Biochimica et Biophysica Acta, 1068 (1991) 237–244
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ADONIS 000527369100351F

BBAMEM 75349

An energy-dependent efflux system for potassium ions in yeast

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(Received 13 February 1991)
(Revised manuscript received 17 May 1991)

Key words: Ion transport; Potassium ion transport; Potassium efflux; Potassium ion–proton exchange; Ion exchange; (Yeast)

An efflux of potassium ions was demonstrated in mutants of yeast cells lacking a functional high affinity carrier system for monovalent cations. This efflux showed the following characteristics: (a) It was stimulated by the presence of a substrate, either glucose or ethanol. (b) It was stimulated by several cationic organic molecules, such as ethidium bromide, dihydrostreptomycin, diethylaminoethyldextran, and also by trivalent cations, such as Al^{3+} and lanthanides; this stimulation also depended on the presence of a substrate. (c) K^+ efflux was decreased in yeast mutants with decreased ATPase activity, which generated a lower membrane potential. (d) Although the efflux appeared to be of an electrogenic nature, producing hyperpolarization of cells, it was accompanied by the efflux of phosphate, probably as an anion partially compensating for the large amount of cations leaving the cell. (e) K^+ efflux was also accompanied by an uptake of protons. (f) The efflux appeared more clearly in cells grown in YPD medium, and not in more complex media nor in the same YPD medium if supplemented with Ca^{2+} or Mg^{2+}. Efflux of monovalent cations produced by Tb^{3+} and organic cationic agents was also demonstrated in wild type strains. This efflux system appears to be, at least partially, electrogenic, but seems to be also an exchange system for protons and to function as a symport with phosphate; it may be involved in the regulation of the internal pH of the cell, and appears to be regulated by its link to the energetic status of the cell, probably through the membrane potential.

Introduction

Since 1958, Rothstein and Bruce [1] described a system for the efflux of potassium observed under conditions in which the cation was absent from the external medium. Conway et al. also described a system for the efflux of sodium ions from yeasts that have been loaded with this cation [2]. Similar studies have been performed by Rodríguez Navarro [3] for the efflux of different cations. The question arises whether the uptake system, characterized kinetically by Conway [4] and Rothstein [5] could be responsible for the efflux of the cation. More recently, Van de Mortel et al. [6,7] described an efflux system for potassium ions in yeast, which can be blocked by divalent cations and La^{3+}. Ramos et al. [8] also analyzed the regulation of potassium influx and efflux in yeast.

Since the work of Brunner et al. [9] and Rodríguez-Navarro and Ramos [10], and Ramos et al. [11], mutants defective in K^+ transport have been obtained. This kind of mutants led Gaber [12] to the cloning and sequencing of a gene that conferred yeast the capability to transport the cation with high affinity. The same author also prepared a yeast strain lacking the gene for the carrier. These findings prompted studies on the efflux system in cells lacking a functional high affinity carrier for potassium. Other mutants with H^+-ATPase alterations in the plasma membrane [13,14], which produce a lower membrane potential, were used, since they may provide further information on the energetic requirements of the efflux system.

Materials and Methods

Yeast cells of different strains were cultured in several media; one of them was YPD, either as such (2% glucose, 1% peptone, 1% yeast extract), or complemented with different salts; other media were that described by De Kloet [15], and yeast nitrogen base [16] with 10 mM proline instead of ammonia as the nitrogen source. The usual system consisted in growing

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Abbreviations: TEA, tri-ethanolamine; bromcresol purple, 5,5'-dibromo-*o*-cresolsulfonphthalein

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the cells from a slant into the indicated medium for 18 h at 30 °C in a gyrotory shaker. Yeast was then harvested by centrifugation and washed twice with distilled water. The final pellet was resuspended in 50 ml of water and aerated in the same shaker for 4 h at 30 °C. After aeration, the cells were centrifuged and washed twice again, and finally resuspended in water to a concentration of 0.5 g of cells per ml. The suspensions were kept overnight in the refrigerator (4 °C) and used the next day.

Ion movements were measured by continuously recording the concentration using a cationic electrode (Corning 476220) or a pH electrode, connected to two pH meters and recorders. The particular conditions and incubation mixtures are indicated under each experiment. The pH changes were measured by following the absorbance changes of bromcresol purple at 487–586 nm in a dual wavelength spectrophotometer, with a low buffering incubation mixture.

