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Low-affinity potassium uptake by *Saccharomyces cerevisiae* is mediated by NSC1, a calcium-blocked non-specific cation channel

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Abstract

Previous descriptions by whole-cell patch clamping of the calcium-inhibited non-selective cation channel (NSC1) in the plasma membrane of *Saccharomyces cerevisiae* (H. Bihler, C.L. Slayman, A. Bertl, FEBS Lett. 432 (1998); S.K. Roberts, M. Fischer, G.K. Dixon, D.Sanders, J. Bacteriol. 181 (1999)) suggested that this inwardly rectifying pathway could relieve the growth inhibition normally imposed on yeast by disruption of its potassium transporters, Trk1p and Trk2p. Now, demonstration of multiple parallel effects produced by various agonists and antagonists on both NSC1 currents and growth (of *trk1Δtrk2Δ* strains), has identified this non-selective cation pathway as the primary low-affinity uptake route for potassium ions in yeast. Factors which suppress NSC1-mediated inward currents and inhibit growth of *trk1Δtrk2Δ* cells include (i) elevating extracellular calcium over the range of 10 μM–10 mM, (ii) lowering extracellular pH over the range 7.5–4, (iii) blockade of NSC1 by hygromycin B, and (iv) to a lesser extent by TEA^+. Growth of *trk1Δtrk2Δ* cells is also inhibited by lithium and ammonium; however, these ions do not inhibit NSC1, but instead enter yeast cells via NSC1. Growth inhibition by lithium ions is probably a toxic effect, whereas growth inhibition by ammonium ions probably results from competitive inhibition, i.e. displacement of intracellular potassium by entering ammonium.

Keywords: Yeast; Non-specific channel; Cation uptake; Calcium inhibition; Salt tolerance; Membrane voltage

1. Introduction

Mutant screening [1,2] and subsequent complete sequencing of the *Saccharomyces* genome have made possible the identification of multiple membrane-associated proteins, which mediate movement of potassium ions into or out of yeast cells. All of these have emerged as distinct entities having strong sequence homologies with K^+ transporters in bacterial, plant and animal cells. Best understood are two carrier-like proteins responsible for K^+ uptake and accumulation: Trk1p, mediating uptake of potassium with high affinity ($K_m \approx 30$ μM), and Trk2p, mediating uptake with moderate affinity ($K_m = 10$ mM). In addition, a bona fide K^+ channel, Duk1p or Tok1p, has been identified as an efflux pathway, opening at positive [3–5] and low negative membrane voltages [6,7]. Energy-dependent K^+ efflux has been described [8] but not yet identified with a specific gene or protein, although a putative Na^+-pumping enzyme, Ena1p, may be responsible [9]. Two ion ex-

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changer homologues, Kha1p and Nha1p, also mediate K^+ movements under some conditions [10–12].

Growth of yeast on medium containing only micromolar K^+ (or Rb^+) requires Trk1p, while brisk growth of TRK1 knockout strains (*trk1Δ*) on moderate K^+ concentrations (1–10 mM) requires Trk2p, and brisk growth of double knockout strains, TrkΔΔ (*trk1Δtrk2Δ*), requires high extracellular K^+ ($\sim$50 mM). Potassium entry into double knockout cells follows simple Michaelis–Menten kinetics ($V_{max} \approx$ 4 nmol min^{-1} mg^{-1}, $K_m \approx 50$ mM in haploid cells [13]). However, despite the plethora of additional K^+ transporters, listed above, the low-affinity pathway actually used for potassium entry into TrkΔΔ cells has not previously been demonstrated.

Recently, two laboratories [14,15] described an additional mechanism for K^+ permeability in yeast: a non-specific 'channel' which is blocked by divalent metal ions, but which can conduct huge currents (1–3 nA per cell) through the yeast plasmalemma, when membrane voltage is clamped near −200 mV and when Ca^{2+}, Mg^{2+}, etc. are absent from the external solution. This channel, designated NSC1, has not yet been cloned or sequenced.

In the present study, we used growth of a TRK double knockout strain for qualitative assessment of potassium uptake via NSC1, under a wide range of conditions. The results parallel patch-clamp measurements of NSC1-mediated inward currents, made in wild-type cells under closely related conditions, and clearly identify NSC1 as the TRK-independent low-affinity K^+ pathway.

