RESEARCH ARTICLE

Potassium deprivation is sufficient to induce a cell death program in *Saccharomyces cerevisiae*

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Abstract

Cell culture in low potassium (K^+) media has been associated to programmed cell death (PCD) in metazoans. In this study, deprivation of K^+ led *Saccharomyces cerevisiae* cells to a death process that involved phosphatidylserine externalization, changes in chromatin condensation, DNA and vacuole fragmentation as well as enhanced accumulation of reactive oxygen species. During the course of K^+ starvation, plasma membrane hyperpolarization and increased accumulation of calcium (Ca^{2+}) took place. The presence of rubidium (Rb^+), a K^+-analogue element, in the K^+-deprived medium was accompanied by Rb^+ accumulation but did not fully prevent the appearance of PCD markers. This argues for a specific effect of K^+ on the course of cell death. While the absence of the YCA1 metacaspase did not have a major effect, the absence of TRK (transport of K^+) K^+-transporters led to changes in the pattern of annexin V/propidium iodide labeling. This change paralleled a fast accumulation of Ca^{2+}. Addition of ethylene glycol tetraacetic acid improved growth and reduced cell death in *trk1Δtrk2Δ* cells. These findings reveal that K^+ deprivation is sufficient to induce PCD in a cell-walled eukaryotic organism and suggest that the phenotype attributed to the lack of *TRK* genes is partially due to the effect of the encoded transporters on Ca^{2+} homeostasis.

Introduction

Complex multicellular organisms such as metazoans and vascular plants are furnished with specialized cell death programs that allow them to complete their life cycle. Over the last decade, there has been unequivocal evidence (Madeo *et al.*, 1997; Ludovico *et al.*, 2001) that fungi can also undergo programmed cell death (PCD). Although breakdown of potassium (K^+) homeostasis is considered a critical event for the progression of apoptosis in animals (Bortner & Cidlowski, 2007) the possible relevance of K^+ as a player in fungal PCD processes has received less attention. A possible link between loss of K^+ homeostasis and fungal PCD emerges from the observation that fungal cells exposed to conditions leading to apoptotic-like PCD (Huh *et al.*, 2002; Leiter *et al.*, 2005; Pozniakovsky *et al.*, 2005; Reiter *et al.*, 2005) also display defective K^+ nutrition (Martinac *et al.*, 1990; Mulet *et al.*, 1999; Kaiserer *et al.*, 2003; Peña *et al.*, 2009). Furthermore, it has been reported recently that exposure to human lactoferrin causes PCD in the pathogenic fungus *Candida albicans* and the antimicrobial activity

of that protein is prevented by a prior treatment of cells with the K^+-channel blocker tetraethylammonium (Andrés *et al.*, 2008). Although these observations indicate that a breakdown of K^+ homeostasis takes place and may be necessary for the course of apoptotic-like fungal PCD, the hypothesis that K^+ depletion by itself is sufficient to induce PCD has been not assessed. In the present work, this question has been examined for the yeast *Saccharomyces cerevisiae*.

Maintenance of K^+ concentration within a fairly narrow range in living cells involves the concerted activity of several transporters and regulatory components. Among the structural components contributing to yeast K^+ homeostasis, the TRK transporters are considered to be the main pathway for K^+ acquisition from low external K^+ concentrations (Gaber *et al.*, 1988; Bertl *et al.*, 2003; Vargas *et al.*, 2007). These transporters most likely evolved from minimal bacterial K^+ channels, being found ubiquitously, although under highly divergent forms, in bacteria, fungi and plants (Rodríguez-Navarro, 2000). Recent evidence has shown that TRKs are effectors in the cell death process that takes place in *C. albicans* exposed to the cationic protein histatin V (Baev

et al., 2004). Given the pivotal role of these transporters in K^+ homeostasis, we examined the possibility that an intensification of the K^+ imbalance generated by the lack of TRK transporters could influence the dynamics of the cell death process that takes place in K^+-deprived cells.

Materials and methods

Strains, media and culture conditions

Saccharomyces cerevisiae strain W303.1A (*Mat a his3 leu2 trp1 ade2 ura3*) and the derivatives WΔ3 (*Mat a trk2::HIS3 trk1::LEU2 trp1 ade2 ura3*) and *yca1Δ::kanMX* were used in most of the experiments reported here. In addition, in some experiments the LMB 02 (*Mat a ena1::HIS3::ena4 nha1::LEU2 kha1::kanMX::tok1*) and LMM 04 (*Mat a ena1::HIS3::ena4 nha1::LEU2 trk1::LEU2 trk2::HIS3 kha1::kanMX::tok1*) strains were used (Maresova & Sychrova, 2005). Yeast cells were grown in an arginine phosphate medium (APm) modified by raising the PO_4H_3 concentration to 32 mM and adding the amount of arginine necessary to reach a pH of 6.00 ± 0.05. This procedure allowed an adequate maintenance of the external pH, thus avoiding the effect of lowering pH on yeast cell death. The APm medium contained glucose 2%. The K^+-free medium contained only a trace of K^+ (approximately 1 μM). The media were renewed every 12 h, which helped to maintain an essentially constant growth rate for K^+-rich cells (Supporting Information, Fig. S1).

Flow cytometry studies

To estimate the membrane potential at the plasma membrane, changes in the fluorescence of the dye 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] were studied as described by Madrid *et al.* (1998). Briefly, cells were suspended in 2-(*N*-morpholino)ethanesulfonic acid-calcium (Ca^{2+}) buffer and exposed to 1 nM $DiOC_6(3)$ for 30 min at 28 °C in the dark. To assess the reliability of this measurement, the effect of 1 min exposure to carbonyl cyanide *m*-chlorophenylhydrazone 40 μM on $DiOC_6(3)$ fluorescence was determined. Accumulation of reactive oxygen species (ROS) was assessed using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). In this case, cells were washed with phosphate-buffered saline (PBS) and exposed for 15 min to a buffer containing 100 μM H_2DCFDA at room temperature in the dark. The accumulation of intracellular free Ca^{2+} was estimated with the cell permeant indicator Fluo-3-acetoxymethyl ester (Fluo-3 AM) following the protocol described by Zheng *et al.* (2007). Flow cytometry analyses were performed in a FACSCalibur (Becton-Dickinson). In all, 4000–12 000 cells were measured for each independent replicate and data were analyzed using WINMDI 2.9 software.

