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Regulation and mechanism of potassium release from barley roots: an *in planta* $^{42}K^+$ analysis

Devrim Coskun, Dev T. Britto and Herbert J. Kronzucker

Department of Biological Sciences, University of Toronto, 1265 Military Trail, Toronto, ON, Canada M1C 1A4

Author for correspondence:
Herbert J. Kronzucker
Tel: +1 416 2877436
Email: herbertk@utsc.utoronto.ca

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Summary

- Potassium (K^+) flux into plant cells is a well-characterized ion transport phenomenon. By contrast, little is known about the mechanisms and regulation of K^+ flux *from* the cell. Here, we present a radioisotopic analysis of K^+ fluxes from roots of intact barley (*Hordeum vulgare*), in the context of recent discoveries in the molecular biology and electrophysiology of this process.
- Plants were labelled with $^{42}K^+$, and kinetics of its release from roots were monitored at low (0.1 mM) or high (1.0 mM) external K concentration, $[K^+]_{ext}$, and with the application of channel modulators and nutrient shifts.
- At 0.1 (but not 1.0) mM $[K^+]$, where K^+ efflux is thought to be mediated by K^+-outward-rectifying channels, $^{42}K^+$ efflux was inhibited by the channel blockers barium (Ba^{2+}), caesium (Cs^+), tetraethylammonium (TEA^+), and lanthanum (La^{3+}). Ammonium and nitrate (10 mM) stimulated and inhibited $^{42}K^+$ efflux, respectively, while 10 mM $[K^+]_{ext}$ or $[Rb^+]_{ext}$ decreased it. No evidence for the involvement of ATP-binding cassettes, nonselective cation channels, or active K^+-efflux pumps was found.
- Our study provides new evidence for the thermodynamic transition between high- and low-affinity transport, from the efflux perspective, identifying the operation of channels at low $[K^+]$, and the cessation of transmembrane efflux at high $[K^+]$.

Introduction

Potassium (K^+), a major macronutrient, is the most abundant cation in plant cells, and is required for a wide range of functions including osmotic balance, electrical regulation, and enzyme activation. Its uptake by plant roots has been studied extensively for decades, and a robust two-system model is widely used that describes the distinct kinetics of K^+ acquisition at low external K^+ concentrations ($[K^+]_{ext}$; a 'high-affinity' system) and at higher external concentrations (a 'low-affinity' system), the demarcation between the two typically falling in the range of 0.5–1.0 mM $[K^+]_{ext}$ (Britto & Kronzucker, 2008). This model was initially developed on the basis of uptake kinetics, as measured by radiotracers, particularly in the pioneering work of Epstein (e.g. Epstein *et al.*, 1963). The two systems differ from each other in a number of fundamental ways, including their regulation, thermodynamics, saturation kinetics and response to nitrogen source (for a review see Britto & Kronzucker, 2008).

More recently, specific transport proteins have been identified that catalyse K^+ fluxes in both the high- and low-affinity ranges. The saturable, high-affinity system has been linked to the KUP/HAK/KT family of transporters, while the linear, low-affinity system is associated with the activity of inwardly-rectifying K^+-specific ion channels. The molecular physiology of these transporters has been discussed in several recent reviews (including Véry & Sentenac, 2003; Gierth & Mäser, 2007; Lebaudy *et al.*, 2007; Britto & Kronzucker, 2008; Dreyer & Blatt, 2009; Szczerba *et al.*, 2009). It should be pointed out that there is some overlap in function between these two types of transporters, for example with AtKUP1 from *Arabidopsis* catalysing some degree of transport in the low-affinity range (Fu & Luan, 1998; Kim *et al.*, 1998), and the *Arabidopsis* AKT1 channel conducting K^+ fluxes in the high-affinity range (Hirsch *et al.*, 1998). However, because channel-mediated transport takes place passively, AKT1 can only facilitate K^+ fluxes in the high-affinity range when the electrical state of the plasma membrane is highly polarized.

In contrast to the influx of K^+, little is known about the physiology and molecular biology of K^+ efflux from plant roots. Physiological studies examining the efflux of K^+ have

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largely been restricted to two areas: compartmental analysis, in which efflux kinetics are used to determine subcellular pool sizes as well as other fluxes such as influx into the cell, and fluxes to the vacuole and shoot (Pitman & Saddler, 1967; Macklon, 1975; Memon *et al.*, 1985; Kronzucker *et al.*, 2003); and the analysis of K^+ loss from roots in response to salt or ionic stress (Nocito *et al.*, 2002; Shabala *et al.*, 2006; Britto *et al.*, 2010). Similarly, the molecular biology of K^+ release from the root is poorly understood, although several channels involved in the release of K^+ from root cells have been identified. These include SKOR, which facilitates K^+ release into the xylem from neighbouring parenchyma cells (Gaymard *et al.*, 1998), and GORK, which normally functions in K^+ release from guard cells in leaf tissue, but has also been localized in root hairs of *Arabidopsis* (Ivashikina *et al.*, 2001). In addition, assembly of AKT1 homotetramers in *Arabidopsis* mutants lacking the accessory channel protein *At*KC1 can result in the formation of functional channels that conduct 'leak' fluxes from the cell (Geiger *et al.*, 2009). However, SKOR and GORK are not known to mediate K^+ efflux from the root to the external environment, and AKT1 homotetramers are the products of mutagenesis and do not occur naturally. Thus, the molecular identity of ion channels mediating K^+ loss from the plant root into the external environment, like their physiological characteristics, remains largely unknown. Further complicating the issue is the apparent diversity of outwardly directed channel activity among plant species and cell type (Diatloff *et al.*, 2004). In addition to leaving the cell via ion channels, K^+ may also be driven outwardly via cation/proton exchangers such as those of the CHX (cation-proton exchanger) family (Pardo *et al.*, 2006), although the direction of the flux catalysed by these exchangers has been recently questioned (Zhao *et al.*, 2008).