2. Materials and methods

2.1. Strains

Whole-cell patch recording from *Saccharomyces cerevisiae* was carried out by standard procedures [17], using a tetraploid wild-type (WT) (Y588: **a/a/α/α** *ura3 ade2*) provided by M. Snyder [18]. Potassium-dependent growth tests were carried out on a haploid double knockout strain TrkΔΔ = PLY240 (**a** *his3-Δ200 leu2-3,112 trp1-Δ901 ura3-52 suc2-Δ9 trk1-Δ51 trk2-Δ50::kanMX*). All growth tests were controlled by comparison with an isogenic 'wild-type' strain WT = PLY232 (**a** *his3-Δ200 leu2-3,112 trp1-Δ901 ura3-52 suc2-Δ9*). Both strains were provided by P. Martinez and P. Ljungdahl, Ludwig Institute, Stockholm (a manuscript is in preparation, detailing construction and physiological characterization of these strains; Bertl et al., 2001).

2.2. Media and special conditions

YPAD medium is the standard rich medium for yeast [19], containing 1% (w/v) yeast extract, 2% bactopeptone, 2% dextrose, supplemented with 50 mg/l adenine sulfate to compensate the *ade2-101* mutation in the WT strain (Y588). SDAP medium [20] is a synthetic medium containing 2% dextrose, 10 mM arginine as the nitrogen source titrated to the desired pH (usually 6.5) with phosphoric acid, 0.2 mM $CaCl_2$, and 0.2 mM $MgSO_4$, supplemented with nutritional requirements for the particular yeast strains in use: here, potassium, standard vitamins and trace elements, plus 50 mg/l adenine, uracil, histidine, tryptophan, and leucine. High-calcium SDAP was made by simple addition of $CaCl_2$ (10 mM). Additional supplements to SDAP on the test plates are listed in the figure legends.

2.3. Protoplast preparation

Cell walls of late log phase cells grown in YPAD medium at 30°C were partly digested by a 45 min treatment with 0.01–0.03% (w/v) zymolyase-20T, in 50 mM K^+-phosphate buffer (pH 7.2) containing 0.2% β-mercaptoethanol as antioxidant and 1.2 M sorbitol as osmoprotectant. To release the majority of protoplasts, enzyme-treated cells were harvested by centrifugation, then washed and resuspended in slightly hypotonic buffer (220 mM KCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM MES–Tris at pH 7.2, and 0.5% glucose). After 30–60 min incubation in this buffer, 3–5 μl of the suspension were transferred to the recording chamber filled with the same buffer, and cells were allowed to settle and to attach slightly to the chamber bottom.

2.4. Electrophysiology

Patch pipets were pulled from borosilicate glass (Kimax-51, Kimble/Kontes No. 38500), fire-polished, and filled with buffer containing 175 mM KCl, 4 mM

$MgCl_2$, 4 mM K-ATP and 1 mM EGTA, titrated to pH 7.0 (with KOH) and pCa 7.0 (with $CaCl_2$); total $[K^+]_{pipet}$ = 195 mM. The principal recording solutions were made up with 1–5 mM MES, titrated to pH 7.5 with Tris base, and contained the following salts: high-Ca^{2+} buffer: 150 mM KCl, 10 mM $CaCl_2$, and 5 mM $MgCl_2$; low-Ca^{2+} buffer ('zero-Ca^{2+}' solution): 150 mM KCl, 1 mM EGTA. After tight seals (5–15 GΩ) had been formed [17], the enclosed membrane patch was broken, by simultaneous application of light suction and a brief high-voltage pulse (~900 mV, 100 μs), to attain the whole-cell recording configuration. Inward and outward currents through the yeast cell membrane were assessed via a voltage-clamp protocol consisting of square pulses of 2.5 or 3 s duration, starting from a holding voltage of −40 mV: to +100 mV, then in 20 mV decrements to −200 mV, as diagramed in Fig. 2A (right lower panel). All whole-cell current recordings, except for the data in Fig. 5, have been corrected for linear leak conductance. Data were recorded at 10 kHz, filtered in most cases at 330 Hz, and sampled at 1 kHz.

2.5. Growth tests

Growth dependent upon low-affinity K^+ uptake was assessed at 30°C in the double knockout strain, and in the isogenic wild-type strain by means of a drop test, in which cells were harvested by centrifugation from K^+-replete growth medium (YPD+50 mM KCl), washed and resuspended in sterile distilled water, adjusted to an OD of 1.0, serially diluted 10-, 100-, and 1000-fold with sterile distilled water, and dropped (7 μl) onto plates of SDAP medium in 2% purified agar. Plates were incubated for 48 h at 30°C, and imaged on a flat-bed scanner at 600 dpi.

3. Results

3.1. Basic measurements

Potassium-dependent growth of yeast is demonstrated in Fig. 1, by the drop test comparisons of WT with TrkΔΔ on SDAP agar supplemented with 1 mM, 15 mM, and 100 mM KCl. In the presence of 200 μM extracellular Ca^{2+}, wild-type yeast grew almost equally well at all three values of $[K^+]_o$, whereas the TRK double knockout strain grew well at 100 mM K^+, weakly at 15 mM K^+, and not at all at 1 mM K^+.