Tests for PCD markers and visualization of vacuolar morphology

Phosphatidylserine externalization was determined by FITC-coupled annexin V labeling (BD Pharmingen) using flow cytometry. Cells were washed in sorbitol buffer (1.2 M sorbitol, 0.5 mM $MgCl_2$, 35 mM potassium phosphate, pH 6.8) and digested with 120 U mL^{-1} lyticase in sorbitol buffer for 2 h at 28 °C. The protoplasts were washed and resuspended in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, 1.2 M sorbitol, pH 7.4). Cell viability was estimated by internalization of propidium iodide (PI). Annexin V (2 μL) and PI were added to 50 μL of the protoplast suspension and incubated for 30 min at room temperature. Cells fixed in 3.7% formaldehyde at room temperature for 30 min were used for TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling), which was carried out similarly to descriptions by other authors (Madeo *et al.*, 1997; Ludovico *et al.*, 2001). Briefly, protoplasts were generated from fixed cells as described above, washed with PBS and attached to a poly-lysine coated slide, washed again with PBS and permeabilized with a mix of 0.1% sodium citrate and 0.1% Triton X-100 in ice for 2 min. After another washout with PBS, the protoplasts were labeled in TUNEL reaction mix with the *In Situ* Cell Death Detection Kit (Roche, Mannheim, Germany) in a humidity chamber for 1 h at 37 °C following the manufacturer's instructions. Protoplasts treated with RQI RNAse-Free DNAse (Promega, Madison, WI) for 30 min at 37 °C were used as a positive control, and protoplasts incubated in the absence of terminal deoxynucleotidyl transferase as negative controls. Two parallel independent experiments were performed and at least 800 cells were counted for each treatment. For the detection of chromatin changes, fixed cells were rinsed in PBS, resuspended in 70% EtOH for 30 min and stained with 1 μg mL^{-1} 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI). To study the morphology of the vacuoles, cells were washed and resuspended in 10 mM HEPES buffer pH 7.4 containing 5% glucose. Vacuole membranes were studied by incubating the cells for 3 min in the presence of 10 μM MDY-64 marker probe (Molecular Probes, Eugene, OR), and then washing with 10 mM HEPES buffer pH 7.4. For the observation of the lumen, cells were incubated for 25 min with 100 μM CMAC (7-amino-4 chloromethylcoumarin, Molecular Probes). A Nikon Eclipse E 600 microscope was used and images were acquired with a Nikon DS-Fi1 high definition camera. Photographs were digitally processed to enhance their quality.

Measurements of ion concentration

Following the treatments described above, cells were collected onto a membrane cellulose filter (0.8 μm) and washed twice with 4 g L^{-1} solution of $MgCl_2$. Filters were incubated for 24 h in 0.5 N HCl and 2 g L^{-1} $MgCl_2$. The concentrations

of K^+, rubidium (Rb^+), sodium (Na^+) and Ca^{2+} in the samples were determined by atomic absorption spectrometry with an AAnalyst 100 Spectrometer (Perkin Elmer). To determine the concentration of chloride, cells were collected by centrifugation, lyophilized and weighed. H_2O_2 30% 1 mL, HNO_3 1 mL and 50 μL isoamylic alcohol were added to the sample for 15 min. The supernatant was used to determine chloride by the mercuric thiocyanate and ferric ion method. Samples of a supernatant obtained from the growth media were filtered through a 0.22-μm filter and used for determinations of amino acids, which were performed using a BIOCHROM 30 Amino Acid Analyzer (GE-Healthcare Life Sciences). Conductivity was determined with an HI 255 combined meter (Hanna Instruments).

Except where indicated, results were analyzed by ANOVA using the STATISTICA 6.0 program (StatSoft*). *Post hoc* comparisons were performed by Duncan's test.

Results

Reduced growth rate of a yeast population exposed to K^+ deprivation involves PCD