In the present study, we have sought to address the lack of knowledge about K^+ efflux from plant roots, by using radiotracer analysis to examine in detail its physiological properties in intact barley seedlings. In particular, we have posed the following questions: What mechanisms underlie and regulate K^+ efflux from roots to the external environment? How does K^+ efflux respond to shifts in external [K^+] from high- to low-affinity conditions? How do the principal nitrogen sources for plants (ammonium and nitrate) differ in their effects upon K^+ efflux? Throughout, we have sought to answer these questions as a means of augmenting emerging discoveries in the molecular biology and electrophysiology of K^+ efflux.

Materials and Methods

Plant culture

Barley seeds (*Hordeum vulgare* L. cv 'Metcalfe') were surface-sterilized for 10 min in 1% sodium hypochlorite, and germinated under acid-washed sand for 3 d before placement in 12 l vessels containing aerated, modified Johnson's solution (0.5 mM $Ca(NO_3)_2$, 0.5 mM NaH_2PO_4, 0.25 mM $MgSO_4$, 0.125 μM Na_2MoO_4, 20 μM FeEDTA, 25 μM H_3BO_3, 2 μM $ZnSO_4$, 0.5 μM $MnSO_4$, and 0.5 μM $CuSO_4$), pH 6.3–6.5 (adjusted with 1 M NaOH), for an additional 4 d. The growth solutions were modified to provide potassium (as K_2SO_4), at 0.1 mM or 1.0 mM (for a few experiments, K^+ was also provided at 0.5 mM or 0.75 mM). To ensure that plants remained at a nutritional steady state, solutions were exchanged on days 5 and 6 (for 0.1 mM grown plants) or on day 5 only (for 1.0 mM grown plants). Plants were grown in walk-in growth chambers under fluorescent lights with an irradiation of 200 μmol photons m^{-2} s^{-1} at plant height, for 16 h d^{-1} (Philips Silhouette High Output F54T5/850HO; Philips Electronics Ltd, Markham, ON, Canada). Daytime temperature was 20°C, night-time temperature was 15°C, and relative humidity was 80%. One day before experimentation (day 6), seedlings were bundled together at the base of the shoot, in groups of three (for direct influx measurements) or five (for efflux analysis) using a plastic collar, 0.5 cm in height.

Direct efflux

Monitoring of unidirectional tracer efflux rates over time was based on methods from compartmental analysis (Lee & Clarkson, 1986; Siddiqi *et al.*, 1991; Kronzucker *et al.*, 1995), but no assumptions were made regarding the identity of compartments releasing tracer. Intact roots of bundled plants were labelled for 60 min in solution identical to growth solution but containing the radiotracer ^{42}K ($t_{1/2}$ = 12.36 h), received as K_2CO_3 from the McMaster University Nuclear Reactor (Hamilton, ON, Canada). Labelled bundles were attached to efflux funnels and eluted of radioactivity with successive 13 ml aliquots of nonradioactive desorption solution (identical to growth solution in the steady-state runs, but containing various flux-modulating agents in other runs; see two paragraphs below). The desorption series for K^+ fluxes was timed as follows, from the first to the final eluate: 15 s (four times), 20 s (three times), 30 s (twice), 40 s (once), 50 s (once), 1 min (25 times), for a total elution period of 29.5 min. Nonsteady-state experiments entailed additional solutes in the final 14 eluates (applied at elution time t = 15.5 min). All solutions were mixed using a fine stream of air bubbles. Each condition was replicated 3–13 times.

Immediately following elution, roots were detached from shoots and spun in a low-speed centrifuge for 30 s, to remove surface water, before weighing. Radioactivity from eluates, roots, shoots and centrifugates was counted, and corrected for isotopic decay, using two gamma counters (PerkinElmer Wallac 1480 Wizard 3″ (Turku, Finland) and Canberra-Packard, Quantum Cobra Series II, model 5003

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(Packard Instrument Co., Meriden, CT, USA)). For comparison charts of $^{42}K^+$ efflux, the specific activities of all replicates were normalized to an arbitrary value of 2×10^5 cpm μmol^{-1}.