Membrane potential was estimated with dithiocarbocyanine ($DiSC_3(3)$) [17,18], obtained from Molecular Probes. The fluorescence of the molecule at a concentration of 0.25 μM was recorded at room temperature at 540–600 nm in a spectrofluorometer with a magnetic stirrer in the sample compartment.

Internal concentrations of cations were measured by boiling the cell suspensions in a water bath for 10 min. After centrifugation and adequate dilution of the supernatant, the cations were measured in a flame photometer. This same supernatant was used to measure inorganic phosphate, following the procedure of Fiske and Subbarrow [19].

Yeast plasma membranes were prepared according to the method of Perlin et al. [20]. The resulting plasma membranes were suspended in 1 mM EGTA-Tris (pH 7.2) and stored at −70 °C. These membranes were reconstituted into liposomes; a plasma membrane suspension was prepared containing (in 2 ml) 10 mg of membrane protein, 20 mM Hepes-TEA (pH 7.2), 1 mM EDTA-TEA, 50 mM sucrose and 30 mg of phosphatidylethanolamine from *Escherichia coli*. Recrystallized deoxycholate was added to this cloudy suspension, to give a final concentration of 0.6%. After vigorous homogenization, the suspension became clear; it was then rapidly applied to a Bio-Gel P-10 column (40 × 1 cm), which was eluted with buffer containing 10 mM Hepes-TEA (pH 7.2), 0.1 mM EDTA-TEA and 50 mM sucrose, at a flow rate of 1 ml/min. The cloudy void volume fractions were pooled, diluted 40-fold with 20 mM Hepes-TEA (pH 7.2) and 50 mM sucrose, and centrifuged at 100 000 × g for 1 h. The pellet of vesicles was suspended in 500 μl of 20 mM Hepes-TEA (pH 7.2) and 50 mM sucrose.

Transport of $^{86}Rb^+$ was assayed as follows. At zero time, the vesicles (100 μl, 2 mg of protein) were diluted with 900 μl of 20 mM Hepes-TEA (pH 7.2), 50 mM sucrose, 5 mM $MgCl_2$ and 25 MM RbCl (^{86}Rb, specific activity, 134 μCi/mmol) in the presence of absence of 25 mM HNO_3-TEA (pH 7.0) and/or 250 μM Na_3VO_4. After 2 min, 5 mM ATP was added, and 5 min later, 250 μM Na_3VO_4. Aliquots of 100 μl were withdrawn at intervals and diluted with 2.5 ml of ice-cold 100 mM LiCl and 800 μg/ml of protamine sulfate. The suspension was filtered through 0.3 μm cellulose nitrate filters (Millipore) and washed once with 2.5 ml of LiCl-protamine. The filters were dried and transferred to scintillation vials with 5 ml of a scintillation cocktail, and their radioactivity was measured in a Packard liquid scintillation counter. To measure Rb^+ transport with whole cells, at zero time, 5 mg of cells were placed in 1 ml of 20 mM Mes-TEA buffer (pH 6.0) and 50 mM glucose. After 2 min, 2.5, 5.0 or 10 μM $GdCl_3$ and/or 0.1, 0.2, 0.3, 0.5, 1.0, 2.5 or 5.0 mM $^{86}Rb^+$ were added. After two more min, an aliquot of 500 μl was taken, filtered through a cellulose nitrate filter of 0.45 μm mean diameter pore (Millipore), and washed once with 10 ml of 20 mM KCl. The filters were then dried and their radioactivity was measured in a Packard scintillation counter.

Results

K^+ movements were measured without any additions either in the absence or in the presence of glucose as substrate, in both the wild type strains XT-300.3A of Rodríguez Navarro [11] and 757 of Gaber [12] and in the PC-1 mutants of Rodríguez-Navarro lacking the ability to transport K^+ with high affinity [10], and TrkΔ of Gaber [11] (Fig. 1). As expected, in

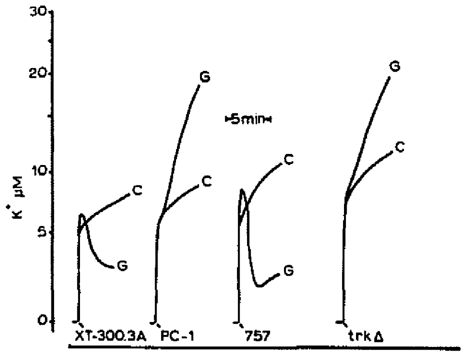

Fig. 1. Movements of K^+ in the presence (G) or absence (C) of glucose in two wild type (XT-300.3A and 757) and two mutant strains without the high affinity transport capacity (PC-1 and TRkΔ). Yeast cells were grown, as described under Materials and Methods, in YPD medium. Incubation was carried out in 4 mM Mes-TEA buffer (pH 6.0), with 5 mM glucose where indicated, in a final volume of 10.0 ml, in a water jacketed chamber under continuous magnetic stirring. Tracings were started by the addition of 50 μl (25 mg, wet weight) of the yeast suspension. Potassium concentration outside the cells was followed by continuous recording.