The growth rate of a yeast cell population remained essentially constant in a high K^+ medium, whereas it was rapidly reduced after transfer to a K^+-deficient medium, being almost nil 50 h after the beginning of the K^+-deprivation treatment (Fig. S1). The growth reduction observed 24 h after K^+ deprivation involved a decrease in cell viability as estimated by the reduced frequency of annexin V-negative/ PI-negative cells (Fig. 1a). The fraction of cells displaying phosphatidylserine externalization, revealed by annexin V-positive/PI-negative labeling and considered a hallmark of PCD, significantly increased over that period (Fig. 1b). Taking into account the results obtained in the eight experiments in which the effect of 24 h K^+ deprivation on annexin V and PI labeling was studied, the increase in the frequency of annexin V-positive/PI-negative cells accounted for a 68% loss of cell viability. As the K^+-deprivation period progressed (48 h), the frequency of double-labeled cells (annexin V-positive/PI-positive) increased, indicating the subsequent dominance of events in which membrane integrity became compromised (Fig. 1c). DAPI staining revealed that after 36 h of K^+ deprivation, nuclear condensation, another criteria for PCD, took place (Fig. 1e). After that period, $18.8 \pm 4.1\%$ of the cells displayed DNA fragmentation as revealed by TUNEL assays (Fig. 1f). It has been reported previously that vacuoles change their morphology (Aerts *et al.*, 2008) and play a major role during the cell death process (Schauer *et al.*, 2009). Through the use of the MDY-64 and CMAC markers, which stain the vacuolar membrane and the lumen, respectively, here it was found that exposure to K^+ deprivation results in vacuole fragmentation (Fig. 2). Enhanced accumulation of ROS, which usually occurs during PCD, was also observed in K^+-starved cells as evaluated by H_2DCFDA fluorescence (Fig. 3). In addition, it was observed that long-term K^+ deprivation results in a rise of
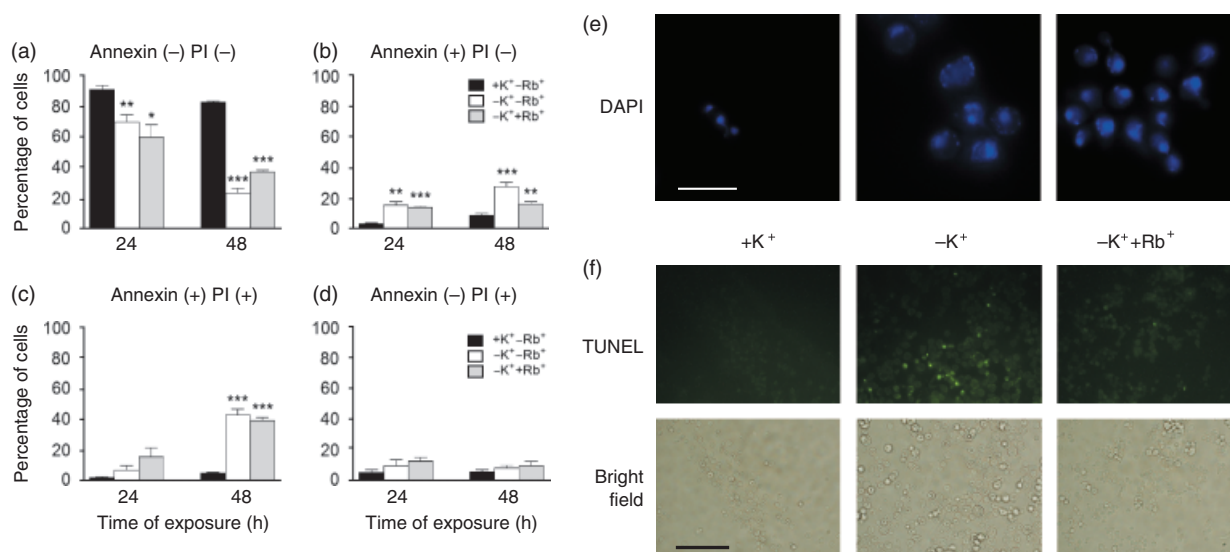

Fig. 1. Yeast cells exposed to K^+ deprivation display phosphatidylserine externalization, changes in chromatin condensation and DNA fragmentation. (a–d) Characterization of cells according to FITC-coupled annexin V and PI staining after culture for 24 or 48 h in a medium containing KCl 15 mM, no KCl added or no KCl added in the presence of RbCl 15 mM. Results shown correspond to pooled data ($n \geq 5$) from two experiments. Error bars correspond to SE. Significant differences relative to the presence of KCl: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (Student's *t*-test). Changes in (e) chromatin condensation as revealed by DAPI staining and (f) DNA fragmentation with TUNEL assay, in cells cultured for 36 h in the abovementioned treatments. Scale bars = 10 μm (e), 40 μm (f).

ion release as estimated by the modification of the electrical conductivity of the media (Fig. S2a), a phenomenon that took place in parallel with a differential change in the $A_{260\,nm}$ (Fig. S2b). In addition, an examination of the changes in the concentration of amino acids in the growth media showed a higher release of lysine in cells deprived of K^+ for 24 h than in cells not deprived of K^+ (Fig. S2c).

Specific effect of K^+ supply on cell death

Although attempts to determine the concentration of K^+ in individual cells with the fluorescent probe PBFI-AM (potassium binding benzofuran isophthalate tetraacetoxy-methyl ester) failed due to the very low number of cells able to incorporate the dye, a rapid and progressive decline of the intracellular K^+ concentration in cells deprived of this element was determined by atomic absorption spectrometry (Fig. S3a). In agreement with previous observations (Camacho *et al.*, 1981), the concentration of Na^+ also increased after K^+
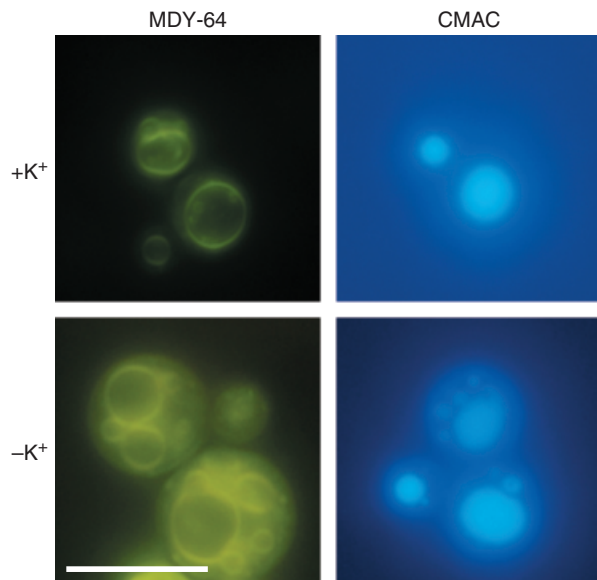

Fig. 2. Vacuole fragmentation takes place during K^+ deprivation. The membrane and lumen of vacuoles were stained with the MDY-64 and CMAC markers, respectively, in yeast cells cultured for 36 h in a media containing KCl 15 mM or without KCl addition. Scale bar = 10 μm.

deprivation (Fig. S3b). To explore the potential specificity of K^+ on the cell death process, the effect of Rb^+, which extensively substitutes for K^+ in fungal cells (Aiking & Tempest, 1977; Camacho *et al.*, 1981), was studied. The growth rate in a medium lacking KCl and containing 15 mM RbCl was reduced until it reached a new steady value, which was midway between values observed in the presence of 15 mM K^+ and those observed in the absence of both K^+ and Rb^+ (Fig. S1). The presence of Rb^+ during a 24-h K^+-deprivation period did not prevent the occurrence of annexin V staining in PI-negative cells (Fig. 1b), or ROS-enhanced accumulation (Fig. 3). Rb^+-treated cells also displayed chromatin condensation (Fig. 1e), the frequency of TUNEL positive events being much lower ($0.58 \pm 0.02\%$) than in the absence of K^+ but still higher than found for K^+-rich cells, which was nil (Fig. 1f). Noticeably, these events took place even though the concentration of Rb^+ in Rb^+-treated cells (Fig. S3c) was similar to that determined for K^+ in K^+-rich cells, and the concentration of Na^+ remained low (Fig. S3b).