Using this general procedure, efflux measurements were conducted in the presence of one or more of the following pharmacological agents, applied at elution time 15.5 min: caesium (as CsCl, 10 mM), lanthanum (as $LaCl_3$, 10 mM), tetraethylammonium (as TEACl, 10 mM), barium (as $BaCl_2$, 5 mM), calcium (as $CaSO_4$, 10 mM), bicarbonate (as $NaHCO_3$, 10 mM), ammonium (as $(NH_4)_2SO_4$, 5 mM), sodium (as NaCl, 10, 25 or 100 mM), rubidium (as Rb_2SO_4, 5 mM), potassium (as K_2SO_4, 5 mM), nitrate (as $Ca(NO_3)_2$, 5 mM), vanadate (as Na_3VO_4, 10 mM) and cyanide (as NaCN, 1 mM). Treatments involving elevation of external pH to 9.2 were prepared with 1 M NaOH. ATP-binding cassette modulators minoxidil sulphate, pinacidil monohydrate, diazoxide, and 4-aminopyridine were all dissolved in dimethyl sulphoxide (DMSO) (0.1% in growth solution), and used at a final concentration of 100 μM.

Efflux studies involving the longer-term application of ATP-binding cassette modulators were conducted with a 2 h pre-equilibration of seedling bundles in growth solution containing the modulator in question. Labelling and elution steps were performed as described, except that all eluates contained the modulator in question.

For the data in Table 1, tracer efflux and retention data were used to estimate unidirectional and net fluxes, according to the procedure described in Kronzucker *et al.* (1995).

Direct influx

Short-term $^{42}K^+$ labelling was performed on barley seedlings to study the effects of various N sources on unidirectional K^+ influx under nonsteady-state conditions. For steady-state and nonsteady-state conditions, seedling bundles were grown as described earlier at either 0.1 mM or 1.0 mM $[K^+]_{ext}$. For steady-state experiments, bundles were pre-equilibrated for 5 min in growth solution, then immersed for 5 min in labelling solution (identical to growth solution but containing the radiotracer $^{42}K^+$). From there, plants were transferred to nonradioactive growth solution for 5 s to reduce tracer carryover and finally desorbed for 5 min in fresh growth solution. All solutions were chemically identical to growth medium. For nonsteady-state conditions, experiments were conducted in the same way with the exception of each solution within a treatment containing either 10 mM NH_4^+ (as $(NH_4)_2SO_4$), 10 mM NO_3^- (as $Ca(NO_3)_2$), or 10 mM NH_4NO_3. Each condition was replicated 9–10 times.

Table 1 Steady-state unidirectional and net fluxes of barley (*Hordeum vulgare*) seedlings grown at 0.1 mM $[K^+]_{ext}$ or 1.0 mM $[K^+]_{ext}$

$[K^+]_{ext}$ (mM)	K^+ fluxes (μmol g^{-1} (root FW) h^{-1})			Efflux : influx ratio
	Influx	Efflux	Net flux	
0.1	7.22 ± 0.23	1.86 ± 0.18	5.36 ± 0.18	0.25 ± 0.02
1.0	5.97 ± 0.77	0.57 ± 0.03	5.40 ± 0.77	0.11 ± 0.01

Errors indicate ± SE of the mean of 11–13 replicates.

Statistics

Each bundle was treated as a single replicate (see the Materials and Methods section, 'Plant culture'). In each efflux graph, traces for treatments (following application of treatment at 15.5 min) were compared with control traces by matching each pair of data points for a given elution time, by the use of Student's *t*-test. The number of paired points that were found to be significantly different ($P < 0.05$) are shown in the legend of each graph. For influx experiments, we performed ANOVA with Bonferroni *post-hoc* corrections.

Results

Unidirectional and net fluxes in control, steady-state plants grown at 0.1 or 1.0 mM $[K^+]_{ext}$ were determined using tracer efflux and retention data, and are shown in Table 1. Efflux, influx and the ratio of the two were all lower in the high-K^+ plants, while the net flux of K^+ was nearly the same under both conditions.

Fig. 1 shows the effects of channel blocking agents on the efflux of $^{42}K^+$ from pre-labelled roots of intact barley seedlings, grown at two concentrations of K^+ ($[K^+]_{ext}$). At low $[K^+]_{ext}$ (0.1 mM), $^{42}K^+$ efflux was reduced by application of TEA^+ and La^{3+}, and blocked by Ba^{2+} and Cs^+ (Fig. 1). Because of the greater efficacy of Ba^{2+} and Cs^+ at 0.1 mM, these agents were used for efflux trials at higher $[K^+]_{ext}$. Nevertheless, we found that neither agent changed the efflux pattern at 1.0 mM (Fig. 1, inset; a small suppressive effect of Ba^{2+} was seen at 0.5 mM, not shown).

In addition to its response to channel blockers, $^{42}K^+$ efflux from roots of 0.1 mM-grown plants was also suppressed by the application of high concentrations (10 mM) of potassium or rubidium, while neither treatment altered efflux from plants grown at 1.0 mM (Fig. 2). In plants grown at 0.1 mM, application of 10 mM Ca^{2+} or 10–25 mM sodium had no effect on $^{42}K^+$ efflux, but efflux rose when 100 mM sodium was applied (Fig. 2a,b).