the presence of glucose, the wild type strains recaptured the potassium that leaked out of them when placed in a K$^+$-free medium; in the absence of glucose, a slow, but constant efflux was observed. In the mutants, in the absence of glucose, the efflux of K$^+$ was similar to that observed in the wild type strains. However, when glucose was present, the mutants showed a significantly increased rate of efflux of the cation.

When the wild type or mutant cells were incubated in the presence of certain concentrations of 20 μM ethidium bromide (Fig. 2), as described before for this and other dyes [21], efflux of the monovalent cation was also observed. Besides, this increased efflux was much higher in the presence of glucose. Similar results were observed also with 2.5 μM dihydroestreptomycin, which, similarly to streptomycin [22] produced a marked efflux of monovalent cations from yeast cells; this efflux was higher in both potassium-transport mutants. DEAE-dextran (12.5 μg, with approx. 2.8 μM of the diethylaminoethyl group), that has been used at higher concentrations to disrupt the plasma membrane of yeast, preserving the vacuolar membrane [23], was found to produce the efflux of K$^+$ (Fig. 2), in an event that also required of glucose, depending on the concentration of the agent. It is important to note that in all conditions, the mutants showed a higher sensitivity to the agents used to stimulate the efflux.

Several ions are known to interact with the yeast cell membrane as found by Armstrong [24], Rothstein [25] and more recently by Ohsumi et al. [26]. Divalent cations, such as Ca^{2+} have also been described as membrane 'stabilizers'; Van de Mortel et al. [6,7], for instance, observed that several divalent cations and also La^{3+} produce a decrease of the spontaneous K$^+$ efflux observed in yeast. The chlorides of Al, La, Ce, Gd and Tb were tested, yielding the results depicted on Fig. 3, showing that specially Tb^{3+} and Gd^{3+}, at a concentration of 5 μM or less, produced a significant efflux of the monovalent cation. Experiments not presented showed that 5 μM TbCl$_3$ also produced the efflux of K$^+$ in all the mutant or wild type strains used in Figs. 1 and 2. This efflux was also found to require either glucose or ethanol, although ethanol did not produce the same rate or extent of efflux (not shown). CaCl$_2$, MgCl$_2$ and BaCl$_2$ at a 200 μM concentration, in the presence of glucose, yielded apparently contradictory results; whereas in the wild type strains, the divalent cations produced a decrease of the uptake of K$^+$; and Ba^{2+} even showed some stimulation of the efflux; in the mutants, however, all divalent cations tested produced at the end (approx. 5 min) a decreased K$^+$ efflux (Fig. 4).

Another point considered worthwhile exploring, was the possible role of the membrane potential as the