Maintenance of cell membrane potential and steady concentrations of total Ca^{2+} require K^+

To further characterize the specific role of K^+ in cell death induced by K^+ deficiency, the effect of RbCl on other cellular attributes in the absence of K^+ was examined. The fluorescence of $DiOC_6(3)$, a validated marker for cell membrane potential in yeast cells (Madrid *et al.*, 1998), was studied first. The inclusion of Rb^+ in the growing media delayed the hyperpolarization that took place in medium where K^+ was not added (Fig. 3). It was also observed that the total concentration of Ca^{2+} in cells remained fairly constant in a medium with K^+, but increased up to 25 times after a period of long-term K^+ deprivation (Fig. S3d). The presence of Rb^+ in the K^+-deprived medium was not sufficient to prevent the enhanced accumulation of Ca^{2+}.

The absence of the *YCA1* gene does not interfere with the growth response induced by K^+ deprivation

In some physiological scenarios or following certain stimuli, the execution of a cell death program depends in part or
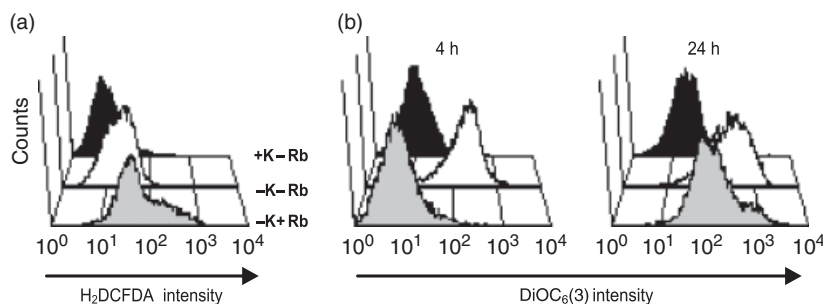

Fig. 3. Rb^+ does not avoid long-term membrane hyperpolarization and enhanced ROS accumulation induced by K^+ deficiency. (a) ROS accumulation was estimated with the fluorescent dye H_2DCFDA after a 24-h culture period in a medium containing 15 mM KCl, no KCl added or no KCl added but with RbCl 15 mM. (b) Membrane potential was estimated by $DiOC_6(3)$ fluorescence after 4 and 24 h of culture in the abovementioned media. Data are representative of one of three independent replicates.

completely on the presence of the YCA1 metacaspase (Pereira *et al.*, 2008; Madeo *et al.*, 2009). Accordingly, the involvement of this protein in the cell death process induced by K^+ deprivation was explored through the use of an *yca1* mutant. Noticeably, the absence of the *YCA1* gene did not modify the growth pattern or the frequency of annexin V-positive/PI-negative staining in K^+-deprived cells (Fig. 4). Additionally, it was observed that nuclear condensation also occurred in *yca1* cells deprived of K^+ (Fig. 4).

The absence of genes coding for TRK transporters modifies the pattern of annexin V/ PI labeling

Results shown above indicate that the cell death process studied here is coupled to intracellular K^+ depletion. This depletion could result from the activity of transport systems that mediate alkali cation entrance and/or exit. It was found that after a period of short-term K^+ deprivation (Fig. S4a), the accumulation of K^+ was similar in wild-type and in a yeast strain (LMB 02) carrying a deletion of genes coding for transporters presumably involved in K^+ efflux, i.e. the alkali ENA1-4 ATPases, the NHA1 antiporter, the TOK1 channel as well as the putative KHA1 transporter. However, for the LMM 04 strain, which in addition to those mutations carries a mutation in the genes coding for the K^+ transporters TRK1 and TRK2 (Maresova & Sychrova, 2005), a reduced K^+ content was observed (Fig. S4b). This finding enabled us to use a *trk1Δtrk2Δ* (WΔ3) yeast strain to explore the consequences of a further intracellular K^+ depletion on the course of the cell death process induced by K^+ deficiency. In the presence of 15 mM KCl, *trk1Δtrk2Δ* and *TRK1TRK2* cell populations showed a similar frequency of viable cells (Fig. 5a). For the *TRK1TRK2* strain the frequency of cells stained only with annexin V increased after a 24-h K^+-deprivation period, whereas that of cells displaying double

staining with annexin V and PI remained low. The frequency of viable cells decreased sharply for *trk1Δtrk2Δ* cells exposed to K^+ deprivation when compared with *TRK1TRK2* cells. This decrease was paralleled by an increase in the frequency of cells displaying PI and annexin V double labeling and of those only stained with PI, whereas the frequency of cells displaying only annexin V labeling was similar to that found in the presence of K^+. These observations led to the notion that loss of TRK transporters influences the cell death process that takes place during a 24-h K^+-deprivation period. DAPI staining, in turn, revealed that nuclear condensation took place in *trk1Δtrk2Δ* cells following K^+ deprivation (Fig. 5b).