Nitrogen source had an immediate effect on $^{42}K^+$ efflux, at both low (0.1 mM) and high (1.0 mM) K^+ provision (Fig. 3). All plants, grown at 1 mM NO_3^-, responded to the addition of 10 mM NH_4^+ with an approximately threefold increase in $^{42}K^+$ efflux. Application of 10 mM NH_4NO_3 also accelerated $^{42}K^+$ efflux, but to a lesser degree than 10 mM NH_4^+. Similar responses to high NH_4^+ and

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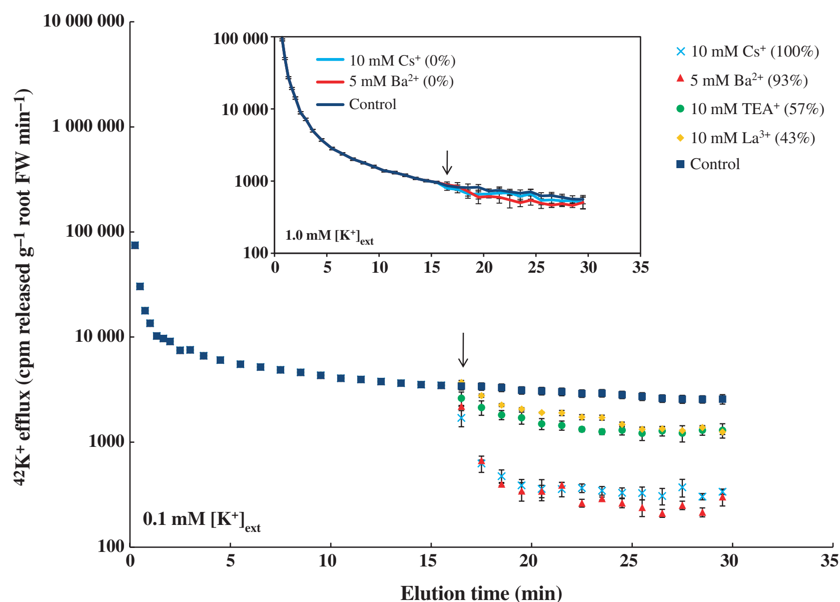

Fig. 1 Response of $^{42}K^+$ efflux from roots of intact barley (*Hordeum vulgare*) seedlings to sudden provision (at elution time = 15.5 min; see arrow) of the channel inhibitors Cs^+ (as CsCl), Ba^{2+} (as $BaCl_2$), TEA^+ (as tetraethylammonium-Cl), or La^{3+} (as $LaCl_3$) at external K concentration, $[K^+]_{ext}$, of 0.1 mM. Inset: response of $^{42}K^+$ efflux to channel inhibitors Cs^+ and Ba^{2+} at $[K^+]_{ext}$ of 1.0 mM. For clarity, data points within the inset were connected with coloured lines. In internal legend, numbers in parentheses following treatment conditions indicate per cent of treated points differing significantly from control (*t*-test, $P < 0.05$). Each plot represents the mean of 3–13 replicates. Error bars indicate ± SEM.

NH_4NO_3 were seen at the intermediate $[K^+]_{ext}$ values of 0.5 mM and 0.75 mM (not shown). The shape of the ammonium-stimulated $^{42}K^+$ efflux trace differed between the 0.1 mM $[K^+]_{ext}$ treatment and the others, however, in that at 0.1 mM it peaked within 2 min, then declined (Fig. 3a). By contrast, $^{42}K^+$ efflux at higher $[K^+]_{ext}$ rose more slowly and reached a plateau at which it was sustained for at least 10 min (Fig. 3b). Another key difference between low and high K^+ conditions was that, at 0.1 $[K^+]_{ext}$, the application of 10 mM NO_3^- suppressed $^{42}K^+$ efflux (Fig. 3a), while this treatment had no effect at 1.0 mM $[K^+]_{ext}$ (Fig. 3b).

Stimulation of $^{42}K^+$ efflux by NH_4^+ was ameliorated by the application of channel-blocking agents, but the efficacy of each agent depended on external $[K^+]$ conditions (insets, Fig. 3a,b). At 0.1 mM $[K^+]_{ext}$, Cs^+ was the most potent, reducing efflux to below control levels (as in Fig. 1a), while TEA^+ was nearly as effective (Fig. 3a, inset). By contrast, at 1.0 mM $[K^+]_{ext}$, Cs^+ and TEA^+ treatments showed a moderate degree of suppression (Fig. 3b, inset). Interestingly, application of K^+ itself (at 10 mM) also suppressed the NH_4^+-stimulated $^{42}K^+$ efflux, as strongly as Cs^+ under the 0.1 mM $[K^+]_{ext}$ conditions, and more moderately (comparable to TEA^+ and Cs^+) under the 1.0 mM condition (not shown). In addition, application of 10 mM Rb^+ was nearly as effective as 10 mM K^+ in both high- and low-K^+-grown plants.

