Ectopic Potassium Uptake in \textit{trk1 trk2} Mutants of \textit{Saccharomyces cerevisiae} Correlates with a Highly Hyperpolarized Membrane Potential*

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Ricardo Madrid‡, María J. Gómez‡, José Ramos‡, and Alonso Rodríguez-Navarro††

From ‡Departamento de Biotecnología, Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica de Madrid, 28040 Madrid, Spain and the †Departamento de Microbiología, Escuela Técnica Superior de Ingenieros Agrónomos, Universidad de Córdoba, 14071 Córdoba, Spain

Null \textit{trk1 trk2} mutants of \textit{Saccharomyces cerevisiae} exhibit a low-affinity uptake of K$^+$ and Rb$^+$. We show that this low-affinity Rb$^+$ uptake is mediated by several independent transporters, and that \textit{trk1} cells and especially \textit{trk1A trk2Δ} cells are highly hyperpolarized. Differences in the membrane potentials were assessed for sensitivity to hygromycin B and by flow cytometric analyses of cellular DiOC$_6$(3) fluorescence. On the basis of the latter analyses, it is proposed that Trk1p and Trk2p are involved in the control of the membrane potential, preventing excessive hyperpolarizations. K$^+$ starvation and nitrogen starvation hyperpolarize both \textit{TRK1 TRK2} and \textit{trk1A trk2Δ} cells, thus suggesting that other proteins, in addition to Trk1p and Trk2p, participate in the control of the membrane potential. The HAK1 K$^+$ transporter from \textit{Schwanniomyces occidentalis} suppresses the K$^+$-defective transport of \textit{trk1A trk2Δ} cells but not the high hyperpolarization, and the HKT1 K$^+$ transporter from wheat suppresses both defects, in the presence of Na$^+$. We discuss the mechanism involved in the control of the membrane potential by Trk1p and Trk2p and the causal relationship between the high membrane potential (negative inside) of \textit{trk1A trk2Δ} cells and its ectopic transport of alkali cations.

Potassium is an indispensable element, which is accumulated against large transmembrane concentration gradients in cells living in diluted environments. Probably because of the central role of this element in all forms of life, different mechanisms mediating K$^+$ uptake have evolved in different types of cells. In plants and fungi, the cellular uptake of K$^+$ is probably always an electrochemical process, which takes place in response to the membrane potential created by the H$^+$-pump ATPase (1, 2), as described for \textit{Neurospora crassa} (3).

Among all eucaryotic non-animal cells, the maximum information about potassium transport has been obtained in \textit{Saccharomyces cerevisiae}. In this fungus, the biochemistry (4–7) and the genetics of K$^+$ transport (8–11) and H$^+$-pump ATPase (12) have been extensively studied. The \textit{TRK1} gene of \textit{S. cerevisiae} encodes a notable K$^+$ transporter, which is adapted to provide the required amount of K$^+$ in many different nutritional conditions. To perform this function, this transporter changes its \textit{Km}, which shows values in the millimolar range in cells growing at millimolar concentrations of K$^+$, and as low as 20–30 \textmu m in K$^+$-starved cells (5, 8, 13). The \textit{Vmax} is also variable, depending on the cellular pH and K$^+$ content of the cells (5, 6). A second gene, \textit{TRK2} (10, 11), encodes a second K$^+$ transporter structurally related to Trk1p (11). However, unlike Trk1p, Trk2p shows a very low Rb$^+$ influx \textit{Vmax} in \textit{trk1A} cells with a low K$^+$ content, and the influx is undetectable in \textit{TRK1} cells or in \textit{trk1Δ} cells with a normal K$^+$ content (7). Using a \textit{trk1Δ} strain overexpressing Trk2p, it has been found that the affinity of the \textit{TRK2} transporter for K$^+$ and Rb$^+$ is also regulated by the K$^+$ content of the cell, and that the \textit{Km} in K$^+$-starved cells is only slightly higher than that of \textit{TRK1} (7). Because the \textit{Vmax} of \textit{TRK2} is very low in comparison to that of \textit{TRK1} and the \textit{Km} is higher than the \textit{Km} of \textit{TRK1}, the function of Trk2p seems to be superfluous as an independent K$^+$ transporter.