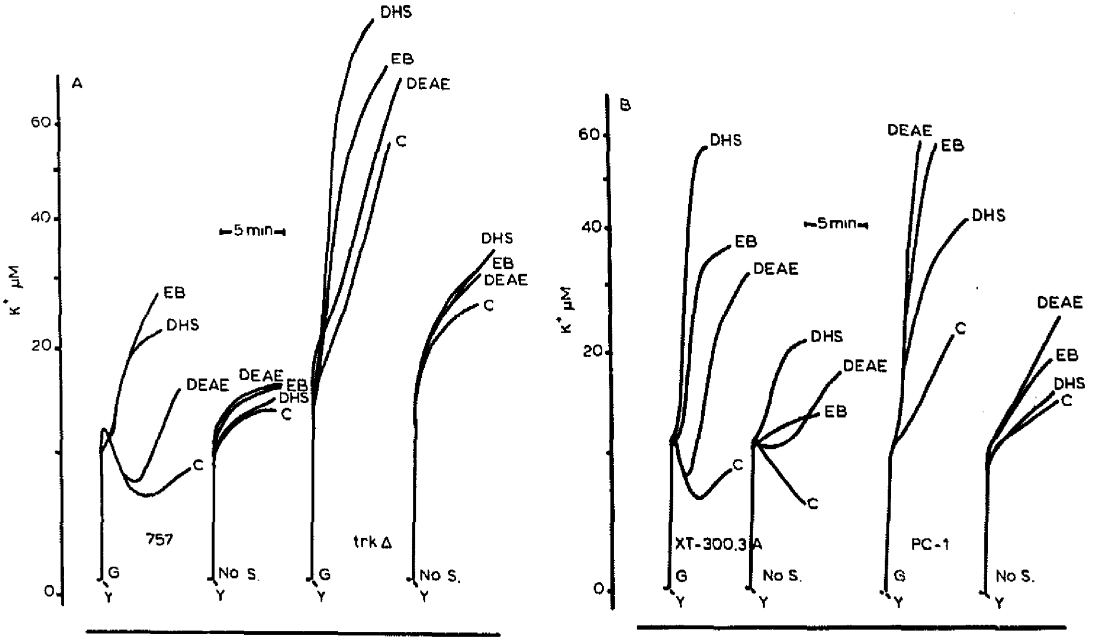

Fig. 2. Efflux of K$^+$ from yeast cells produced by different agents, in the absence (No S) or in the presence (G) of glucose. Incubation conditions were as for Fig. 1. Where indicated, 5 mM glucose, 20 μM ethidum bromide (EB), 2.5 μM dihydrostreptomycin (DHS) OR 12.5 μg of diethylaminoethyldextran (DEAE) with approx. 400 ng of nitrogen (28 nmol to a volume of 10 ml) was added. Tracings in A were obtained from strain 757 and the mutant TrkΔ, and in B, from strain XT-300.3A, and its mutant PC-1.

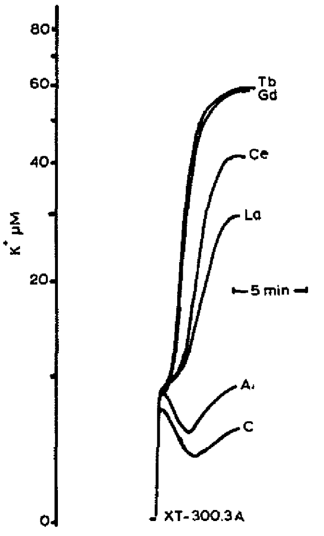

Fig. 3. Efflux of K^+ produced by different trivalent cations in yeast, in the presence of glucose. The experimental conditions were as for Fig. 1, but the concentration of the trivalent cations was 5 μM.

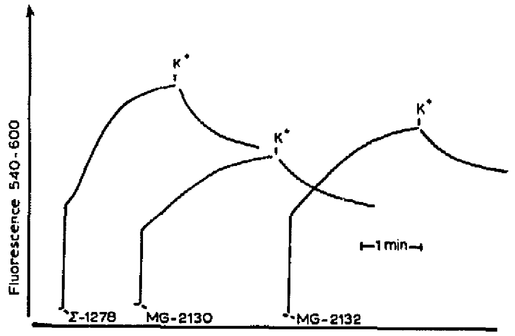

Fig. 5. Membrane potential variations, as indicated by the fluorescence changes of $DiSC_3(3)$ in a wild type strain (Σ 1278.b) and mutants defective in the plasma membrane ATPase (MG-2130 and MG-2132, of Refs. 10 and 11). Incubation conditions: Mes-TEA buffer, 20 mM (pH 6.0; glucose 100 mM; $CaCl_2$, 250 μM, FCCP, 6 μM; cyanine, 0.25 μM; final volume was 2.0 ml. The tracing was started by the addition of 50 μl (25 mg, wet weight) of yeast cells, and fluorescence was recorded at room temperature at 540–600 nm. Where indicated, 5 mM KCl was added. The mutants and the wild type strain were kindly provided by Prof. André Goffeau.

necessary requirement for the increase in the efflux caused by Tb^{3+}. For this purpose, two more mutants with a decreased H^+-ATPase activity of the plasma membrane, kindly provided by Prof. André Goffeau [13,14] were used (Fig. 5). Both mutants showed a lower rate in the installation and in the final level reached by the membrane potential, as indicated by the fluorescence of $DISC_3(3)$, a dye that can be used to estimate the membrane potential of yeast [17,18]. Both mutants with this lower ability to develop a membrane potential showed also a lower K^+ efflux in the presence of Tb^{3+} (Fig. 6).