Varying extracellular free Ca^{2+} affected this picture very dramatically, as is shown in Fig. 2B. In that experiment, plates contained 15 mM K^+, and the control (center panel) contained 200 μM Ca^{2+}, as in Fig. 1. Reducing $[Ca^{2+}]_o$ to 0 (no extra calcium added) had little effect on growth (left panel). Elevating $[Ca^{2+}]_o$ by 50-fold, to 10 mM (right panel), nearly abolished growth of TrkΔΔ, without affecting growth of WT. This result corresponds very closely to what had already been observed by recording of whole-cell inward currents in several strains of yeast [14], and which is demonstrated explicitly in Fig. 2A, for the tetraploid wild-type strain, WT. The 'zero' Ca^{2+} control yielded NSC1 inward currents (left panel, downward traces) as large as 1 nA at −200 mV, and 10 mM Ca^{2+} (right upper panel) completely suppressed these inward currents.

Calcium-dependent blockade of NSC1 current over the concentration range of 10^{-8}–10^{-2} M is shown for the most negative voltages (Fig. 2C), demonstrating that in 0.2 mM external Ca^{2+} – our control conditions – about 35% of the maximum inward current persists, which is clearly sufficient to support growth of TrkΔΔ in K^+-limiting medium.

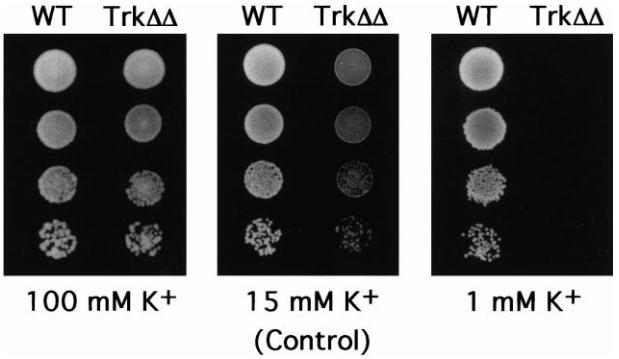

Fig. 1. Drop test demonstration of the potassium requirement for a TRK double knockout strain of yeast (TrkΔΔ, PLY240), compared with an isogenic wild-type (WT, PLY232). Plates prepared with SDAP medium in 2% purified agar, containing 0.2 mM $CaCl_2$, 0.2 mM $MgSO_4$ at pH 6.2, and the indicated concentrations of KCl. (The center panel in this figure is typical of our control growth experiments, and is the reference display used in all the other figures.)

3.2. Inhibition by lowered pH

Many previous investigations on potassium transport in wild-type *Saccharomyces* have been conducted in Ca^{2+}-poor medium, with neither calcium ions nor chelators added, so that free extracellular Ca^{2+}, though undetermined, probably lay in the neighborhood of 10 μM. In that case, the NSC1 pathway would have been partially active (Fig. 2C), as well as the TRK transporters. Armstrong and Rothstein [21], working with such conditions, observed that lowering extracellular pH had two conspicuous effects on the influx of isotopes of monovalent cations: (i) it diminished the influx of all the alkali metal cations, and (ii) it biased the residual influx strongly in favor of K^+ (and Rb^+), over Li^+, Na^+, and Cs^+. The results were analyzed in terms of two sites of action of protons on a presumed single transporter, but they could also be handily accommodated by two transporters functioning in parallel: one, like Trk1p or Trk2p, highly selective for K^+ but active at low pH, and the other, like NSC1, nonselective among cations but strongly inhibited by low pH. The actual response of NSC1 currents to lowered pH_o was noted previously [14] and is demonstrated in Fig. 3A, for direct comparison with growth results. At pH 7.5, the typical time-dependent NSC1-inward currents were elicited by negative voltage pulses. These NSC1 currents were essentially abolished upon acidification of the external medium to pH 4.0. The residual currents at pH 4.0 showed no time dependence, and can be attributed entirely to the TRK potassium transport systems.

The effect of low pH on NSC1 currents corresponds well with the effect of external pH on growth (Fig. 3B). On SDAP plates containing 15 mM KCl, with 200 μM $CaCl_2$ growth of TrkΔΔ cells was essentially identical with that of WT at pH 7.5 and pH 6.5, but it was abolished at pH 4.0, whereas growth