Deletion of the \textit{TRK1} and \textit{TRK2} genes results in cells that grow normally at high concentrations of K$^+$, and slowly at relatively low concentrations, exhibiting a Rb$^+$ influx kinetics with a \textit{Km} of 60 mm and a \textit{Vmax} of 9 and 16 nmol mg$^{-1}$ min$^{-1}$ in the two strains studied so far (7). This low-affinity Rb$^+$ uptake of \textit{trk1Δ trk2Δ} cells also constitutes the major pathway for Rb$^+$ uptake in \textit{trk1Δ TRK2} cells, in which Trk2p mediates a minor pathway, as already mentioned. However, in contrast with the relevance of the low-affinity K$^+$ uptake in \textit{trk1Δ TRK2} and \textit{trk1Δ trk2Δ} cells, this uptake has never been detected in wild strains (4, 5). Because the \textit{Km} of the low-affinity Rb$^+$ influx is much higher than the \textit{Km} of \textit{TRK1} and the \textit{Vmax} not insignificant in comparison to the \textit{Vmax} of \textit{TRK1}, it can be concluded that the low-affinity Rb$^+$ uptake has not been detected in \textit{TRK1} cells because it does not exist in these cells.

Therefore, two important questions remain unanswered about K$^+$ transport in \textit{S. cerevisiae}, the function of Trk2p and the identity, and consequently the function, of the transporter mediating the low-affinity uptake of K$^+$ and Rb$^+$ observed in \textit{trk1Δ} cells. For the latter question two hypotheses have been put forward, that this transport takes place mediated by several non-K$^+$ transporters, which harbor intrinsic K$^+$ transport capabilities (14–16), and that it is mediated by a part of the normal K$^+$ uptake system, which is multimeric and loses its normal properties in the absence of Trk1p (7). The former hypothesis is supported by well documented studies with strains carrying mutations in sugar and amino acid transporters, and with strains overexpressing amino acid permeases (14–16). The latter has been advanced to explain why the low-affinity Rb$^+$ uptake does not exist in wild strains, and to explain why glucose activates the low-affinity Rb$^+$ influx of

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† To whom correspondence should be addressed: Departamento de Biotecnología, Escuela Técnica Superior de Ingenieros Agrónomos, 28040 Madrid, Spain. Tel.: 34-91-336-5751; Fax: 34-91-336-5757; E-mail: arodrignavar@bit.etsia.upm.es.

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trk1Δ mutants in the same fashion as it does in wild strains (7, 17).

In this paper we report the characteristics of Rb⁺ uptake, tolerance to hygromycin B, and cellular DiOC₆(3) fluorescence, as determined by flow cytometry, in strains carrying different combinations of wild and null alleles of the TRK1 and TRK2 genes. The results suggest that Trk1p and Trk2p are involved in the regulation of the electrical membrane potential. Deletion of these proteins brings the cells to a very high membrane potential, negative inside, which could drive an ectopic uptake of alkali cations.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, Growth Conditions, and Methods—**The S. cerevisiae strains used in this study are all isogenic to W303.1A (Table I).

| Strains and plasmids | Relevant characteristics | Reference or source |
|----------------------|--------------------------|---------------------|
| **S. cerevisiae**    |                          |                     |
| W303.1A              | Mat a his3 leu2 trp1 ade2 ura3 | R. Haro (this laboratory) |
| W59                  | W303.1A trk1::LEU2        | R. Haro (this laboratory) |
| W19                  | W303.1A trk2::HIS3        | R. Haro (this laboratory) |
| W3Δ                  | W303.1A trk1::LEU2 trk2::HIS3 |                     |
| UCP9                 | W303 expressing the mutant version UCP9 of the UCP1 cDNA | Ref. 18 |
| Y806                 | Mat a leu2 ade2 ade3 ura3 lys2 ϕ⁺ | Ref. 19 |
| **Plasmids**         |                          |                     |
| pYPGE15              | Yeast expression vector. PGK1 gene promoter and CYC1 terminator. 2 μm, URA3 yeast marker and Amp⁸ | Ref. 20 |
| pYES2                | Yeast expression vector. GAL1 gene promoter and HIS3 terminator. 2 μm, URA3 yeast marker and Amp⁸ | Invitrogen |
| YEp352               | Yeast episomal vector. 2 μm, URA3 yeast marker and Amp⁸ | Ref. 21 |
| pRS404               | Yeast integrative plasmid; TRP1 yeast marker and Amp⁸ | Ref. 22 |
| pPGKHAK1             | pYPGE15 derivative, expressing the 2.3-kb coding region of the gene HAK1 of S. occidentalis | M. A. Bañuelos (this laboratory) |
| pAG8                 | pYES2 derivative, expressing the 2.3-kb coding region of the gene HAK1 of S. occidentalis | M. A. Bañuelos (this laboratory) |
| pRSHAK               | pRS404 derivative containing the 5-kb SnaB/MluI fragment of pAG8 including the HAK1 gene of S. occidentalis the GAL1 gene promoter and the HIS3 terminator | This study |
| pPMAHKT1             | pDR195 derivative expressing the HKT1 cDNA | F. Rubio |
| YEpHNM1              | YEp352 derivative containing the HNM1 gene | This study |
| YEpHXT3              | YEp352 derivative containing the HXT3 gene | This study |