It was considered that Tb^{3+} could be altering the normal K^+-transport system of the cells, or producing the leakage of the cation. To clarify this point, it was decided to test the effects of the monovalent cation on the level of membrane potential reached in the presence of Tb^{3+}. The trivalent cation, which produced K^+ efflux, gave also rise to a faster increase and a higher

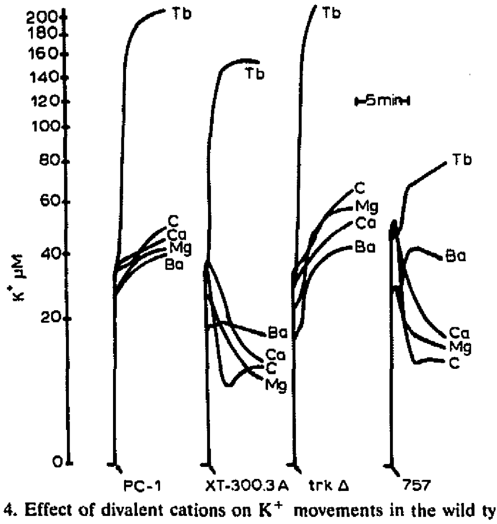

Fig. 4. Effect of divalent cations on K^+ movements in the wild type and mutant strains of yeast with glucose as substrate. Experimental conditions were as for Fig. 1; Tb^{3+} was 5 μM, and chlorides of Mg, Ca and Ba were added at 200 μM, where indicated.

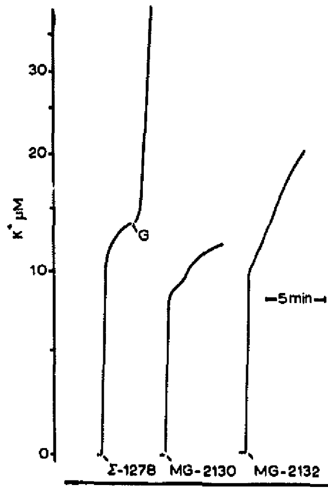

Fig. 6. K^+ efflux in a wild type strain (Σ 1278.b) and two mutants defective in the ATPase of the plasma membrane (MG 2130 and MG 2132). Incubation conditions were as described for Fig. 1; $TbCl_3$ concentration was 5 μM.

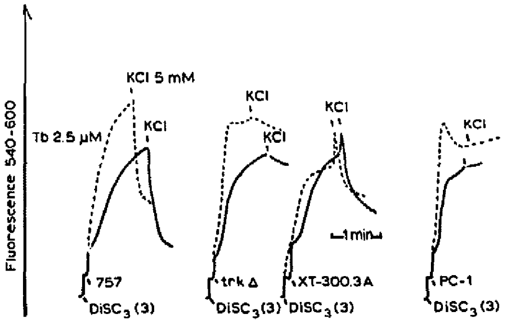

Fig. 7. Membrane potential changes produced by Tb^{3+} and the addition of K^+ in two wild type strains (757 and XT-300.3A), and two mutants (PC-1 and TrkΔ). Incubation conditions were as described for Fig. 6. $TbCl_3$ concentration was 2.5 μM.

level fo fluorescence, or both, indicating a hyperpolarization of the cells. However, addition of K^+ still produced, as expected, only in the wild type, the usual decrease of the membrane potential which is also observed in the absence of the trivalent cation (Fig. 7). This decrease occurred only slightly in the mutants, in agreement with their decreased ability to transport the monovalent cation.

Interestingly, this efflux system could be observed clearly when the cells were grown in YPD medium, but not in yeast nitrogen base with proline as the nitrogen source [16], nor in the medium described by De Kloet et al. [15]; in cells grown in these media, even 5 μM Tb^{3+} failed to produce the efflux (not shown). To define which possible components of the more complex media were responsible for the appearance or not of the efflux system, the cells were grown in YPD medium, to which several additions were made. KCl, $MgCl_2$ and $CaCl_2$, at similar concentrations to those of the medium by De Kloet et al. [15], abolished the efflux, either spontaneous or caused by the different agents tested. The addition of inorganic phosphate to the medium produced an enhancement of this phenomenon when tested with the addition of Tb^{3+} (Fig. 8A and B).

The K^+ efflux produced by the addition of Tb^{3+}, was accompanied by a change in the rate of proton pumping (Fig. 9), which was slowed as long as the efflux of K^+ lasted. After this period, proton expulsion by the cells was faster, indicating that possibly, protons were taken up by the cells during the K^+ efflux. This recovery of the proton pumping rate is also an indication that the efflux was not produced by the disruption of the cells.