Direct influx measurements, complementary to efflux runs, were also conducted in relation to nitrogen source and strength (Fig. 4; values were slightly different from those determined using efflux analysis, but showed similar trends). Switching from the growth concentration of 1 mM NO_3^- to 10 mM NO_3^- had opposite effects on low- and high-K^+ plants, with elevated NO_3^- decreasing influx by *c.* 20% in low-K^+ plants, and increasing it in high-K^+ plants by *c.* 70%. Both NH_4^+ and NH_4NO_3, provided at 10 mM, decreased influx under both growing conditions.

It was of interest to investigate the effect on $^{42}K^+$ efflux of treatments known to disrupt the energy state of the cell and the plasma membrane. Fig. 5 shows that the metabolic inhibitors vanadate (VO_4^{3-}) and cyanide (CN^-) increased $^{42}K^+$ efflux at 0.1 mM $[K^+]_{ext}$ (Fig. 5a), but had little or no effect at the higher $[K^+]_{ext}$ (1.0 mM, Fig. 5, inset). The addition of sodium bicarbonate, which is known to hyperpolarize the plasma membrane (Poole, 1969), at 10 mM caused a suppression of $^{42}K^+$ efflux at the lower $[K^+]_{ext}$ (0.1 mM) but not at the higher $[K^+]_{ext}$ (1.0 mM), while increasing the external pH from 6.3–6.5 to 9.2 had no effect at either $[K^+]_{ext}$.

To test for the involvement of a special class of K^+ channels, those regulated by ATP-binding cassettes (ABCs), in the efflux of K^+, we applied a range of compounds known to stimulate the activity of these proteins (minoxidil, pinacidil, diazoxide; Leonhardt *et al.*, 1997), and one known to inhibit them (4-aminopyridine). We found that their application did not affect $^{42}K^+$ efflux in a statistically significant way, at $[K^+]_{ext}$ of 0.1 mM (not shown).

Discussion

Although $^{42}K^+$ efflux patterns (Fig. 1) and flux parameters (Table 1) were similar among all control plants, regardless of growth condition, treatment of roots with a wide variety of physiologically active agents belies these similarities. With rising K^+ provision, the efficacy of treatments inhibiting $^{42}K^+$ efflux at the lowest $[K^+]_{ext}$ of 0.1 mM was progressively

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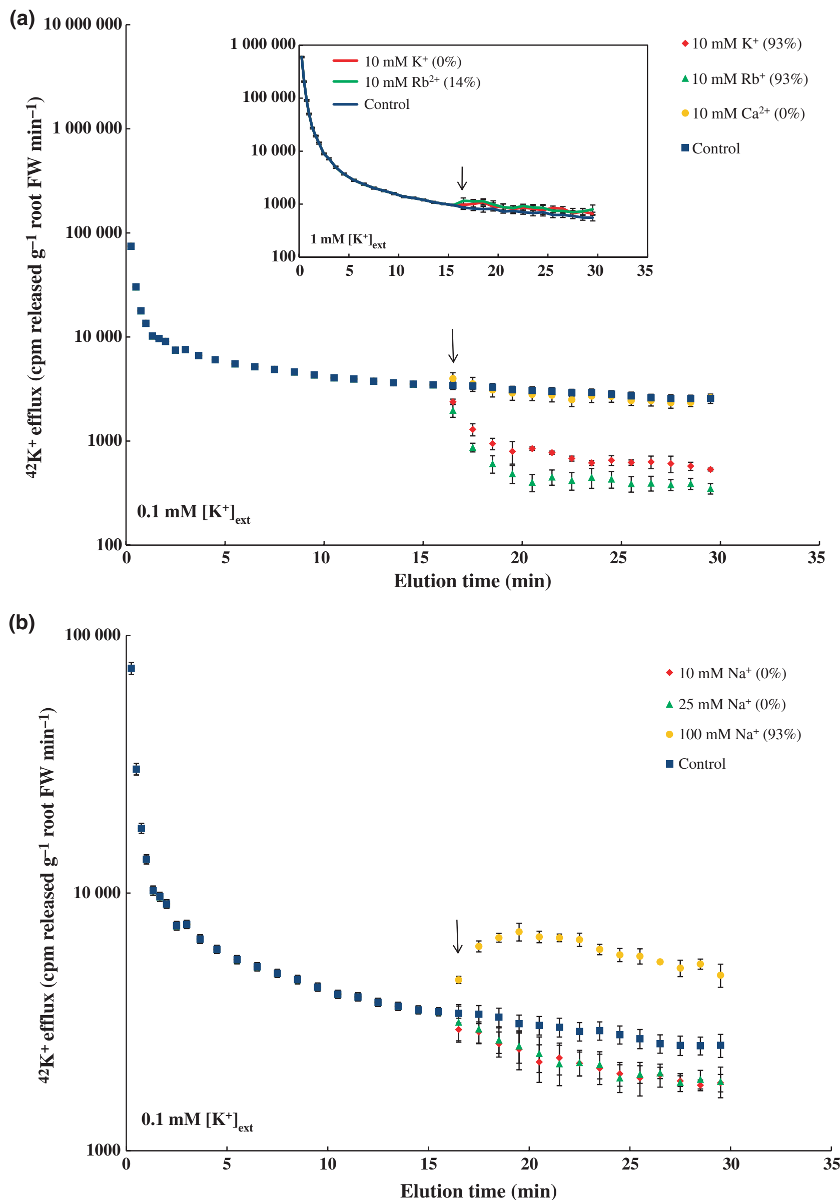