SHAK1 strain carrying the pGAL1-HAK1 gene, galactose-substituted glucose, and in the experiments designed to test the effect of divalent cations on Rb⁺ influx, the pH of the testing buffer was adjusted with NaOH, and MgCl₂ was withdrawn. To start the experiments, Rb⁺ was added to the cell suspension and, at short intervals, samples of cells were removed by filtration from the testing buffer. To determine the Rb⁺ content, the cells were treated with HCl and the extracts analyzed by atomic emission spectrophotometry, as described previously (5). In all cases, we determined the initial rate of Rb⁺ uptake from the time course of the net accumulation.

**Flow Cytometry—**S. cerevisiae and N. crassa cells were grown as described in each case. S. cerevisiae was always grown at low cell density (<0.3 mg dry weight ml⁻¹), and as a consequence the concentration of glucose was very similar in all experiments (close to 2%). Cells were harvested from the culture medium, suspended in testing buffer (2 × 10⁶ cells ml⁻¹), and exposed to DiOC₆(3) cyanine dye (3,3'-dihexyloxacarbocyanine iodide, Molecular Probes, Eugene, OR) at 1 mM for 30 min at 28 °C. The dark, to test yeast cell viability, pip+ was added at the moment of the analysis. Flow cytometric analyses were performed in a FACSscan (Becton Dickinson) and EPICS XL (Coulter Electronics) flow cytometers, equipped with argon lasers. For the determination of the DiOC₆(3) fluorescence, excitation at 488 nm and a 525-nm dichroic LP filter were used. For pip+ iodide, a 590-nm dichroic filter and a 610-nm LP absorbance filter were added. Because the cellular fluorescence was size dependent, we normalized all the results measuring the fluorescence of single small cells (25). In all experiments, a control sample of wild-type cells (W303.1A strain) grown in the arginine medium at 0.5 mM K⁺ was analyzed in parallel with the other samples, and the fluorescence values given by the flow cytometer were always referred to the fluorescence of the control cells and expressed as a percentage. All measurements were repeated at least in three different days.

**RESULTS**

**Characteristics of the Low-affinity K⁺ Transport of trk1Δ trk2Δ Cells—**The kinetic study of the influx of the alkali cations in trk1Δ trk2Δ cells with different K⁺ contents and the competitive inhibitions exerted between them indicated that these cells took up alkali cations with little differences in affinities (we determined Kₘ values of 60 mM K⁺, 60 mM Rb⁺, 110 mM Na⁺, and 100 mM Li⁺ in the W3Δ strain), and without regulation by the K⁺ content. Although this uptake shows homogenous kinetics, experiments with different inhibitors showed that it involves different pathways. Ammonium inhibited 45% of the low-affinity Rb⁺ uptake of trk1Δ trk2Δ cells, but showed

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1 The abbreviations used are: MES, 2-(N-morpholino)ethanesulfonic acid; TAPS, 3-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)ammonium-1-propanesulfonic acid; CCCP, carbonyl cyanide p-chlorophenylhydrazon.
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no effect over the remaining 55%; low concentrations of Mn$^{2+}$, and to a lesser extent other divalent cations (Mg$^{2+}$, Ca$^{2+}$, and Ba$^{2+}$), inhibited 30%, but showed no effect on the remaining 70% (Fig. 1); and ammonium plus Mn$^{2+}$ (20 mm each) inhibited 60% of the uptake. These results suggest the existence of at least three pathways for Rb$^+$ uptake in trk1Δ trk2Δ cells, one inhibited by Mn$^{2+}$, another inhibited by ammonium and insensitive to Mn$^{2+}$, and a pathway insensitive to both ions. It is worth mentioning that the described inhibition of Rb$^+$ uptake by ammonium or Mn$^{2+}$ in trk1Δ trk2Δ cells was different from that found in wild-type cells. In the latter, ammonium was a competitive inhibitor of Rb$^+$ uptake, and Mn$^{2+}$ and other divalent cations did not show any significant effect (not shown).