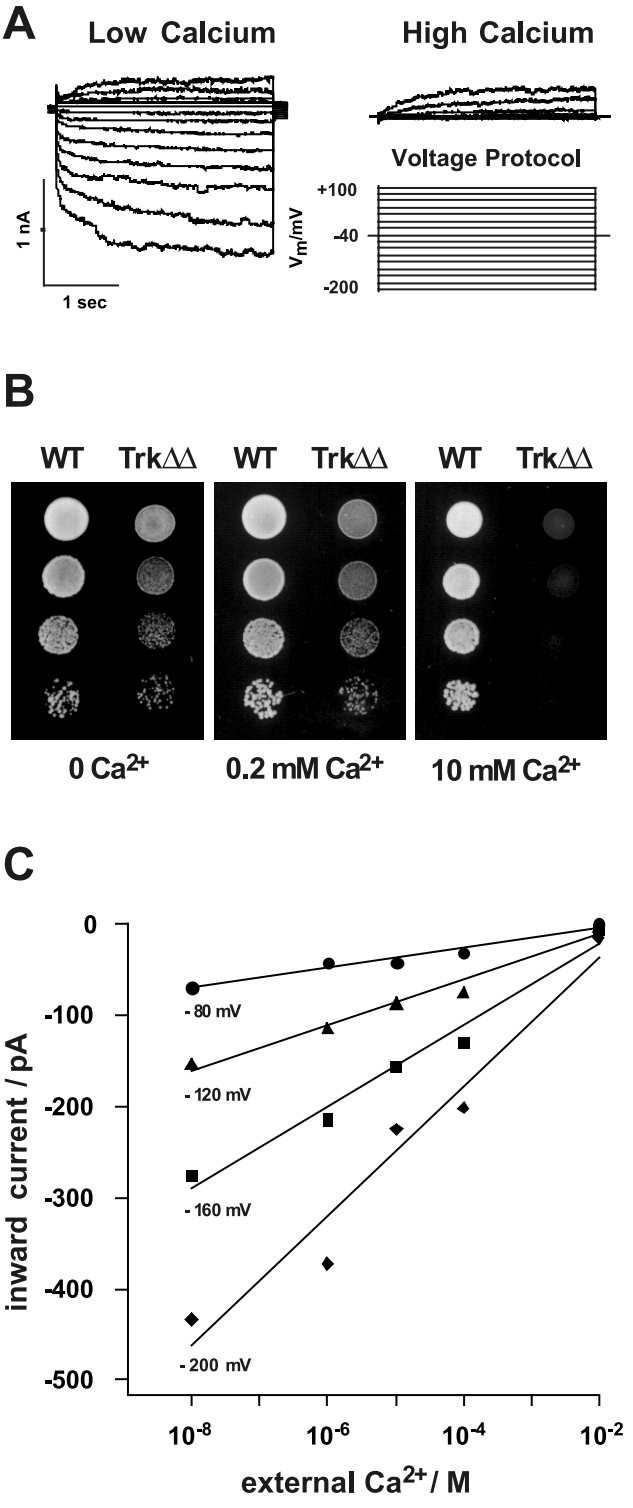

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Fig. 2. Calcium controls both NSC1 inward currents on sufficient K^+, and growth of TrkΔΔ cells on limiting K^+. (A) Inward current (downward traces) via NSC1, for cells bathed in 150 mM K^+, with 1 mM EGTA (left panel) or 10 mM $CaCl_2$ (right panel, top). (B) Corresponding growth tests of TrkΔΔ, compared with the isogenic WT, on SDAP agar containing 15 mM KCl with no $CaCl_2$ added (left panel), 0.2 mM $CaCl_2$ (middle panel), and 10 mM $CaCl_2$ (right panel). (C) Plot of steady-state NSC1 currents as a function of extracellular free [Ca^{2+}]. For 0.2 mM Ca^{2+} (the control value), currents would be 35% of those measured for 10 nM Ca^{2+}, at all voltages. (A) Right lower panel: schematic drawing of the voltage-clamp protocol used in all whole-cell recordings reported in this paper.

of WT was slightly increased by the external acidification. Even 100 mM $[K^+]_o$ was insufficient to support growth of TrkΔΔ at pH 4.0 (data not shown).

3.3. Other monovalent cations

Rubidium supplants potassium in many enzymatic functions, including transport and protein synthesis, and therefore in growth. However, most other monovalent cations are biochemically and physiologically far less K^+-like, or even antagonistic. The non-selectivity of NSC1 [14], then, offers no advantage in support of growth by many permeant cations.

Other than rubidium, the most K^+-like ion in many physiological reactions is ammonium, and Fig. 4A demonstrates that replacement of extracellular K^+ by NH_4^+ did not significantly change the total inward current through NSC1, in the tetraploid WT strain (cf. downward traces in the two sets of records). This would suggest an apparent permeability ratio for the two ions, $P_{NH_4^+}/P_{K^+}$, of ~1.0, which compares with previous estimates of P_{X^+}/P_{K^+} of ~0.9, ~0.6 ~0.4, and <0.2, for X = Rb^+, Cs^+, Na^+, and Li^+, respectively [14,15]. Outward currents (upward in Fig. 4A) mediated by Tok1 are slightly increased in 150 mM external NH_4Cl, which is likely due to small changes in cytosolic pH (<0.5 pH units), resulting from influx and subsequent internal dissociation of NH_4^+. This should have little or no effect on the growth results, since it is well within the range of intracellular pH (6.5–7.2) values found during rapid aerobic and anaerobic growth [16].