Fig. 2 Response of $^{42}K^+$ efflux from roots of intact barley (*Hordeum vulgare*) seedlings to sudden application or elevation (at elution time = 15.5 min; see arrow) of Ca^{2+} (as $CaSO_4$), K^+ (as K_2SO_4), Rb^+ (as Rb_2SO_4) or Na^+ (as NaCl). Growth concentrations of K^+ were 0.1 mM (a, b) or 1.0 mM (a, inset). Internal legend, as in Fig. 1. Each plot represents the mean of 3–13 replicates. Error bars indicate ± SEM.

reduced (not shown), until there was very little effect at the highest $[K^+]_{ext}$ of 1.0 mM. When comparing the bracketing conditions of 0.1 and 1.0 mM $[K^+]_{ext}$ (referred to subsequently as 'low-K^+' and 'high-K^+', respectively), the difference is clear as the following six treatments had effects on $^{42}K^+$ efflux under low K^+-growth conditions, but none under high K^+: Cs^+, Ba^{2+} (Fig. 1), K^+ (stepped up to 10 mM), Rb^+ (Fig. 2), NO_3^- (Fig. 3) and HCO_3^- (Fig. 5). In addition, treatment with the metabolic inhibitors cyanide and vanadate resulted in immediate stimulation of $^{42}K^+$ efflux at low K^+, but only a weak stimulation at high K^+ (Fig. 5).

It is reasonable to conclude from these results that, despite their superficial similarities, the efflux traces under low-K^+ and high-K^+ growth conditions represent two distinct phenomena. This idea is supported by substantial work indicating that the transition between high- and low-affinity K^+ transport modes in plant roots typically lies between 0.1 mM $[K^+]_{ext}$ and 1.0 mM $[K^+]_{ext}$ (Epstein *et al.*, 1963; Glass & Dunlop, 1978; Kochian & Lucas, 1982; Maathuis & Sanders, 1995; Kronzucker *et al.*, 2003). Over this range, the thermodynamic conditions change owing to K-induced alterations in the electrical potential difference across the plasma membrane (Etherton & Higinbotham, 1960; Cheeseman & Hanson, 1979a,b; Kochian *et al.*, 1989; Walker *et al.*, 1996; Szczerba *et al.*, 2006), as well as to changes in the concentration (activity) ratio of K^+ across this membrane. The result is a shift in the mechanism of K^+ influx into the cell, from an energetically

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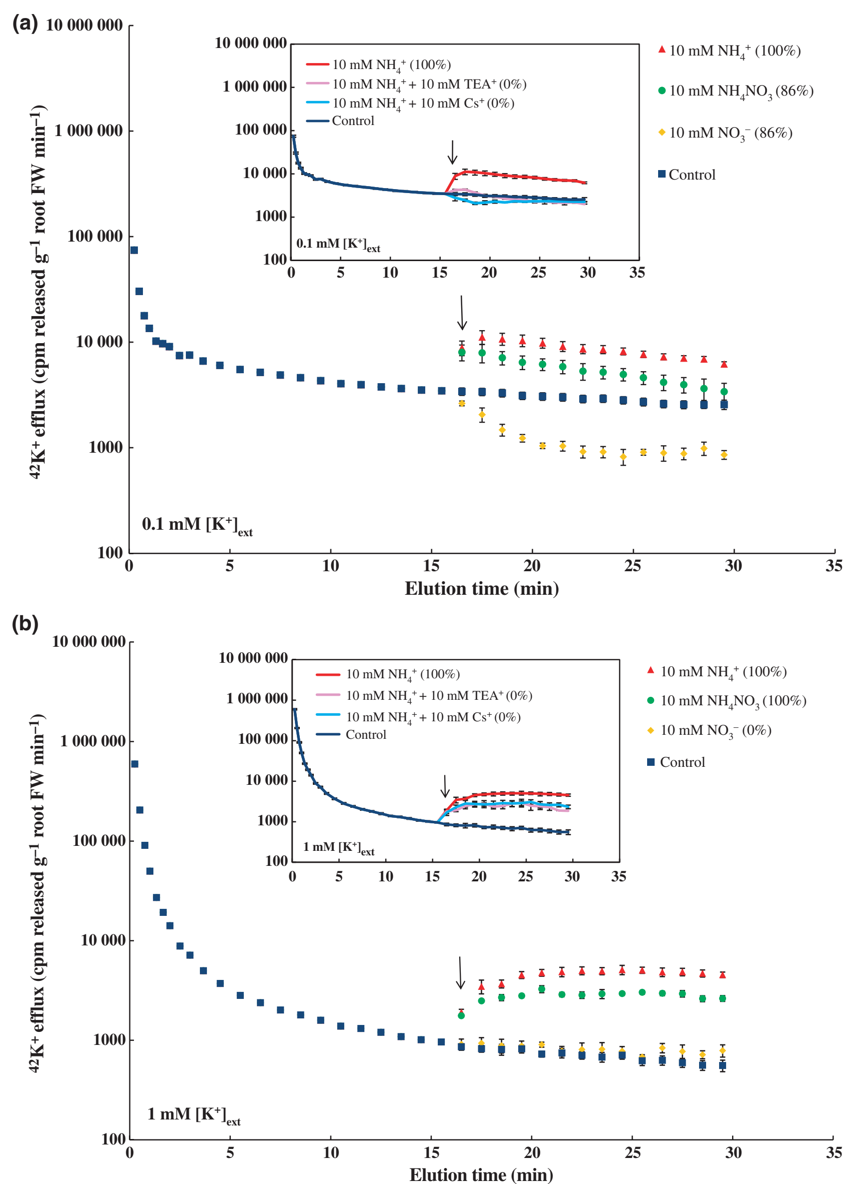