TRK transporters could mediate the inward and outward transport of Na^+ and Cl^-, respectively, in addition to the inward flux of K^+ (Haro & Rodríguez-Navarro, 2002; Kuroda *et al.*, 2004; Corratgé *et al.*, 2007), and eventually influence the course of the cell death process in multiple ways. As the primary events leading to long-term differences between *trk1Δtrk2Δ* and *TRK1TRK2* cells must take place early, we examined the ionic balance 4 h after the beginning of the K^+-deprivation period. In agreement with the pattern mentioned above for LMM 04 cells, it was found that, after that K^+-deprivation period, less K^+ is accumulated in *trk1Δtrk2Δ* than in *TRK1TRK2* cells (Fig. 6a). In turn, the accumulation of Na^+ was significantly higher in *TRK1TRK2* than in *trk1Δtrk2Δ* cells (Fig. 6b). In addition, our results confirmed that a decline of intracellular Cl^- concentration took place in yeast cells deprived of KCl (Jennings & Cui, 2008) and showed that this was similar for *TRK1TRK2* and *trk1Δtrk2Δ* K^+-deprived cells (Fig. 6c). These findings indicate that differences in alkali cation accumulation, but not in Cl^-, could be involved in the different course of the cell death process observed in *TRK1TRK2* and *trk1Δtrk2Δ* cells. A strong decline of Cl^- was observed in studies performed with *TRK1TRK2* cells grown in the presence of
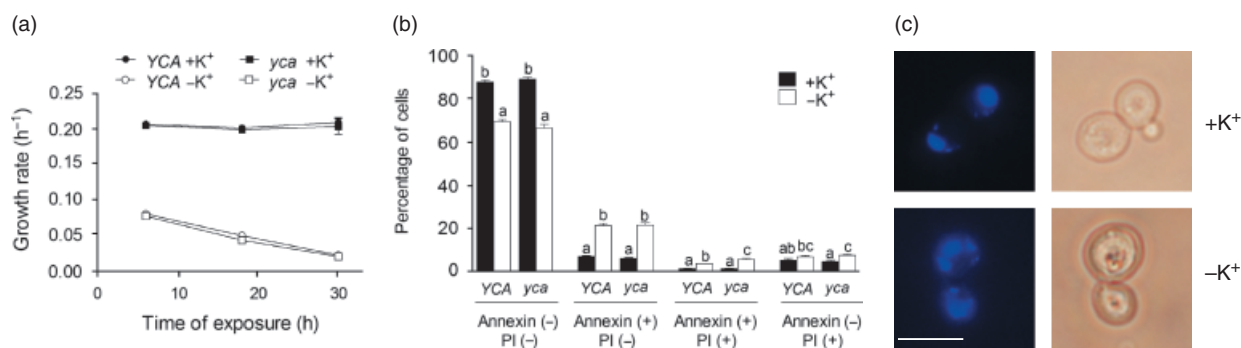

Fig. 4. Effect of the absence of the *YCA1* gene on the growth rate (a) and the pattern of annexin V and PI staining (b). Results correspond to pooled data (*n* = 6) from two experiments. Error bars correspond to SE. For each staining group, results labeled with a different letter are significantly different at *P* < 0.05. (c) Nuclear condensation as revealed by DAPI staining in *yca1* cells cultured for 36 h in a medium containing KCl 15 mM or without KCl addition. Scale bar = 10 μm. Bright fields are shown on the right side of the figure.
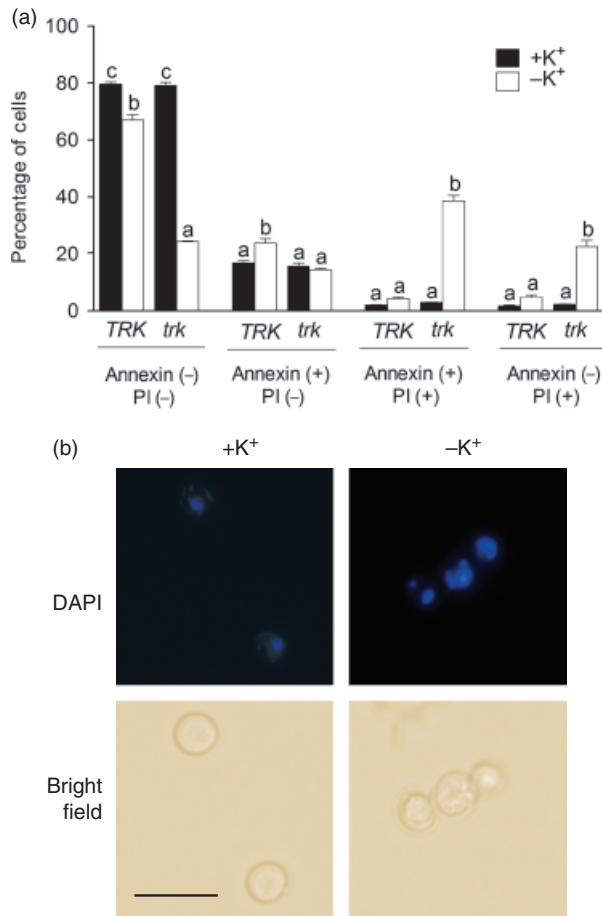
(a)

(b)

Fig. 5. (a) Characterization of *trk1Δtrk2Δ* and *TRK1TRK2* cells according to FITC-coupled annexin V and PI staining after 24 h culture in a medium containing KCl 15 mM or without KCl added. Data are the means of three independent replicates; the bars correspond to SE. For each staining group, results labeled with a different letter are significantly different at $P < 0.05$. (b) Nuclear condensation as revealed by DAPI staining in *trk1Δtrk2Δ* cells grown for 24 h in a medium containing KCl 15 mM or without KCl addition. Scale bar = 10 μm.

K_2SO_4 7.5 mM and then transferred to the same medium without K^+ added (Fig. S5). This supports the hypothesis that the reduction of the intracellular concentration of Cl^- results not only from the change in the Cl^- gradient but also from the change in the external supply of K^+.

