 1975; Seaston *et al.*, 1976) that when yeast cells were incubated without glucose in the presence of 2,4-DNP, K^+ efflux was observed. We repeated these experiments with our cells and compared the extent of K^+ efflux induced by 2,4-DNP in the absence of glucose to that induced by TFP in the presence of glucose. 2,4-DNP (in the absence of glucose) produced a much smaller K^+ efflux than TFP with glucose. 2,4-DNP together with TFP in the absence of glucose had some synergistic effect on K^+ efflux, but the extent of the combined effect was much less than K^+ efflux induced by TFP with glucose and without 2,4-DNP. Obviously, addition of 2,4-DNP to the cells caused membrane depolarization and the calculated K_{in}^+ was smaller than the measured K_{in}^+ , indicating that the K^+ efflux caused by 2,4-DNP was along its electrochemical gradient.

Fig. 3. Effect of TFP and pH on the intracellular concentrations of Na^+ and Mg^{2+} . Cells were incubated for 30 min at 30 °C in media containing glucose (80 mM), TFP as indicated and MES/Tris (20 mM) pH 4.5 ($\blacktriangle$), 5.5 ($\triangle$), 6.0 ($\bullet$), 6.5 ($\circ$), and 7.5 ($\blacksquare$). The cellular contents of Na^+ (a) and Mg^{2+} (b) were measured as described in Methods. Values represent means $\pm$ SEM ($n = 4$).

Table 2. Membrane potential ($\Delta\psi$), the intra- and extracellular concentrations of K^+ (K_{in}^+ and K_{out}^+) and $K_{\dagger}^+$ in the absence and presence of TFP

K_{in}^+ and K_{out}^+ (final) are the concentrations of K^+ within the cells and in the medium at the end of the incubation. The initial concentration of K^+ in the medium was 0.3 mM; the increase in this concentration during the incubation was by K^+ efflux from the cells. $K_{\dagger}^+$ is the theoretical concentration of K^+ within the cells at zero electrochemical gradient. Membrane potential ($\Delta\psi$) was calculated from the equilibrium distribution ratio of TPP $^+$. The medium contained MES/Tris (20 mM) at the indicated pH, glucose (80 mM), KCl (initially) (0.3 mM) and TFP as indicated. Values represent means $\pm$ SEM ($n = 4$).

pH of medium	Control				+ TFP (35 μ M)			
	$\Delta\psi$ (mV)	K_{in}^+ (mM)	K_{out}^+ (final) (mM)	$K_{\dagger}^+$ (mM)	$\Delta\psi$ (mV)	K_{in}^+ (mM)	K_{out}^+ (final) (mM)	$K_{\dagger}^+$ (mM)
4.5	-66.3 ± 1.1	136.1 ± 3.8	0.33	4.4	-90.8 ± 2.3	36.2 ± 2.5	0.44	11.9
5.5	-73.3 ± 1.3	163.8 ± 2.9	0.30	5.3	-134.1 ± 3.4	28.7 ± 1.8	0.49	93.1
6.0	-82.2 ± 2.0	160.4 ± 4.1	0.30	7.5	-139.7 ± 3.1	32.4 ± 2.1	0.48	114.2
6.5	-88.8 ± 2.1	148.3 ± 3.3	0.31	10.3	-115.0 ± 2.8	48.3 ± 2.8	0.44	39.9
7.5	-90.1 ± 2.8	140.2 ± 3.1	0.32	10.9	-112.8 ± 2.3	88.4 ± 3.2	0.37	30.7

Table 3. Effects of TFP on the concentrations of ATP in yeast cells

Cells were incubated for 30 min in media containing MES/Tris (20 mM) at the indicated pH, KCl (0.3 mM) and TFP as indicated. Values represent means $\pm$ SEM ($n = 4$).

pH of medium	Glucose (80 mM)	$10^{16} \times$ ATP concn (mol per cell)	
		Control	+ TFP (35 μ M)
4.5	+	1.170 ± 0.05	0.916 ± 0.05
	—	0.796 ± 0.03	0.646 ± 0.03
6.0	+	1.186 ± 0.04	0.651 ± 0.03
	—	0.766 ± 0.03	0.646 ± 0.04
7.5	+	0.795 ± 0.03	0.706 ± 0.03
	—	0.721 ± 0.02	0.630 ± 0.04

Table 4. *The effects of glucose, 2,4-DNP and TFP on K_m^+*

Media contained MES/Tris pH 6.0 (20 mM), KCl (0.3 mM), $\pm$ TFP and 2,4-DNP as indicated. Cells were incubated for 30 min at 30 °C. Values represent means $\pm$ SEM ($n = 4$).