Ammonium, in the concentration range of 1–15 mM, added on top of 15 mM K^+ had no effect on growth of TrkΔΔ cells, whereas 100 mM ammonium

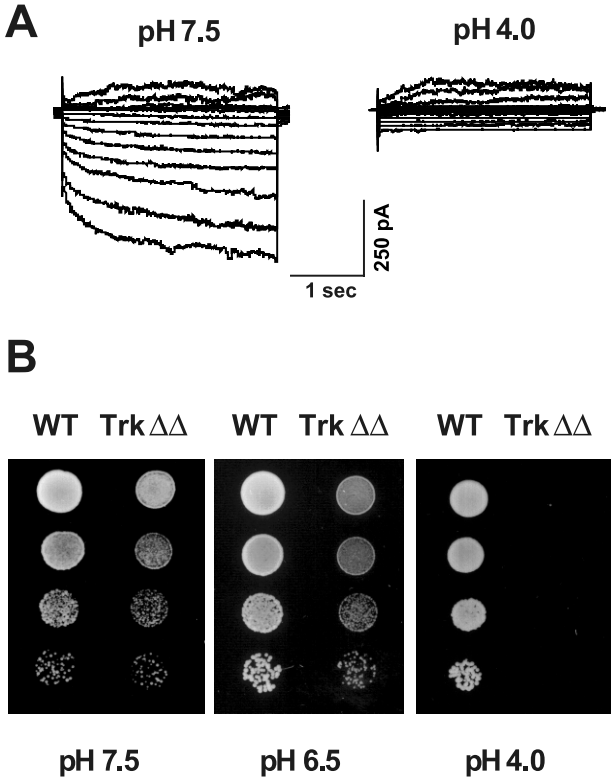

Fig. 3. Low external pH also inhibits both NSC1-mediated inward currents and growth of TrkΔΔ on limiting extracellular potassium. Conditions were generally as in Fig. 2 (see also Section 2), except for extracellular pH as shown, and zero Ca^{2+} in A or 0.2 mM Ca^{2+} in B. Note that growth of WT is even enhanced by the lowered pH_o: cf. left columns of the three panels in B.

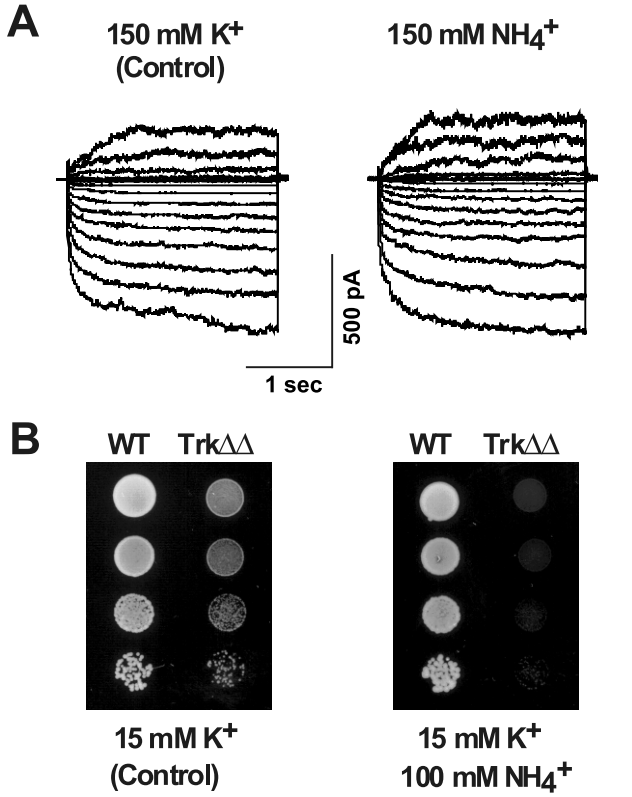

Fig. 4. NSC1-permeable ions which modulate the low-Ca^{2+} effect on growth: inhibition by NH_4^+. (A) No significant change of NSC1 inward currents (downward traces) upon replacement of extracellular K^+ by NH_4^+, in a WT protoplast. (B) Inhibition of TrkΔΔ growth by 100 mM NH_4^+, with little effect – or perhaps slight enhancement – of WT growth.