Fig. 3 Response of $^{42}K^+$ efflux from roots of intact barley (*Hordeum vulgare*) seedlings to sudden provision (at elution time = 15.5 min; see arrow) of (a,d) NH_4^+ (as $(NH_4)_2SO_4$), NH_4NO_3, or NO_3^- (as $Ca(NO_3)_2$). Inset: response of $^{42}K^+$ efflux to sudden provision of NH_4^+ in combination with the channel inhibitors TEA^+ (as TEACl) or Cs^+ (as CsCl). Plants were grown at an external K concentration, $[K^+]_{ext}$, of 0.1 mM (a) or 1.0 mM (b). Internal legend, as in Fig. 1. Each plot represents the mean of 3–13 replicates. Error bars indicate ± SEM.

active system to one catalysing transport down an electrochemical potential gradient as $[K^+]_{ext}$ increases. It is to be expected, then, that the opposite thermodynamic conditions will apply to the transport of K^+ in the efflux direction, from the cell to the external medium. Because many passive transport functions in biological systems are mediated by ion channels, it is reasonable to suggest that the $^{42}K^+$ efflux traces at 0.1 mM $[K^+]_{ext}$ represent efflux of K^+ from the cell down its electrochemical potential gradient, via outwardly-rectifying, Shaker-type K^+ (KOR) channels that have been identified at a molecular level and have been localized to the plasma membrane (for a review see Szczerba *et al.*, 2009). This idea is supported by the efficacy of 'classic' K^+-channel-blocking agents TEA^+, La^{3+}, Ba^{2+} and Cs^+ (Krol & Trebacz, 2000; White & Broadley, 2000) in reducing $^{42}K^+$ efflux at low $[K^+]_{ext}$, as depicted in Fig. 1. This inhibition profile parallels that of inward-rectifying, Shaker-type K^+ (KIR) channels (White & Broadley, 2000), and agrees with the study by Roberts & Tester (1995), who showed that TEA^+ is not as effective as Cs^+ or Ba^{2+} in blocking outward, whole-cell, K^+ currents in maize root protoplasts. Similarly, the observation (Fig. 2a) that K^+ and Rb^+ (when either was provided at 10 mM) suppressed $^{42}K^+$ efflux in plants grown at 0.1 mM $[K^+]_{ext}$ supports KOR channel involvement, as K^+ efflux channels in plants, rather uniquely, are known to be inhibited by elevated external K^+ or Rb^+, through cooperative binding of these ions at regulatory sites distinct from the channel pore (Blatt, 1999; Johansson *et al.*, 2006; Gajdanowicz *et al.*, 2009). Importantly, the observation that Rb^+ inhibited $^{42}K^+$ efflux

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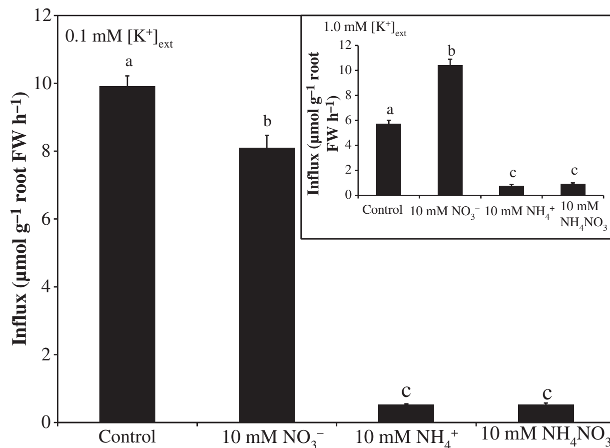

Fig. 4 Direct K^+ influx determined by short-term (5 min) $^{42}K^+$ labelling of intact barley (*Hordeum vulgare*) seedlings, grown at either 1.0 (inset) or 0.1 mM external K concentration, $[K^+]_{ext}$, and introduced to sudden provision (10 min) of elevated NO_3^- (as $Ca(NO_3)_2$), NH_4^+ (as $(NH_4)_2SO_4$), or NH_4NO_3. Letters indicate significantly different means (one-way analysis of variance with Bonferroni post-test, $P < 0.05$). Error bars indicate ± SEM.

to the same extent as elevated K^+ indicates that it is not the transmembrane concentration gradient of K^+ that brings about this channel closure, but more likely the regulatory binding of K^+, or its analogue Rb^+.