Deletion of *TRK1TRK2* genes causes increased Ca^{2+} accumulation in K^+-deprived cells

Our results prompted us to examine the possible mechanisms linking the change in the intracellular ionic environment with the change observed in the cell death process derived from the disruption of genes coding for the TRK transporters. In agreement with previous observations (Madrid *et al.*, 1998;

Mulet *et al.*, 1999), a 4-h K^+-deprivation period led to a more pronounced membrane hyperpolarization in cells lacking the TRK transporters (Fig. S6a). This enhanced hyperpolarization coincided with an enhanced accumulation of Ca^{2+} in K^+-starved *trk1Δtrk2Δ* cells (Fig. 6d). To assess the possibility that differences in total Ca^{2+} are accompanied by a difference in the intracellular free Ca^{2+} concentration, the fluorescence of the cell permeant fluorescent dye Fluo-3 AM was studied. Fluo-3 AM fluorescence was similar for both isogenic lines grown at a high external K^+ concentration. After a 4-h K^+ deprivation period, the frequency of *trk1Δtrk2Δ* cells displaying enhanced Fluo-3 AM fluorescence increased (Fig. S6b). The presence of 2.5 mM ethylene glycol tetraacetic acid (EGTA) reduced this enhanced accumulation of Ca^{2+}.

Long-term exposure to K^+ deprivation (24 h) led to enhanced Fluo-3 AM fluorescence in both *TRK1TRK2* and *trk1Δtrk2Δ* cells, the increase being more pronounced for the mutant strain (Fig. 7). The difference in the fluorescence of the dye between K^+-starved and K^+-non-starved cells was diminished by the presence of 2.5 mM EGTA in the growth media, indicating that the long-term enhancement of Ca^{2+} accumulation should be, at least partially, ascribed to the inward flux of Ca^{2+} from the external solution.

Exposure to EGTA attenuates cell death in *trk1Δtrk2Δ* cells

Our results suggested a possible link between growth restriction and the rise of Ca^{2+} concentration under K^+-deprivation conditions. Therefore, the effect of EGTA on the growth of *TRK1TRK2* and *trk1Δtrk2Δ* cells was studied. The presence of 2.5 mM EGTA partially relieved the negative growth rate operating under K^+-deprivation conditions for *trk1Δtrk2Δ* cells, but did not modify the growth rate displayed by K^+-deprived *TRK1TRK2* cells (Fig. 8a). Therefore, the possible effect of EGTA on the frequency of annexin V and PI labeling in *trk1Δtrk2Δ* cells was examined (Fig. 8b). The presence of EGTA in a medium to which K^+ had not been added led to a significant increase of viable cells as well as to a reduction of PI-stained cells (Fig. 8b).

Discussion

The presence of apoptotic-like PCD in fungi has become well established (Sharon *et al.*, 2009), with budding yeasts considered to be a model for the regulation of PCD at the cellular level (Madeo *et al.*, 2002). The occurrence of intracellular K^+ depletion during apoptotic-like fungal cell death processes induced by different stimuli has been shown previously (Martinac *et al.*, 1990; Mulet *et al.*, 1999; Huh *et al.*, 2002; Kaiserer *et al.*, 2003; Leiter *et al.*, 2005; Pozniakovsky *et al.*, 2005; Reiter *et al.*, 2005; Mangano *et al.*, 2008; Peña *et al.*, 2009) and a possible role for the lack of this element in the progression of apoptotic-like PCD has

Fig. 6. Effect of short-term K^+ deprivation on the concentration of K^+, Na^+, Cl^- and Ca^{2+} in $trk1\Delta trk2\Delta$ and $TRK1TRK2$ cells. Cells were grown for 4 h in the presence of KCl 15 mM or in a medium without KCl added. Data in (a), (b), (c) and (d) correspond to K^+, Na^+, Cl^- and Ca^{2+}, respectively. The data are from measurements done in three (for Cl^-), five (for K^+ and Na^+) and six (for Ca^{2+}) experiments performed in triplicate; the bars correspond to SE. Significant differences between $trk1\Delta trk2\Delta$ and $TRK1TRK2$ cells for each growth condition: $^*P < 0.05$ and $^{**}P < 0.01$ (Student's t-test). Given that absolute values were not entirely comparable among experiments, the trend of ion accumulation was expressed on a relative base. The absolute average values, in $nmol\,mg^{-1}$ DW, are given between brackets within each bar.
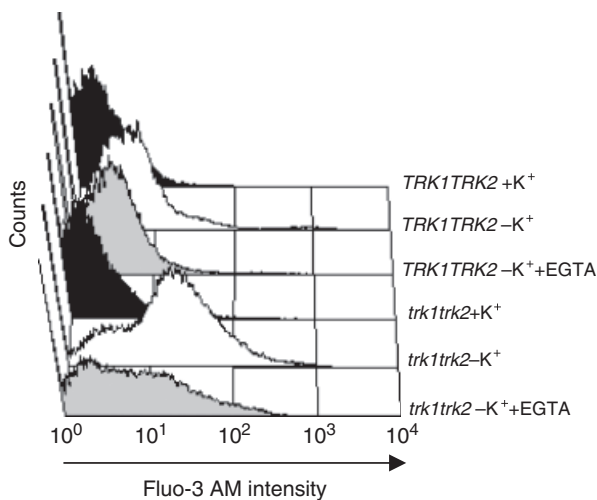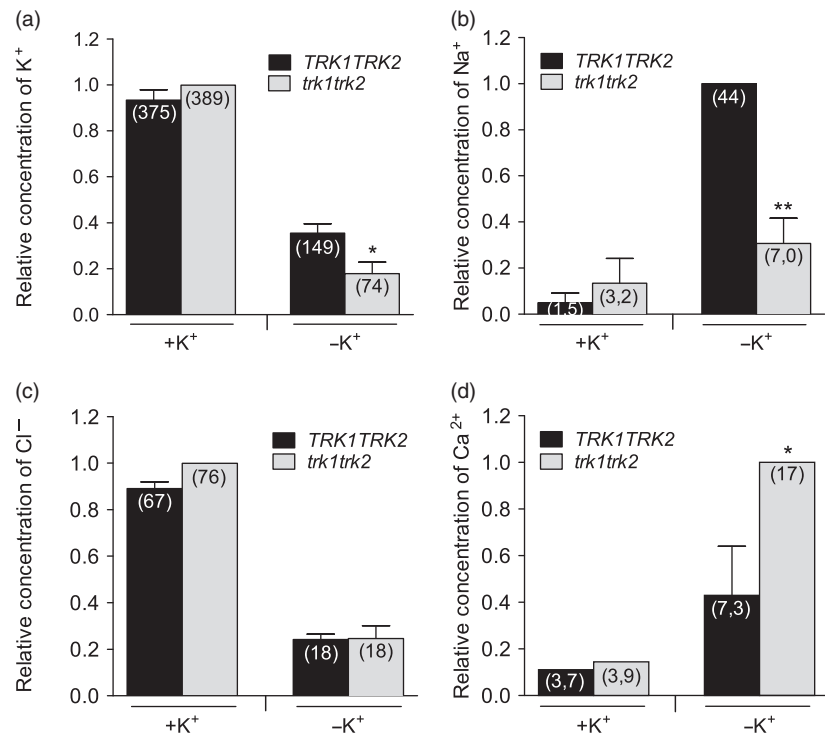