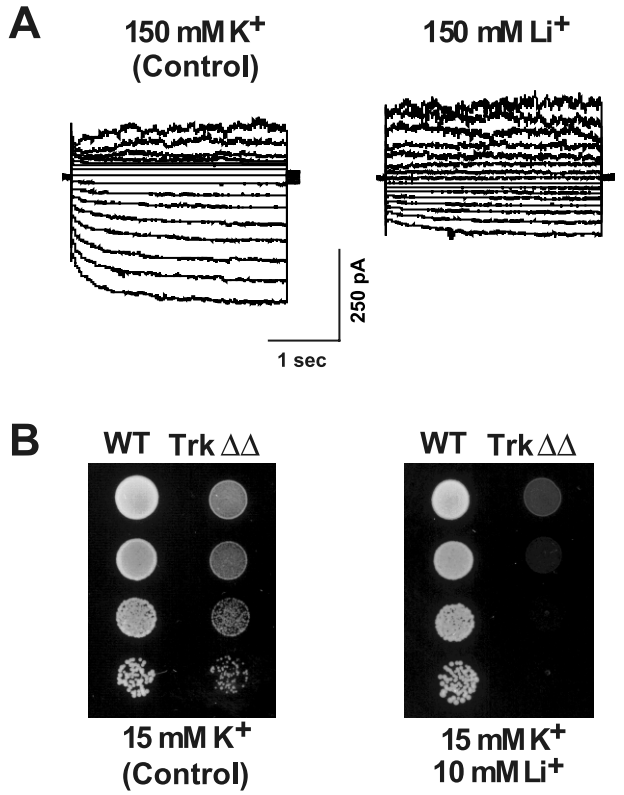

Fig. 5. NSC1-permeable ions which modulate low-Ca^{2+}-dependent, K^+-limited growth: strong inhibition by lithium. (A) Reduction by approx. 50% of inward currents upon replacement of extracellular K^+ by Li^+ in a WT protoplast. (B) Strong inhibition of growth in TrkΔΔ upon addition of 10 mM Li^+ to the 15 mM K^+ medium, with little effect on WT.

clearly inhibited growth of TrkΔΔ cells in 15 mM K^+ with little affect on WT (Fig. 4B).

Lithium ions could also carry significant current through NSC1, though usually less than 50% of the control K^+ current when totally replacing extracellular K^+ (Fig. 5A). However, as little as 10 mM Li^+, added on top of 15 mM K^+, strongly inhibited growth of TrkΔΔ strains (Fig. 5B). These results are consistent with the general understanding of deleterious effects of lithium ions on yeast (see, e.g. [22–24]), and confirm that NSC1 is a physiologically significant pathway for Li^+ accumulation under low-Ca^{2+} conditions.

3.4. Inhibitors of NSC1

Inhibitors of the low-Ca^{2+} cation pathway, as distinct from competing permeant ions, can be identified operationally by reduced inward currents, when an agent is presented in addition to the normal, high, test concentration of extracellular K^+. Without exception, such agents also inhibited low-Ca^{2+}, K^+-limited growth in the TrkΔΔ strain, although the mechanisms for growth inhibition can be more complicated than simple blockade of NSC1. Two examples are the tetraethylammonium ion (TEA^+) and the protein synthesis inhibitor hygromycin B (a divalent cation), whose effects on NSC1 current and on growth are shown in Figs. 6 and 7, respectively.

As expected according to Bertl et al. [17], 10 mM extracellular TEA^+ proved to be a strong inhibitor of the outwardly rectifying K^+ channel Duk1p (Tok1p), indicated by the strong reduction of time-dependent outward currents (upward in the records) in the