Another possibility is that the cells were exchanging potassium for some of the components of the medium; of these, triethanolamine was the only possibility, since it is a cation at the pH of the medium. The efflux of K^+ was measured in the presence of different concentrations of the buffer, and it occurred at approximately the same rate even in the absence of the buffer (results not shown).

It is also possible that the efflux took place, at least partially, accompanied by the efflux of an anion, especially during the initial times of incubation with a substrate. Inorganic phosphate is the most abundant anion inside the cell. Experiments were performed in which, after a brief incubation of different concentrations of yeast, either in the presence or absence of Tb^{3+}, the cells were centrifuged and the concentration of inorganic phosphate was measured in the supernatant. Table I shows that, in fact, Tb^{3+} produced a marked increase of inorganic phosphate in the incubation medium (Table I).

Experiments not presented, in which the uptake of $^{86}Rb^+$ was measured at short times (2 min), showed that the efflux is not due to the effect of the trivalent cations on the influx, since these cations, at concentrations up to 10 μM, tested against concentrations of 100 μM to 5 mM $^{86}Rb^+$ did not show an important effect on the influx of the monovalent cation.

Since the results indicated the existence of an efflux system capable of extruding K^+ from the cells, experiments were performed in vesicles obtained from the plasma membrane of the cells. Vesicles were prepared from the wild type strain 757; a membrane potential, positive inside, and a pH gradient, acid inside, were produced as described previously [25] by the addition of ATP, which can be used by the membrane ATPase to pump protons into the vesicles. Under these conditions, the experiment showed (Fig. 10) that the vesicles were able to take up $^{86}Rb^+$ when ATP was added to the medium, which is in agreement with the existence

TABLE I

Efflux of inorganic phosphate from yeast produced by the addition of 5 μM Tb^{3+}

Yeast cells were grown in YPD medium with the addition of 15 mM phosphoric acid. After preparation of the medium, the pH was adjusted to 6.8, which is the same pH obtained when the same amount of KH_2PO_4 was added. The cells were incubated as indicated for Figs. 1 and 2, with the addition of 5 μM $TbCl_3$. After 7 min, when the efflux of K^+ was maximum, the cells were centrifuged and inorganic phosphate was measured in an aliquot of the supernatant.

Addition	Inorganic phosphate (nmol)	
	efflux into the medium	total content in cells
50 mg of yeast		
none	38.9	1005
5 μM $TbCl_3$	188.4	1005
100 mg of yeast		
none	74.8	2010
5 μM $TbCl_3$	245.2	2010
10 μM $TbCl_3$	293.1	2010