Rb$^+$ uptake in trk1Δ trk2Δ cells was also found to be very sensitive to uncouplers, much more than in TRK1 TRK2 or TRK1 trk2Δ cells. These experiments were carried out in K$^+$-starved cells, in order to test cells in identical physiological conditions, finding that at 20 μM CCCP, Rb$^+$ influx (initial rate of uptake at 100 mM Rb$^+$) in trk1Δ trk2Δ cells was inhibited by 80–100%, whereas this concentration showed no effect on TRK1 TRK2 cells (Fig. 2).

Overexpression of Hxt3p and Hnm1p Partially Suppresses the Phenotype of trk1Δ trk2Δ Cells—In a different part of the study of the low-affinity K$^+$ uptake of S. cerevisiae, we had isolated mutants suppressing the trk1Δ trk2Δ phenotype, and found that many of them mapped on the HXT3 gene, as is consistent with previous reports (14, 15, 26). The sequence of the HXT3 mutated genes revealed that some of these mutations were located in the 5′ non-coding region. This suggested that these mutations produced overexpression of the encoded protein, and hence that the wild HXT3 transporter was able to transport K$^+$. To test this possibility, we inserted the HXT3 gene in a multicopy plasmid and transformed it into the trk1Δ trk2Δ strain, finding that the growth at low K$^+$ was improved significantly (not shown).

Considering this result, we searched for other genes which, when overexpressed, suppress the trk1Δ trk2Δ phenotype. For this purpose, we transformed a trk1Δ trk2Δ strain with a library of genomic DNA in a multicopy plasmid and screened for clones suppressing the high K$^+$ requirement of trk1Δ trk2Δ cells. Using the ammonium medium with 2 mm K$^+$, we isolated a clone with a 9-kilobase insert containing the YGL077c open reading frame, which encodes the HNM1 choline transporter (27). By subcloning this gene alone in a multicopy plasmid, we found that HNM1 partially suppressed the trk1Δ trk2Δ phenotype (not shown). Although the overexpression of Hxt3p or Hnm1p improved the growth rate of the mutant, it increased less than 10% the rate of Rb$^+$ uptake, suggesting that these proteins made a small contribution to the total K$^+$ uptake of trk1Δ trk2Δ cells.

The finding that the overexpression of the HXT3 and HNM1 transporters partially suppressed the trk1Δ trk2Δ phenotype, as it is the case of some amino acids and the inositol transporters (16), gave clear support to the notion that multiple K$^+$ pathways, possibly making small individual contributions, composed the low-affinity K$^+$ uptake in trk1Δ and trk1Δ trk2Δ mutants. Because the low-affinity K$^+$ uptake does not exist in wild strains, the question was how the lack of one protein, Trk1p, could open all these pathways.

The Lack of Trk1p Triggers the Low-affinity Uptake of Rb$^+$—Two different reasons could trigger the general response that furnished several different transporters with the capacity of transporting K$^+$, the loss of Trk1p by itself or the K$^+$ deficiency generated by its loss. To distinguish between these two possibilities, we used the trk1Δ trk2Δ strain transformed with the HAK1 gene of Schwanniomyces occidentalis (28). This gene encodes a K$^+$ transporter not related to Trk1p and Trk2p, that restores K$^+$ uptake, and consequently a normal growth at low K$^+$, in trk1Δ trk2Δ cells (28). Therefore, if trk1Δ trk2Δ cells expressing Hak1p still kept the low-affinity K$^+$ and Rb$^+$ uptake, the mediation of the K$^+$ deficiency in this uptake could be ruled out.

For a high expression of Hak1p in S. cerevisiae, we used two different constructs, placing the coding region of the HAK1 gene under the control of the PGK1 or GAL1 gene promoters. Both constructs transformed into the trk1Δ trk2Δ strain allowed the transformants to grow at K$^+$ concentrations even lower than those needed for TRK1 strains. In contrast, in these experiments the low-affinity uptake was present or absent depending on the testing conditions. In the pPGK1-HAK1 transformant, Rb$^+$ influx in K$^+$-starved cells showed only one kinetic component, corresponding to HAK1, whereas in normal K$^+$ cells, Rb$^+$ influx showed both the HAK1 and the low-affinity kinetic components. In the pGAL1-HAK1 transformant, cells grown on galactose (YPD) and tested on galactose showed only the Rb$^+$-influx kinetics of HAK1, but shortly after the transfer to glucose (YPD) they showed both the HAK1 and the low-affinity kinetic components. A longer incubation in glucose progressively decreased the former without affecting the latter (not shown).
**Table II**