TFP (μ M)	2,4-DNP (mM)	K_m^+ (mM)	
		+ Glucose (80 mM)	– Glucose
–	–	164.7 $\pm$ 3.2	157.6 $\pm$ 2.9
–	0.5	159.4 $\pm$ 2.8	157.4 $\pm$ 3.0
–	1.0	163.8 $\pm$ 3.1	156.5 $\pm$ 3.2
–	2.0	156.0 $\pm$ 3.0	149.2 $\pm$ 3.0
35	–	48.9 $\pm$ 1.2	153.4 $\pm$ 2.8
35	0.5	113.9 $\pm$ 2.1	160.7 $\pm$ 2.8
35	1.0	118.2 $\pm$ 1.9	138.8 $\pm$ 2.5
35	2.0	130.0 $\pm$ 2.2	127.4 $\pm$ 2.2
50	–	25.5 $\pm$ 0.8	135.7 $\pm$ 2.4
50	0.5	69.7 $\pm$ 1.3	153.9 $\pm$ 1.9
50	1.0	70.7 $\pm$ 1.9	110.2 $\pm$ 2.2
50	2.0	93.6 $\pm$ 2.0	82.6 $\pm$ 2.0

Table 5. *Effects of TFP and valinomycin on $\Delta\psi$, K_m^+ and K_f^+ at different concentrations of K^+ in the medium*

Cells were incubated for 30 min at 30 °C in media containing MES/Tris (20 mM) pH 6.0, glucose (80 mM), KCl (0.3 mM), $\pm$ TFP (35 μ M) and valinomycin (18 μ M) as indicated. K_{out}^+ , initial and final, are the concentrations of K^+ in the media at the beginning and at the end of the incubation. The increase during the incubation was by K^+ efflux. K_f^+ and $\Delta\psi$ are as in Table 2. $\Delta\psi$ and K_m^+ were always measured in the same experiment. Values represent means $\pm$ SEM ($n = 4$).

Addition	K_{out}^+ (mM)		$\Delta\psi$ (mV)	K_m^+ (mM)	K_f^+ (mM)
	Initial	Final			
–	0.3	0.30	–82.2 $\pm$ 2.0	163.8 $\pm$ 2.9	7.5
TFP	0.3	0.49	–135.3 $\pm$ 4.8	25.1 $\pm$ 1.7	98.0
Valinomycin	0.3	0.32	–72.3 $\pm$ 3.7	151.4 $\pm$ 2.8	5.4
Valinomycin + TFP	0.3	0.45	–107.2 $\pm$ 4.1	51.5 $\pm$ 2.7	29.9
–	0.6	0.60	–81.8 $\pm$ 2.3	167.1 $\pm$ 2.8	14.8
TFP	0.6	0.78	–133.6 $\pm$ 3.9	34.1 $\pm$ 1.1	145.9
Valinomycin	0.6	0.61	–76.1 $\pm$ 2.9	156.7 $\pm$ 2.1	12.0
Valinomycin + TFP	0.6	0.76	–108.5 $\pm$ 3.1	54.1 $\pm$ 1.8	53.2
–	0.9	0.9	–80.4 $\pm$ 2.3	169.6 $\pm$ 2.4	20.9
TFP	0.9	1.10	–134.6 $\pm$ 4.1	24.2 $\pm$ 1.7	214.0
Valinomycin	0.9	0.91	–71.7 $\pm$ 3.8	158.5 $\pm$ 2.2	15.1
Valinomycin + TFP	0.9	1.06	–108.9 $\pm$ 3.7	56.0 $\pm$ 2.1	75.4

Table 6. *Effects of TFP and valinomycin on the cellular concentrations of ATP*

Media contained MES/Tris (20 mM) pH 6.0, KCl (0.3 mM), glucose (80 mM), $\pm$ TFP (35 μ M) and valinomycin (18 μ M) as indicated. Cells were as incubated for 30 min at 30 °C. Values represent means $\pm$ SEM ($n = 4$).

Addition	$10^{16} \times$ ATP concn (mol per cell)
–	1.20 $\pm$ 0.05
TFP	0.55 $\pm$ 0.03
Valinomycin	0.78 $\pm$ 0.04
Valinomycin + TFP	0.42 $\pm$ 0.03

A different way to determine whether TFP-induced K^+ efflux was along or against the electrochemical gradient for K^+ by the use of valinomycin in combination with TFP and several concentrations of K^+ in the medium (at pH 6.0). If K^+ was translocated along its electrochemical gradient, valinomycin would either increase the efflux of K^+ or have no effect. Alternatively, if the TFP-induced K^+ efflux was against its electrochemical gradient, valinomycin would increase the rate of K^+ influx (along its electrochemical gradient) and thus increase the steady state concentration of K^+_{in} and decrease $\Delta\psi$. The results in Table 5 showing a higher K^+_{in} and a lower $\Delta\psi$ in the presence of TFP together with valinomycin as compared with TFP alone, support the second possibility.