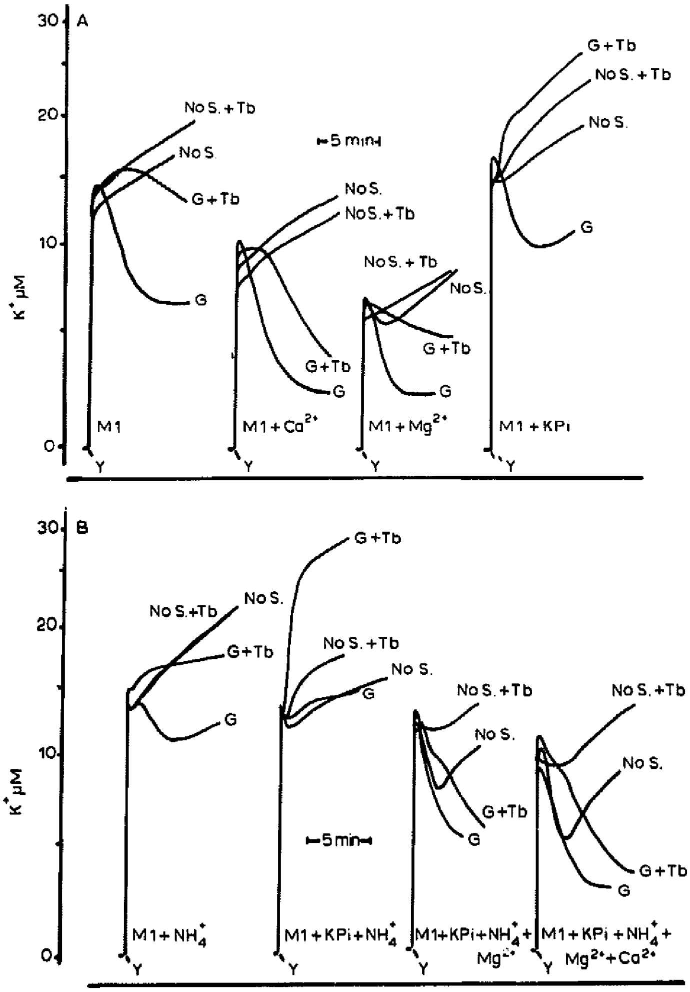

Fig. 8. (A and B). Movements of K^+ in the wild type strain XT-300.3A, grown in YPD medium without additions (M1), or with 2.6 mM $CaCl_2$; 2.1 mM $MgSO_4$; 15 mM monobasic potassium phosphate; 45 mM $(NH_4)_2SO_4$; 45 mM ammonium plus 15 mM potassium phosphate; 45 mM ammonium sulfate plus 15 mM potassium phosphate, plus 2.1 mM magnesium sulfate; ammonium, plus potassium phosphate, plus magnesium, plus calcium chloride. Incubation conditions of the cells were as for Fig. 1.

of an exchange system of K^+ for H^+. This uptake was prevented by adding of vanadate before ATP, and reverted if the inhibitor was added several minutes after ATP.

Discussion

The data presented show that under certain growth conditions, a system appeared capable of producing an increased efflux for potassium and requiring glucose or other substrates. This system, besides, can be stimulated by the presence of several cationic substances of diverse nature, such as ethidum bromide, dihydrostreptomycin and diethylaminoethyl dextran, or by the addition of polyvalent cations, such as those of the lanthanide series. A similar, but slower system, was described by Rothstein [1], Conway [2], Ortega et al. [3], and has also been shown by Van de Mortel et al. [6,7], to be inhibited by divalent cations and lanthanum, and has been ascribed to the ionic channels present in yeast [28–30]. It is difficult at this moment to define its nature, specially because, under our experimental conditions, it can be stimulated precisely by trivalent cations, such as gadolinium, which has been described as an inhibitor of ionic channels [31,32], including those of the yeast plasma membrane [28].

The efflux system was observed with glucose or ethanol as energy sources, indicating that the require-

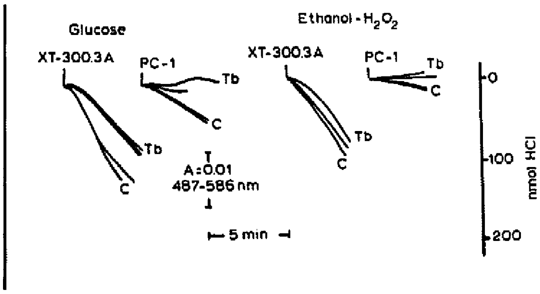

Fig. 9. Changes of the pH of cell suspensions grown on YPD medium supplemented with 15 mM phosphoric acid-TEA. Incubation conditions were as follows: 1 mM Mes-TEA buffer (pH 6.0), 10 mM glucose, or 54 mM ethanol plus 1 μl of 3% hydrogen peroxide; bromcresol purple, 8 μg; yeast, 10 mg, wet weight; final volume, 2.0 ml. The reaction was started by the addition of yeast, and the absorbance at 487–586 nm was recorded against time with continuous stirring at room temperature. The strain used was XT-300.3A as the wild type, and PC-1 as the mutant.

ment is not for an intermediary of metabolism of any of the two substances, which have very different metabolic pathways. The common factor is ATP, and probably the generation of the membrane potential [33] by the membrane H^+-ATPase. This suggestion is supported, in part, by the experiments performed with ATPase mutants, which show that, as expected, both of them have a decreased capacity to generate the potential, and at the same time, also showed a decreased efflux of K^+ when stimulated by Tb^{3+}. It seems possible that the efflux system is similar to that described for the exchange of Na^+ for H^+ in other membranes [34,35]. The experiments also show that this system could be a K^+-H^+ exchanger. It is possible that the slowing of the acidification of the medium, which is more or less coincident with the efflux of K^+ produced by Tb^{3+}, is due to the entrance of H^+ in exchange for K^+. The experiments with the membrane vesicles in which addition of ATP, producing acidification of the interior of the vesicles, resulted in the influx of Rb^+, also agree with the existence of a monovalent cation-H^+ exchange system. However, the finding that there was an efflux of inorganic phosphate during the efflux of potassium ions, indicates that, at least, a movement of compensatory anions out of the cells might be occurring. The hyperpolarization observed by the addition of Tb^{3+}, in agreement with other authors [6] may also indicate that at least part of the K^+ leaves the cell without any accompanying anions or is exchanged for cations, hence producing a diffusion potential. This finding agrees with the suggestion of Van den Mortel et al. [6,7], according to which, the efflux may occur through the ionic channels described in the yeast plasma membrane [28–30].