| Strains (genotypes) | YPD   | YPD + 50 ms KCl |
|---------------------|-------|-----------------|
| TRK1 TRK2           | 50    | 100             |
| TRK1 trk2           | 50    | 100             |
| trk1 TRK2           | 15    | 50              |
| trk1 trk2 pPG15     | 10    | 50              |
| trk1 trk2 PppalmHAK1| 20    | 50              |
| trk1 trk2 PppalmHKT1| 75    | 100             |

**Ectopic Potassium Uptake in trk1 trk2 Mutants**

The sensitivity to hygromycin B depends on the K⁺ transport functions of Trk1p, but that only in very special conditions did it prevent the low-affinity Rb⁺ uptake. Among all the possible causes for this behavior, changes in the membrane potential were the most likely. The working hypothesis was that trk1Δ trk2Δ cells could be hyperpolarized, and that the low-affinity Rb⁺ uptake required such hyperpolarization. This hypothesis gave a satisfactory explanation to the high sensitivity of the low-affinity Rb⁺ uptake to uncouplers (Fig. 2), and was testable by determining the resistance of the different strains to hygromycin B, an amino glycoside antibiotic for which, as in the case of Dio-9 (29, 30), the resistance of the cells depends on their membrane potential (31, 32). The tests were performed in YPD, which contains sufficient K⁺ (15 mM) for normal growth of the trk1Δ trk2Δ strain, using the different mutants in the TRK1 and TRK2 genes, and the trk1Δ trk2Δ strain transformed with the pPG15-HAK1 gene or the pPG15-HKT1 gene or the TRK2 promoter (Table II).

**TABLE II**

| Strains (genotypes) | YPD   | YPD + 50 ms KCl |
|---------------------|-------|-----------------|
| TRK1 TRK2           | 50    | 100             |
| TRK1 trk2           | 50    | 100             |
| trk1 TRK2           | 15    | 50              |
| trk1 trk2 pPG15     | 10    | 50              |
| trk1 trk2 PppalmHAK1| 20    | 50              |
| trk1 trk2 PppalmHKT1| 75    | 100             |

**Fig. 3. Flow cytometric analyses of TRK1 TRK2 cells.** A. plot of the DiOC₆(3) fluorescence as a function of the forward scatter. The vertical line divides two subpopulations of cells, depending on their size, single cells, or cells with small buds, and large single cells or cells with large buds. B, distribution (counts) of the cells according to their cellular DiOC₆(3) fluorescence. The two subpopulations shown in A have been plotted independently, and higher fluorescence correspond to larger cells. A1 and B1, cells with a normal K⁺ content; A2 and B2, K⁺-starved cells.

The electrical membrane potential of yeast cells is not known, but comparative assessments of membrane potentials in different conditions may be obtained with fluorescent cyanine dyes (37, 38). The cyanine dye DiOC₆(3) has been used to stain internal membranes in yeast (39) and to detect dysfunctional mitochondria (18, 25, 40). To use this dye as a probe for the membrane potential, avoiding the interference of the fluorescence of the internal membranes, we reduced the concentration of the dye in the staining medium (testing buffer) to 1 nM and tested only fermenting cells in which the function of the mitochondria is highly reduced (41). Fermenting cells stained with 1 nM DiOC₆(3) and analyzed by flow cytometry showed size-dependence fluorescence (Fig. 3A). Taking the whole population or dividing it into two subpopulations, roughly corresponding to single cells and cells with small buds, and large cells and cells with large buds, K⁺-starved cells showed a much higher fluorescence than cells with a normal K⁺ content (Fig. 3, A and B).

To investigate the contribution of the mitochondria to the overall fluorescence of the cells, we performed some preliminary experiments with rho⁻ and rho⁰ strains, finding that in cells growing at 2% glucose and stained with 1 nM DiOC₆(3) the difference in fluorescence between the rho⁻ and rho⁰ strains was negligible. Then we used a yeast strain expressing a mutant version (UCPΔ9) of the uncoupling protein of brown adipose tissue, when induced with galactose. The expression of this mutant protein produces a complete uncoupling of the mitochondria, thus abolishing the contribution of the mitochondria to cell fluorescence (18, 42). In the first experiment, we grew the cells in 2% glucose or 2% galactose, finding no differences in fluorescence (Table III). In the second experiment, the cells were first grown on lactate (respiratory conditions) and then galactose was added at a concentration (1%) that could be fermented when the mitochondria was not functional. The results showed that the fluorescence of the cells on lactate was reduced to a half upon the addition of galactose (Table III). From these results we concluded that, using 1 nM DiOC₆(3), the mitochondria did not make any significant contribution to the cell fluorescence in fermenting conditions, and it contributed...
Neither NaCl nor KCl showed any appreciable effect.