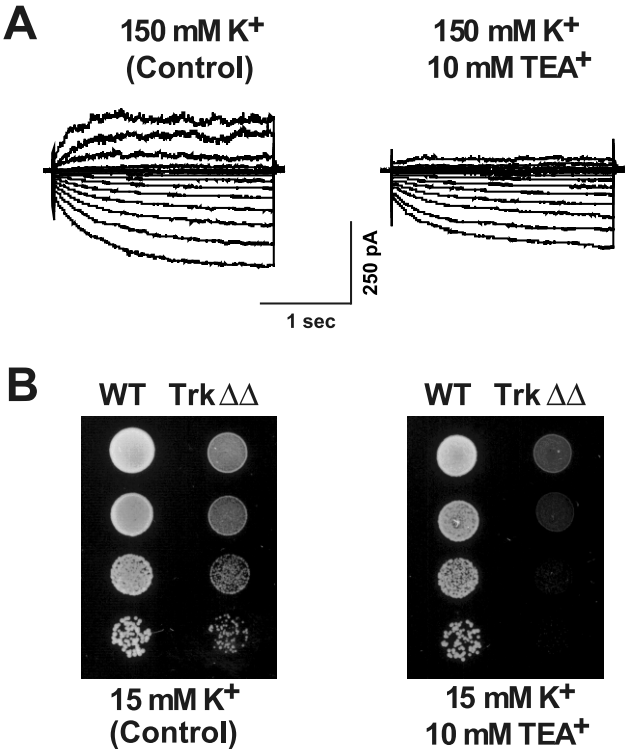

Fig. 6. NSC1-blocking ions which modulate K^+-limited growth: weak inhibition by TEA^+. (A) 20% reduction of low-Ca^{2+} inward currents (downward traces) produced by addition of 10 mM TEA^+ to 150 mM extracellular K^+. Note also the almost total disappearance of time-dependent outward (upward) currents, due to the K^+ channel Duk1p, in the presence of 10 mM TEA^+. (B) Modest inhibition of K^+-limited growth of TrkΔΔ by 10 mM TEA^+ with no effect on WT growth.

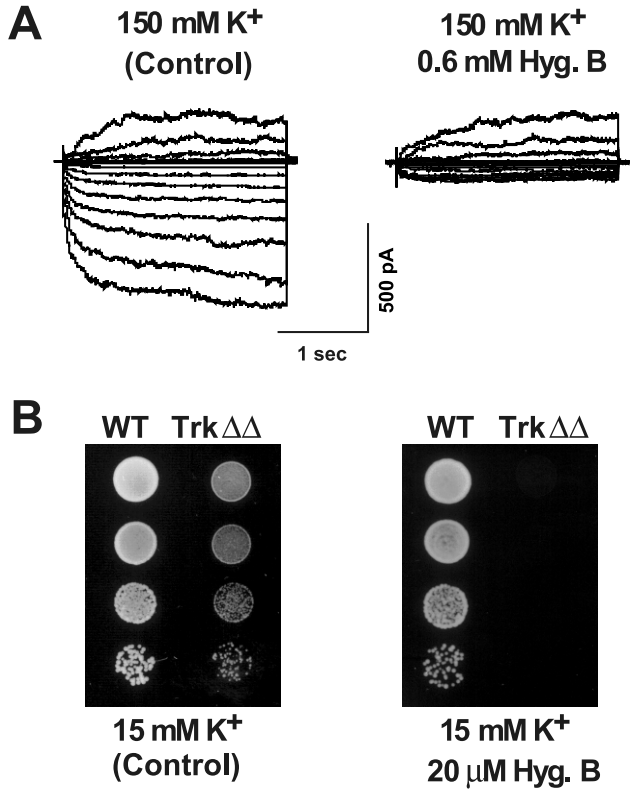

Fig. 7. NSC1-blocking ions which modulate K^+-limited growth. Strong inhibition by hygromycin B, a divalent cation. (A) Blockade of time-dependent NSC1 currents by 0.6 mM hygromycin, effecting 90% inhibition of total NSC1 currents. (B) Drastic growth inhibition by ~20 μM hygromycin, affecting the Trk∆∆ strain, but not the isogenic wild-type.

TEA^+ traces compared with the control in Fig. 6A. Inhibition of NSC1, however, amounted to only ~20% reduction of inward (downward) currents in the presence of TEA^+. Growth inhibition by 10 mM TEA^+ was also modest for the Trk∆∆ strain in 15 mM K^+ and 200 μM Ca^{2+}, as is seen by comparing the two right columns of Fig. 6B.

Hygromycin B (0.6 mM), by comparison, blocked the low-Ca^{2+} inward currents in the WT strain (by 90–95%), but had little effect on the Duk1 channel currents (Fig. 7A). Hygromycin B at still lower concentrations (~20 μM) also blocked K^+-limited growth of the Trk∆∆ strain, as shown by the drop tests in Fig. 7B. The simultaneous demonstration that hygromycin B had little or no growth effect on wild-type cells suggests that its critical action on the Trk∆∆ strain is indeed blockade of K^+ entry via NSC1.

4. Discussion

The monovalent cation entry path opened in low-Ca^{2+} medium was put to practical use very early in the study of membrane transport in yeast [25], especially for the purpose of demonstrating viability of the organism even after more than 99% of normal intracellular K^+ had been displaced. Much later, nearly stoichiometric exchange of extracellular Na^+ for intracellular K^+ (potassium efflux) was demonstrated under similar conditions [26], and glucose-fueled exchange of extracellular K^+ for metabolic protons (potassium influx) was postulated to occur in zero-Ca^{2+} medium via ionic 'channels' that were shut down by low extracellular pH [27,28]. The first unequivocal demonstration of their electrical effect and postulation of an important physiological function, i.e. low-affinity K^+ uptake, came only recently [14,15].