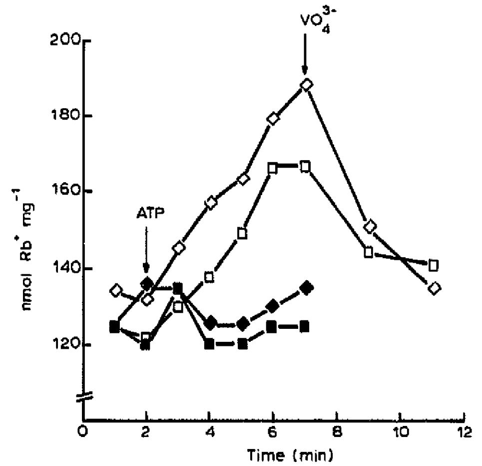

Fig. 10. Uptake of ^{86}Rb by plasma membrane vesicles of yeast upon the addition of ATP, and the effect of vanadate. The experiment was performed as described under Materials and Methods. □, No addition; ◇, 250 μM vanadate; ■, nitrate; ◆, nitrate plus vanadate. ATP and vanadate were added where indicated.

The stimulation mechanism for the efflux system by the diverse agents used, is not clear; all them were of cationic nature, probably acting on this putative exchanger present in the membrane, but based on the present evidence, no further explanation is possible, specially due to the inhibitory characteristics of Gd^{3+} on ionic channels [31,32], including those of yeast [28]. It is interesting to point out that the efflux of potassium produced hyperpolarization of the cells, as indicated by the experiments to estimate the membrane potential with the fluorescent cyanine, an event that should not occur, if the efflux of the cation were due to cell rupture.

Specially in view of previous reports of an all or none damage to the cells by different agents, such as mercury ions [36], methylene blue [37], ethidium bromide itself [38] and some other compounds [36], it is not possible to discard, at least, some disruption of the cells, due to the observed efflux of phosphate ions. It may even be possible that some combined mechanism, working at different concentrations of the agents took place, as demonstrated by Eilam for the cationic agent trifluoperazine [40] or as described for ethidium bromide [41,44]; our findings with Tb^{3+} may be similar to those found with these agents. It is also possible that the stimulation mechanism of the efflux is different for each of the different cationic agents used.

The fact that the efflux system can be observed in the mutants, even in the absence of the stimulating agents, indicates that it is not related to the functional state of the high affinity influx system of the cell. Finally, although an efflux system has been described in yeast grown under different conditions [1–3,6,7], it is worth mentioning that it is more clearly observed in the cells grown in the simple YPD medium. Besides, the fact that the cells grown in the simple YPD medium. Besides, the fact that the cells grown in the medium complemented with phosphate show a larger efflux, agrees with the results showing that a significant amount of phosphate efflux also accompanies that of K^+. The behavior of the efflux system could also be related to the charge balance at the insertion site of the protein responsible for the K^+ efflux in the membrane, since its working rate can be modified significantly by the addition of divalent cations. Ca^{2+} or Mg^{2+}, to the growth medium. The results obtained with the divalent cations appear difficult to interpret, because of the differences observed between the wild type and the mutant strains. However, one possible explanation of the inhibition of the efflux in the wild type cells is the known effect of divalent cations on monovalent cation uptake through the changes produced on the surface potential of the cells, described by Theuvenet and Borst-Pauwels [45]. It seems also, from the experiments in the mutant strains, that divalent cations produced a decrease of the efflux, in agreement with the results of the experiments in which the cells were grown in the presence of either Mg^{2+} or Ca^{2+}.

The role of the described system is difficult to define based on the present results; however, the data suggest an important participation of divalent cations in the behavior and regulation of ion fluxes in yeast.

Acknowledgements

This work was supported by grants from the 'Consejo Nacional de Ciencia y Tecnologia', and the 'Dirección de Asuntos del Personal Académico' (UNAM). The technical assistance of Miss Norma Sánchez is acknowledged.

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