The presence of the HAK1 promoter, indicated that K⁺ uptake can
be increased by 100% in the absence of NaCl or Na⁺ depolarization. When the HKT1 transformant was grown in the presence of Na⁺ the cells were strongly depolarized consistent with the high tolerance of this transformant to hypoxycyanin B (YPD contains both K⁺ and Na⁺).

Considering that Trk1p and Hakt1p are very active K⁺ transporters, that Hkt1p is a K⁺-Na⁺ transporter, which may also transport only Na⁺ in some conditions (34), and that Trk2p has negligible activity as a K⁺ transporter in the strains used, the most important conclusions that can be drawn from the results summarized in Table IV are the following: (i) both Trk1p and Trk2p are involved in the control of the membrane potential, even when neither K⁺ nor Na⁺ are present in the external medium; (ii) the heterologous transporters did not suppress the high membrane potential of trk1Δ trk2Δ cells in the absence of K⁺ and Na⁺; (iii) electrophoretic K⁺ and Na⁺ uptake can explain the depolarizing effects shown by KCl and NaCl in those cells expressing an active K⁺ or Na⁺ transporter; (iv) in the absence of Trk1p and Trk2p, KCl and NaCl showed hyperpolarizing effects, especially in K⁺-starved cells.

Chloride Is Not Involved in the Hyperpolarizing Effects of NaCl and KCl—One intriguing result among those summarized in Table IV is the hyperpolarizing effect of KCl and NaCl in the trk1Δ trk2Δ strain. This effect could result from the high diffusion potential created by the increase in the external concentration of chloride if a chloride channel exists in the plasma membrane. To address this possibility we repeated the experiments summarized in Table IV substituting Na⁺-MES for NaCl or K⁺-MES for NaCl and KCl, respectively. The change of the anion did not change the results, thus indicating that Na⁺ and K⁺, but not chloride, were involved in the hyperpolarizing effect.

Trk1p and Trk2p Are Involved in the Response of the Membrane Potential to Changes in the External pH—Two reasons could explain why trk1Δ trk2Δ cells were hyperpolarized with reference to TRK1 TRK2 cells, that the H⁺-pump ATPase was more active in the mutant than in the wild strain, because Trk1p and Trk2p exercise some degree of control on the pump, or that Trk1p and Trk2p depolarized the membrane by mediating a flux of ions at high membrane potentials. Because the difference between the membrane potentials of these strains persisted in a buffer with only MES, Ca²⁺, and H⁺, the most likely ion fluxes were H⁺ inward or anions outward. To test if H⁺ influx was involved, we studied the response of the cellular DiOC₆(3) fluorescence of the different genotypes to changes in the external pH and to the addition of uncouplers. These tests were performed with K⁺-starved cells in the complete absence of K⁺, thus eliminating the differences in depolarizing currents that this cation may introduce in genotypes with different K⁺ uptake capacities. The data summarized in Table V (data at pH 6.0 correspond to basal conditions, K⁺-starved cells, in Table IV) show clearly that the membrane potential decreased with the decrease of the external pH only when Trk1p or Trk2p are present. In contrast, the response to the addition of CCCP, which should increase unspecific H⁺ conductance, was not appreciably different in the different genotypes, producing in all cases a progressive decrease in the cellular DiOC₆(3) fluorescence with the increase in the CCCP concentration. Taken together, these results suggest that Trk1p and Trk2p somehow increase the permeability to H⁺.