Fig. 7. Fluorescence of the Ca^{2+} permeant dye Fluo-3 AM in $trk1\Delta trk2\Delta$ and $TRK1TRK2$ cells after 24 h culture in a medium containing KCl 15 mM or without KCl added. The effect of 2.5 mM EGTA on the fluorescence of Fluo-3 AM in K^+-starved cells was also examined. Data are representative of one of three independent replicates, except for the EGTA treatment, which was performed in duplicate.

been advanced (Andrés et al., 2008). Results presented here show that the initial loss of viability observed in yeast cells exposed to K^+ deprivation is mainly associated to a cell death process that displays features typically found in apoptosis-like PCD, such as changes in chromatin condensation, DNA fragmentation and phosphatidylserine externalization. These data support the hypothesis that depletion of K^+ is sufficient to induce PCD in the cell-walled eukaryotic organism S. cerevisiae.

A convergence of different stimuli into a common PCD pathway in yeast is suggested by the observation that both intracellular Ca^{2+} elevation and a burst of ROS take place during the yeast PCD induced by amiodarone, Al^{3+} and acetic acid (Pozniakovsky et al., 2005; Zheng et al., 2007; Pereira et al., 2008) and, according to our results, by K^+ deprivation. This apparent convergence is likely to be preceded by other phenomena. In animal cells, early events for the progression of PCD are loss of K^+ and Cl^-, and gain of Na^+ (Bortner & Cidlowski, 2007). Our results indicate a decrease of Cl^- and K^+ as well as an increase of Na^+ concentrations in S. cerevisiae cells deprived of K^+. The resulting ionic imbalance drives a hyperpolarization of the plasma membrane (Madrid et al., 1998; this work), which has been linked to a decrease of internal pH, allowing electrical neutrality to be maintained (Rodríguez-Navarro, 2000). That hyperpolarization must modify the driving force for ion movement and could also influence the opening of Ca^{2+} channels (Eilam & Chernichovsky, 1987), thus leading to massive Ca^{2+} influx. The observation that the enhanced accumulation of Ca^{2+} during the course of K^+ deprivation is preceded and correlates with a more negative
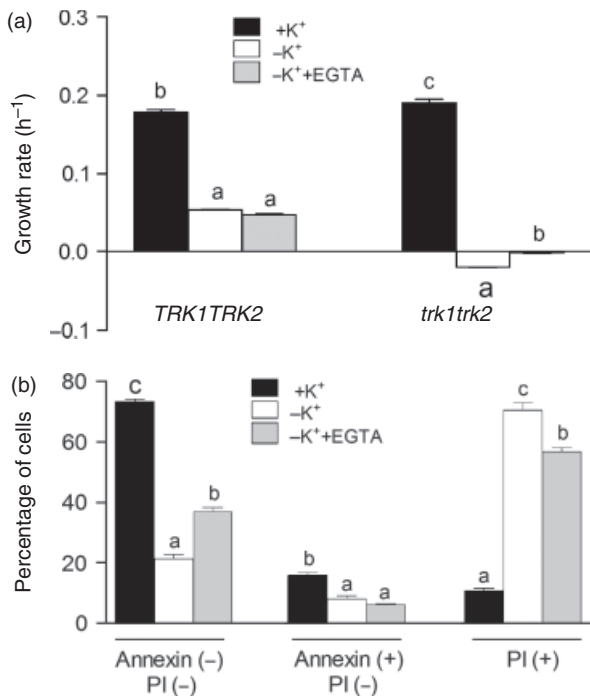
Fig. 8. The presence of EGTA during K^+ deprivation affects the growth rate and the frequency of FITC-coupled annexin V and PI staining in $trk1\Delta trk2\Delta$ cells. (a) $trk1\Delta trk2\Delta$ and $TRK1TRK2$ cells were grown for 24 h in a medium containing KCl 15 mM, without KCl added or without KCl added but in the presence of EGTA 2.5 mM. The growth rate was estimated over the last 12 h of treatment, and is the mean of three independent replicates; bars correspond to SE. (b) The frequency of FITC-coupled annexin V and PI staining in $trk1\Delta trk2\Delta$ cells cultured for 24 h in the above-described media is the mean of three independent replicates; the bars correspond to SE. For each staining group, results labeled with a different letter are significantly different at $P < 0.05$.

membrane potential supports the existence of a causal link between both phenomena. Plasma membrane hyperpolarization associated to loss of K^+ has proved to lead to enhanced Ca^{2+} influx after exposure to amiodarone (Maresova *et al.*, 2009; Peña *et al.*, 2009). In that case it was observed that high concentrations of salts, among them KCl, block drug toxicity. In turn, the loss of cell viability that takes place in yeast cells exposed to strong acid stress has been linked to membrane depolarization (dos Santos Sant'Ana *et al.*, 2009). Those observations, along with the results reported here, suggest that maintenance of membrane potential within a relatively narrow range is critical to avoid the progression of cell death in fungi.