Measurements of cellular ATP levels following incubation with TFP and valinomycin showed that valinomycin decreased the cellular ATP level beyond the decrease caused by TFP alone. However, an effect of valinomycin on cellular ATP level was observed also in the absence of TFP (Table 6).

DISCUSSION

The phenothiazines have been used for years as tranquillizers and antipsychotic drugs. Recently, we have found that yeast cells are also susceptible to these drugs, which induce substantial K^+ efflux and membrane hyperpolarization together with inhibition of the proton translocating ATPase (Eilam, 1983, 1984). The mechanism of these effects remained unknown. A simple interpretation could have been that K^+ efflux was caused by increased permeability of membranes to K^+ , leading to K^+ leakage and thereby increased $\Delta\psi$. However, metabolic substrate is required for the induced K^+ efflux but not for the uptake of TFP into the cells (Eilam, 1984). These findings led us to investigate whether K^+ efflux proceeded along or against its electrochemical gradient, i.e. whether it was a passive or an active process.

In the present work it was found that at least two different mechanisms of TFP effect on yeast cells can be recognized. At higher concentrations (above 75 μM), or at acidic pH (4.5) even at low concentrations (35 μM), TFP caused non-specific damage to the barrier function of cell membranes, leading to the efflux of K^+ , Na^+ and Mg^{2+} , and to proton-influx, which dissipated the increase in $\Delta\psi$ (negative inside) formed by cation efflux. At the alkaline pH of 7.5, proton influx was also observed, but the cation efflux was somewhat reduced. At pH values of 5.5–6.0, and at a low TFP-concentration range (30–50 μM), another mechanism could be detected: specific and substantial efflux of K^+ , not balanced electrically by H^+ influx, caused membrane hyperpolarization. This efflux required metabolic energy and led to a decrease in the cellular ATP level. Our results indicate that this efflux can proceed against the electrochemical gradient of K^+ .

When the cells were incubated at pH 5.5–6.0, in the presence of glucose, 0.3–0.9 mM-KCl and 35 μM -TFP, the concentration of K^+ within the cells at the end of the incubation was below the equilibrium concentration ($K^+_{in} < K^+_f$). Several difficulties may be involved in this calculation: the concentration of K^+ within the cytosol (K^+_{in}) may be affected by the compartmentalization of K^+ within the cells, in particular the distribution of K^+ between the cytosol and the vacuoles, or K^+ binding. In addition, changes in cellular water volume during K^+ efflux may lead to an underestimation of K^+_{in} and so invalidate the conclusion. However, our previous measurements of the concentration of K^+ in the cytosol and the vacuoles following incubation with TFP (35 μM) at pH 6.0 using methods based on differential extraction (Eilam *et al.*, 1985) revealed that most of the TFP-induced K^+ efflux originated from the cytosolic pool while the concentration of K^+ in the vacuoles changed less: at $K^+_{out} = 0.9$, and 35 μM -TFP, K^+_{in} was calculated from the amount of K^+ in the cytosol as determined by differential extraction and the cytoplasmic volume determined previously (Eilam *et al.*, 1985); the value obtained for K^+_{in} was 21.8 mM. This value is somewhat below the value in Table 5. Thus the calculated K^+_{in} might be somewhat overestimated, but certainly not underestimated. Possible shrinkage of the cells during K^+ efflux would lead to underestimation of K^+_{in} , but it would also lead to underestimation of K^+_f since the same value of cell water volume was used both for the calculation of the TPP⁺ distribution ratio (and hence for $\Delta\psi$ determination) and for the calculation of the concentration

of K^+ within the cells (K_{in}^+). Consequently, the difference between K_{out}^+ and K_{in}^+ does not depend on the actual volume of the cells. The difference between K_{out}^+ and K_{in}^+ was particularly pronounced at an initial K_{out}^+ of 0.9 mM. We did not use higher concentrations of K^+ since a high concentration of cations in the medium prevented the effect of TFP; the mechanism of this interaction is not yet understood (Eilam, 1983, 1984).

The calculation of K_{in}^+ relies on the use of TPP^+ as a quantitative probe for measurements of the membrane potential of the yeast cells. This method has been examined in several studies which showed that conditions which led to membrane depolarization, such as high concentrations of monovalent cations, the presence of uncouplers or metabolic inhibitors, or the absence of metabolic substrate, led to a marked decrease in TPP^+ accumulation. It was concluded that when appropriate correction for TPP^+ binding is made, the distribution ratio of TPP^+ at equilibrium may be used for the determination of $\Delta\psi$ in yeasts (Hauer & Hofer, 1978; Boxman *et al.*, 1982, 1984; Van Den Broek *et al.*, 1982; De La Peña *et al.*, 1982).