Nitrogen Starvation Also Produces Hyperpolarization—Before addressing the question of whether Trk1p and Trk2p are H⁺ transporters or only regulate the H⁺ permeability of another membrane protein, we addressed the more general question of whether hyperpolarization of the plasma membrane was a specific response to K⁺ starvation or a more general response to any kind of starvation. With this purpose we studied the
TABLE IV

Cellular DiOC₆(3) fluorescence of S. cerevisiae cells expressing different K⁺ transporters

Expressed as percentage of the cellular fluorescence of TRK1 TRK2 cells growing in 0.5 mM K⁺ arginine medium. The values are mean ± S.D. The number of repetitions were: 8 for TRK1 TRK2 and trk1 trk2, 5 for the other strains in basal conditions, 3 in all other cases.

| Strains (genotypes) | Cells with normal K⁺ content | K⁺-starved cells |
|---------------------|------------------------------|------------------|
|                     | Basal | 10 mM KCl | 10 mM NaCl | Basal | 0.5 mM KCl | 10 mM KCl | 10 mM NaCl |
| TRK1 TRK2           | 100   | 100 ± 17  | 127 ± 22  | 232 ± 29 | 126 ± 10  | 100 ± 9  | 254 ± 22  |
| TRK1 trk2           | 93 ± 11 | 110 ± 24  | 128 ± 22  | 201 ± 39 | 130 ± 12  | 100 ± 21 | 241 ± 23  |
| trk1 TRK2           | 185 ± 32 | 180 ± 35  | 197 ± 13  | 316 ± 47 | 327 ± 23  | 333 ± 33 | 345 ± 19  |
| trk1 trk2           | 246 ± 44 | 250 ± 40  | 344 ± 50  | 403 ± 30 | 553 ± 44  | 620 ± 81 | 628 ± 60  |
| trk1 trk2 HAK1      | 192 ± 33 | 206 ± 35  | 189 ± 19  | 382 ± 67 | 267 ± 33  | 292 ± 10 | 575 ± 30  |
| trk1 trk2 HKT1      | 235 ± 49 | 243 ± 40  | 175 ± 21  | 421 ± 51 | 230 ± 21  | 275 ± 28 | 230 ± 47  |
| trk1 trk2 HKT1 Na⁺  | 25 ± 5  | 30 ± 2    | 25 ± 4    | 30 ± 5  | 25 ± 2    | 25 ± 4   | 28 ± 4    |

* K⁺ contents in the growing media were: 0.5 mM for TRK1 TRK2, TRK1 trk2, trk1 trk2 HAK1, and trk1 trk2 HKT1, 2.5 mM for trk1 TRK2, and 10 mM for trk1 trk2 strains.

ACCOUNTING FOR THE HIGHER Membrane Potential of TRK1 TRK2 Cells

We have described that trk1Δ trk2Δ cells hyperpolarized in response to K⁺ starvation, increasing their cellular DiOC₆(3) fluorescence over the already high normal value. Unlike this response, nitrogen starvation did not show any effect on the DiOC₆(3) fluorescence of trk1Δ trk2Δ cells.

DISCUSSION

The results presented in this report indicate that the growth of trk1Δ trk2Δ mutants of S. cerevisiae is supported by a ectopic K⁺ uptake mediated by several transporters, which, as a whole, resembles a low-affinity single transporter for K⁺, Rb⁺, Na⁺, and Li⁺. The low-affinity uptake is inhibited by ammonium and by divalent cations (Fig. 1), and by the number of different and unrelated transporters (amino acids, sugar, inositol, and choline transporters) (this report and Refs. 14-16) that by over-expressing partially suppress the defective growth of trk1Δ trk2Δ strains at low K⁺. Our results also indicate that in the absence of Trk1p, or Trk1p and Trk2p, the membrane potential is exceptionally high, negative inside. Taken together, these results pose two questions, whether there is any causal relationship between the ectopic low-affinity K⁺ and Rb⁺ uptake and the high membrane potential, and what the cause of this high membrane potential is? Regarding the former question, it is clear that both the low-affinity Rb⁺ uptake and the high membrane potential are the consequences of the deletion of the TRK1 gene. Furthermore, the ectopic K⁺ uptake must result from a change in a general property of the membrane, because it is unlikely that the deletion of the TRK1 gene can modify the specific properties of many different transporters, making all of them permeable to alkali cations. Considering these observations and the high sensitivity of the low-affinity Rb⁺ uptake to CCCP, the most likely cause for the ectopic uptake of K⁺ is the unusually high membrane potential exhibited by trk1Δ strains.