As the decrease in K^+ and Cl^- concentrations can also lead to water imbalance, and osmotic stress induces apoptotic-like cell death (Silva *et al.*, 2006), the involvement of elements critical in the adaptation to osmotic shock during the response of yeast cells to K^+ deprivation cannot be ruled out. In the same way, the decrease in the intracellular pH

that takes place following K^+ deprivation could be *per se* a key component of the cell death process studied here. It was observed that a sharp reduction in the intracellular concentration of K^+ takes place after exposure to the cell permeant acetic acid (data not shown), a finding that parallels the observations made by dos Santos Sant'Ana *et al.* (2009) with a strong acid, indicating a reciprocal interaction between the reduction of intracellular K^+ concentration and pH. In this context it is worth mentioning that cell survival and the execution of a cell death program after exposure to acetic acid (Guaragnella *et al.*, 2006) and osmotic shock conditions (Silva *et al.*, 2006) probably involve both *YCA1*-dependent and *YCA1*-independent pathways. The results reported in this study indicate that the cell death process induced by K^+ deprivation seems to occur exclusively through an *YCA1*-independent pathway, as reported for other cell death scenarios (Madeo *et al.*, 2009). Whether this *YCA1*-independent pathway is similar to that induced by other stress conditions needs to be examined further.

As a primary role of K^+ is to contribute to charge balance (Rodríguez-Navarro, 2000), the effect of K^+ depletion could be mainly a quantitative one. In that case, Rb^+, which is accumulated to the same extent as K^+, should completely replace K^+ for the control of membrane potential. However, our data indicated that inclusion of Rb^+ was capable of fully substituting for K^+ in the control of membrane potential only at the beginning of the K^+-deprivation period. In turn, the increased accumulation of ROS observed in the presence of Rb^+ is also compatible with a specific requirement of K^+ in the control of redox status that probably is not fulfilled because of the almost complete exchange of K^+ by Rb^+. The precise role of K^+ in that control deserves to be explored further. Although the presence of Rb^+ sharply reduced the frequency of cells affected by DNA fragmentation, it was not able to prevent either the early externalization of phosphatidylserine or the changes in chromatin condensation that take place during K^+-induced cell death. These results argue for the notion that the influence of K^+ on cell death is not only quantitative but qualitative as well.

An important observation made during early research on TRK transporters was that cells lacking TRK activity were unable to grow in low K^+ media (Gaber *et al.*, 1988; Bertl *et al.*, 2003). The absence of growth in $trk1\Delta trk2\Delta$ but not in $TRK1TRK2$ cells observed in the non-K^+-enriched medium, must be linked to the fact that cells without functional TRK transporters undergo a death process that involves chromatin condensation, and rapidly compromise plasma membrane integrity. Our data indicated that those changes were anteceded by a pronounced cationic imbalance and the subsequent enhanced plasma membrane hyperpolarization, as observed previously (Madrid *et al.*, 1998; Mulet *et al.*, 1999). This enhanced hyperpolarization was accompanied by a rapid increase in intracellular Ca^{2+}. Addition of the

Ca^{2+} chelating agent EGTA led to a significant decrease in the frequency of PI-stained cells and exerted a positive effect on the growth rate of K$^+$-deprived $trk1\Delta trk2\Delta$ yeast cells. This indicates that the course of the cell death process imposed by the lack of TRK transporters during K$^+$ deprivation is driven in part by a fast elevation of intracellular Ca^{2+}. This detrimental effect of intracellular Ca^{2+} helps to explain the observation that high external CaCl$_2$ concentrations restrict the growth of $trk1\Delta trk2\Delta$ cells (Mulet *et al.*, 1999). The results obtained here with EGTA are compatible with either a direct relief of the negative effect of Ca^{2+} on the contribution of low-affinity inward K$^+$ transporters (Navarre & Goffeau, 2000; Ruíz *et al.*, 2004), which could be marginal at the low external K$^+$ concentrations here assayed, or more likely with an effect of Ca^{2+} on signaling. The possibility that calcineurin (CNB1) could play a role in Ca^{2+} signaling induced by K$^+$ deprivation is suggested by the observation that a $cnb1\Delta$-deficient mutant suffers more pronounced PCD symptoms after exposure to salt stress, and these symptoms are also related to a profound ionic disequilibrium (Huh *et al.*, 2002). However, if CNB1 is actually involved, it could not implicate a modulation of the TRK1 transporter whose affinity is regulated via CNB1 by salt stress but not by K$^+$ deprivation (Mendoza *et al.*, 1994).

TRK transporters have been found to act as effectors for the cell death that takes place in the presence of histatin V and other antifungal peptides (Baev *et al.*, 2004; Vylkova *et al.*, 2006). They also participate in cell cycle progression (Yenush *et al.*, 2002) and are required for the resistance to a wide range of chemicals (Mulet *et al.*, 1999). In turn, Ca^{2+} has been proposed as an important player in the control of multiple yeast cellular processes, among them cell death (Pereira *et al.*, 2008). Suggesting a mechanistic link among those observations, our results indicate that, at least under conditions of K$^+$ deficiency, the activity of the TRK transporters is necessary for the control of intracellular Ca^{2+} homeostasis and, subsequently, for the control of cell death in a partially Ca^{2+}-dependent mode.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Yeast cells exposed to K^+ deprivation display a reduced growth rate which is partially restored by the presence of the K^+-analogue element, Rb^+.

Figure S2. K^+ deprivation generates differential changes in the electrical conductivity (a), $A_{260\,nm}$ (b) and lysine concentration (c) in the growth media.

Figure S3. Time course of K^+ (a), Na^+ (b), Rb^+ (c) and Ca^{2+} (d) concentrations in yeast cells cultured in a media containing KCl, no KCl added or no KCl added in the presence of RbCl.

Figure S4. Effect of the deletion of multiple transport systems on the short-term accumulation of K^+ in K^+-deprived cells.

Figure S5. Effect of K^+ deprivation on intracellular Cl^- retention in a media where K^+ was added as K_2SO_4.

Figure S6. Short-term effect of K^+ deprivation on cell membrane potential and free Ca^{2+} accumulation in *trk1Δtrk2Δ* and *TRK1TRK2* cells.

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