However, the possibility that TPP^+ might accumulate in cellular organelles, such as mitochondria or vacuoles, was not examined in these studies. In an attempt to determine the extent by which metabolically driven uptake of TPP^+ into the mitochondria may lead to overestimation of $\Delta\psi$, we measured the TPP^+ distribution ratio in the respiratory deficient mutant (ρ^0) derived from our strain (Eilam *et al.*, 1985). The membrane potential determined from TPP^+ accumulation was somewhat lower than in the wild-type strain (at pH 6.0, $\Delta\psi$ was 76.4 mV in the ρ^0 mutant as compared with 82.2 mV in the wild-type strain). This difference may result in part from the uptake of TPP^+ into the mitochondria in the wild-type strain, but in part is probably due to the differences in metabolism between the strains. The effect of TFP on TPP^+ accumulation in the ρ^0 mutant was similar to that in the wild-type strain, and with 0.9 mM-KCl and 35 μ M-TFP in the medium at pH 6.0, we obtained the values of $K_{in}^+ = 194.2$ mM and $K_{out}^+ = 25.4$ mM. These values indicate that the possible accumulation of TPP^+ in the mitochondria does not invalidate our conclusion.

The vacuolar membrane potential in yeast is positive in sign owing to an inwardly directed H^+ -ATPase (Kakinuma *et al.*, 1981). It is unlikely therefore that a significant amount of TPP^+ accumulates in the vacuoles. But if TFP inhibits the vacuolar H^+ -ATPase, the vacuolar membrane potential may become more negative, and the uptake of TPP^+ into the vacuoles may increase, thus invalidating the calculation of K_{in}^+ . However, TFP induced a marked increase in Ca^{2+} uptake (Eilam, 1983); the Ca^{2+} was concentrated in the vacuoles as determined by differential extraction (Eilam *et al.*, 1985). Incubation in a medium containing 10^{-6} M- $CaCl_2$ and 40 μ M-TFP led to an increase in the concentration of Ca^{2+} in the vacuoles from 2.2×10^{-4} M in the control to 1.27×10^{-3} M in the TFP treated cells. Since Ca^{2+} uptake into the vacuole is driven by the $\Delta\bar{\mu}_H$, formed by the vacuolar H^+ -ATPase (Ohsumi & Anraku, 1983), it is unlikely that this enzyme is inhibited by low concentrations of TFP.

These arguments indicate that our calculation showing that $K_{out}^+ > K_{in}^+$ in the presence of TFP is not invalidated by possible changes in the accumulation of TPP^+ in cellular organelles.

The results indicate, therefore, that under a particular set of conditions (0.3–0.9 mM- K^+ , pH 6.0, 80 mM-glucose and 35 μ M-TFP in the medium) there was an active electrogenic K^+ efflux. The same set of conditions led to a decrease in the cellular ATP level beyond the decrease observed when active K^+ efflux was not induced. Since TFP inhibits the plasma membrane ATPase (Eilam, 1984) which consumes ATP, it might have been expected that TFP would increase the cellular ATP level. The results showing a decrease in cellular ATP concentration by TFP may indicate activation of an energy consuming process.

Additional evidence for the translocation of K^+ against its electrochemical gradient was obtained by adding valinomycin in combination with TFP. Valinomycin, a K^+ ionophore, would be expected to increase the passive K^+ flux along its electrochemical gradient. The higher concentration of K^+ within cells (K_{in}^+) incubated with valinomycin and TFP together, as compared with TFP alone, indicated that the electrochemical gradient for K^+ in TFP treated cells was directed towards the intracellular water; thus K^+ efflux induced by TFP proceeded against its electrochemical gradient.

Many investigations have been made and several theories have been proposed to explain the

mechanism of the energy dependent K^+ uptake by yeast cells (Rothstein, 1974; Peña, 1975; Borst Pauwels, 1981; Eddy, 1982; Boxman *et al.*, 1984). The mechanism of energy dependent electrogenic K^+ efflux, revealed by the use of TFP (under a specific set of conditions), has not been previously described. The question which may be asked is whether and when such a mechanism is active in the absence of TFP. We suggest that the energy dependent K^+ efflux system may be activated when the cells are incubated in a medium containing a high concentration of K^+ , so that K_{in}^+ would be below K_{out}^+ . The function of such a K^+ efflux system may be to prevent an increase in K_{in}^+ when the passive K^+ influx is high. A different mechanism may be that a TFP induced change in the affinity of the energy dependent Na^+ efflux carrier (Dee & Conway, 1968) causes the transport of K^+ .

The molecular basis of the TFP induced electrogenic K^+ efflux, whether it is mediated by a primary K^+ pump, or linked in some way to the fluxes of other substance(s) is as yet unknown. We also do not understand the mechanism of activation of this flux by TFP, and whether the TFP effect is mediated by inhibition of calmodulin dependent function(s). In this connection it is of interest that Plishker (1984) has reported that phenothiazines also induce K^+ efflux in human red blood cells.

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