Non-selective cation channels and low-affinity carriers mediate a wide variety of physiological functions in most cells and tissues where they have been identified [29], whether activated by mechanical stress, by signaling ligands, or by free calcium ions. However, the particular class of non-selective channels which is either insensitive to calcium, or is inhibited by it, is poorly understood. Such channels have been described most carefully in amphibian oocytes and secretory epithelia and cloned from mammalian cultured cells (see brief summary in [14]), and have also been described both in plant cells [30,31], in fungi and in bacteria [32]. It is clear that these channels can play maladaptive roles, such as K^+ depletion or Na^+ accumulation under saline conditions, particularly when divalent metal ions are scarce [14,33,34].

However, their persistence and wide phylogenetic distribution imply a positive function during evolution. Two likely functions, at least for free-living cells, are (i) as demonstrated above, rescue from K^+ starvation when the primary K^+ uptake systems are missing or poisoned (see also [32]), and (ii) controlled entry of K^+ from potassium-rich environments. Both of these positive functions require that the proposed low-affinity K^+ uptake system, NSC1, is indeed a distinct molecular entity and that the activity of this transport system can be regulated under physiological conditions.

It remains to be determined whether yeast has such mechanisms for positive regulation of NSC1, either via repression/derepression or via functional inhibition/disinhibition, when extracellular divalent ions are adverse. That determination should become easier after the gene or genes encoding the channel have been identified. The pharmacological properties of NSC1 and its associated growth phenotypes should help design screening conditions for the isolation of the gene. Hints about possible regulatory interactions have come from several sources, most recently and interestingly from a study of the gene *PMP3*, which encodes a small hydrophobic polypeptide (55 amino acids) analogous to plant polypeptides that are overexpressed under salt stress [35]. Disruption of *PMP3* mimics activation of NSC1, in that it increases yeast's sensitivity to Na^+ and to hygromycin B, but reduces the K^+ dependence of *trk1Δtrk2Δ* strains, and all of these effects caused by *PMP3* disruption are reversed by elevation of extracellular calcium, again supporting a link between the Pmp3 peptide and NSC1.

Madrid et al. [36] argued, on the basis of Rb^+ uptake experiments and fluorescence measurements, that the *Saccharomyces* plasmalemma in *trk1Δtrk2Δ* cells is highly hyperpolarized, which drives low-affinity ($K_m \approx 60$ mM), 'ectopic' K^+ uptake. Although this low-affinity uptake follows simple Michaelis–Menten kinetics, indicative of a single transporter mechanism, experiments with different inhibitors led to the conclusion that the Trk-independent K^+ uptake is mediated by several transporters, which as a whole resemble a low-affinity single transporter [36]. This conclusion arose from the observations that ammonium and divalent cations inhibited different fractions of Rb^+ uptake (45% and 30%, respectively) in *trk1Δtrk2Δ* cells, and that addition of both agents had an almost additive inhibitory effect.

Such observations, however, could also be explained – and *quantitatively* – by separate modes of action of the two agents on a single molecular species of transporter. As shown in Fig. 4A, complete replacement of K^+ by NH_4^+ does not significantly change NSC1-mediated inward current, demonstrating that NH_4^+ can substitute for potassium through the low-affinity pathway, NSC1. Inhibition of K^+ (Rb^+) uptake by high concentrations of NH_4^+ would be explained by partial displacement of K^+ (Rb^+) by NH_4^+: effectively, *competitive* inhibition. Divalent cations, on the other hand, block the permeation pathway (cf. Fig. 2 above), thus reducing the availability of transporter molecules: effectively, *non-competitive* inhibition. Added on top of the 30% inhibition by divalent cations, ammonium inhibition should be another 31% (= 45% of the 70% flux not inhibited by divalent ions), for a total inhibition of 61%, close enough to the 60% actually reported by Madrid et al. [36].

The present characterization of NSC1 should also be useful for functional expression in yeast of heterologous inwardly rectifying K^+ channels. TrkΔΔ strains have already proven to be important tools for cloning and analysis of such channels, by complementation of their growth defect [37–39], but many types of K^+ channels are inhibited by high extracellular concentrations of divalent cations, so that for their functional analysis in yeast a low-Ca^{2+} environment would be very advantageous. This condition, however, also activates NSC1, thus interfering with, or masking, signals from heterologous channels in growth, flux, and electrophysiological experiments, but external divalent cations can be reduced virtually to zero if the external pH is reduced to < 4, which inactivates NSC1 (Fig. 3). An additional benefit of lowered pH_o, in the case of many plant inwardly rectifying K^+ channels, is that they are *activated* by extracellular acidification [40]. Therefore, simultaneously removing external Ca^{2+} and reducing the external pH to below 4 should silence NSC1 and maximize signals from expressed plant K^+ channels.

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