Concerning the high membrane potential of trk1Δ and trk1Δ trk2Δ cells, our results reveal that Trk1p and Trk2p are not only K⁺ transporters but also essential regulators of the membrane potential. To fulfill this function they must either counter the activity of the pump or control the conductance of the plasma membrane. Before going further in the discussion of the function of Trk1p and Trk2p, it is worth mentioning that a pitfall in the use of the cellular fluorescence of the DiOC₆(3) probe to assess differences in membrane potentials is extremely unlikely, considering the conditions for our assessments: (i) we used the cellular DiOC₆(3) fluorescence only for comparative purposes in entirely isogenic cells, except for the genes considered in each case; (ii) the contribution of the mitochondria to these changes in fluorescence has been ruled out; (iii) we did a control using N. crassa cells, for which the membrane potential has been measured using intracellular electrodes (3). Furthermore, the hyperpolarized state of the trk1Δ and trk1Δ trk2Δ cells can be also deduced by contrasting the phenotype of these mutants with the phenotype of pma1 mutants. The latter are more resistant to hygromycin B than the wild type, whereas trk1Δ and trk1Δ trk2Δ mutants are strikingly more sensitive (Table II). Assuming that the phenotype of pma1 mutants is the result of a lower membrane potential (32), it can be concluded that the phenotype of trk1Δ and trk1Δ trk2Δ mutants is the result of a higher membrane potential.

Nitrogen starvation hyperpolarized TRK1 TRK2 cells but not trk1Δ trk2Δ cells, whereas K⁺ starvation hyperpolarized both types of cells. This suggests that at least two parallel and additive routes control the membrane potential, one dependent on Trk1p and Trk2p, and the other independent of these proteins. Apparently, K⁺ starvation activates both routes, whereas nitrogen starvation activates only that dependent on Trk1p and Trk2p. The mechanisms involved in the control of the membrane potential cannot be established at this moment. However, there are only two ways to achieve this control, either modifying the activity of the pump or triggering a "safety
depolarizing current when the membrane potential reaches a certain value. In the latter case, because the function can be performed in the absence of K\(^+\), or any other alkali cation (Table IV), it is likely that the ions moving are either H\(^+\) inward or anions outward. The Trk1p-Trk2p dependence of low-pH depolarization (Table V) indicates that an inward movement of H\(^+\) may be involved in the route of control depending on Trk1p and Trk2p.

The capacity of Trk1p and Trk2p to control the membrane potential is not a general property of K\(^+\) transporters, because Hak1p cannot perform these functions (Tables III and IV). Although the results supporting this conclusion have been obtained using a heterologous expression, the high level of conservation in proteins and functions among fungi, and even among fungi and higher plants, suggests that it is correct. It is very interesting that in S. occidentalis\(^2\), in N. crassa\(^3\), and in *Debaryomyces hansenii*\(^4\) HAK1 type K\(^+\) transporters coexist with TRK2 K\(^+\) transporters. Whether the function of these TRK2 transporters in these species is more related with the control of the membrane potential than to K\(^+\) uptake is now under study. Interestingly, in barley plants K\(^+\) uptake is mediated by HAK1 transporters (43–46), and wheat plants have a HKT1 transporter whose function is not clear (47). Hkt1p shows homology with Trk1p and Trk2p (36) and, when expressed in S. cerevisiae, it produced strong depolarization in the presence of Na\(^+\) (Table IV). Therefore, the involvement of Hkt1p in the control of the membrane potential in higher plants is an attractive possibility.

The conclusion of this and previous reports (14–16) identifying independent mechanisms for Rb\(^+\) influx in *TRK1 TRK2* and *trk1A trk2Δ* cells indicates that the mechanisms involved in the activation of the V\(_{\text{max}}\) of Rb\(^+\) influx in both types of cells (17) may be also different. Interestingly, we found that the addition of glucose hyperpolarized both wild type and trk mutant cells (not shown), which is consistent with the known activating effect of glucose on the H\(^+\)-pump ATPase (48). The possibility that this hyperpolarization brings about the increase of the V\(_{\text{max}}\) of Rb\(^+\) influx in all cases is an attractive idea. Unfortunately, this cannot be tested with the techniques used in this report.

Finally, *trk1Δ trk2Δ* mutants have been extensively used for cloning heterologous K\(^+\) transporters. Our results indicate that genes expressing low-rate, low-affinity K\(^+\) uptake in these mutants, may encode non-K\(^+\) transporters that support ectopic K\(^+\) uptake.

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\(^{2}\) R. Madrid and A. Rodriguez-Navarro, unpublished results.

\(^{3}\) R. Haro, L. Sainz-Pastor, F. Rubio, and A. Rodriguez-Navarro, unpublished results.

\(^{4}\) C. Prista, M. C. Loureiro-Dias, and J. Ramos, unpublished results.