Interestingly, the application of high (10 mM) external Ca^{2+} had no effect at 0.1 $[K^+]_{ext}$ (Fig. 2), in agreement with the relative Ca^{2+}-insensitivity of K^+ channels seen in most electrophysiological studies (White & Broadley, 2000), but in contrast to one class of nonselective, outward-rectifying channels (NORCs), a hallmark of which is inhibition by low to intermediate concentrations of Ca^{2+} (Demidchik & Maathuis, 2007). The lack of NORC involvement in the present study is further supported by the insensitivity to Cs^+ and TEA^+ of all classes of NORCs (White & Broadley, 2000; Demidchik & Maathuis, 2007).

The number of molecular candidates that could account for the observed flux patterns at 0.1 mM $[K^+]_{ext}$ is limited. While several outward-rectifying K^+ channels have been identified at the molecular level in plant roots, none matches the $^{42}K^+$ efflux profiles reported here. The SKOR protein, while having an inhibition and gating profile suggestive of the present observations (Gaymard *et al.*, 1998; Johansson *et al.*, 2006), is an unlikely match because it is localized to pericycle and xylem parenchyma cells, consistent with its proposed role as a xylem-loading transporter. Owing to the immature Casparian strip in these young seedlings, however, it is possible that at least some of the $^{42}K^+$ is released from stelar cells and may diffuse to the external medium via the apoplast. The guard-cell channel GORK, while predominantly expressed in leaves, has also been detected in epidermal root hairs, but was found to be unaffected by 10 mM Cs^+ (Ivashikina *et al.*, 2001), thus also disqualifying it as a major conductor of the observed flux in the present study. Similarly, Diatloff *et al.* (2004) examined outward-rectifying channels in epidermal cells from roots of *Arabidopsis thaliana*, but these displayed a reduction in current density as extracellular K^+ activity was reduced, contrary to the present findings. In addition, guard-cell outward rectifiers have been shown to be ATP-regulated via ATP-binding cassettes (ABC) (Leonhardt *et al.*, 1997), but in our study, several chemical agents known to either stimulate or inhibit the activity of ABC proteins had little or no effect on $^{42}K^+$ efflux (not shown). Finally, the KCO1 channel, which has a pharmacological profile similar to the fluxes in the present study, is localized

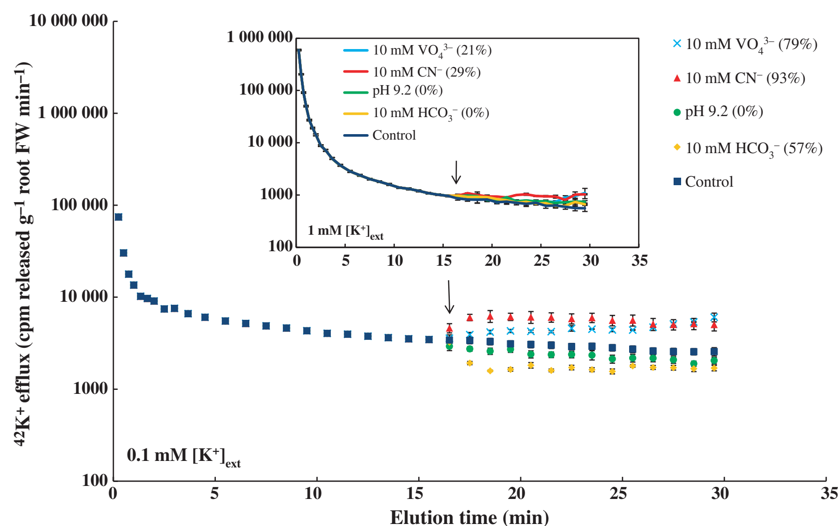

Fig. 5 Response of $^{42}K^+$ efflux from roots of intact barley (*Hordeum vulgare*) seedlings to sudden provision (at elution time = 15.5 min; see arrow) of VO_4^{3-} (as Na_3VO_4), CN^- (as NaCN), pH 9.2, or HCO_3^- (as $NaHCO_3$) at an external K concentration, $[K^+]_{ext}$, of 0.1 mM and 1.0 mM (inset). Internal legend, as in Fig. 1. Each plot represents the mean of 3–13 replicates. Error bars indicate ± SEM.

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in the tonoplast and appears only to be activated episodically, in response to transient changes in cytosolic Ca^{2+} (Czempinski *et al.*, 2002). In summary, our results suggest that the transport system dominating the mediation of efflux of K^+ from roots of plants grown in low K^+ has not as yet been identified at the molecular level. We further suggest that this study should complement and guide molecular and physiological K^+ transport studies, which are typically performed at a less intact level.

Unlike the effects observed at 0.1 mM $[K^+]_{ext}$, $^{42}K^+$ efflux from plants grown at 1.0 mM was scarcely altered by the application of channel blockers (Fig. 1) or by elevated external K^+ or Rb^+ (not shown). In addition, the reduction of $^{42}K^+$ efflux observed upon application of elevated (10 mM) NO_3^- to roots of plants grown at 0.1 mM $[K^+]_{ext}$ was not seen in plants grown at 1.0 mM $[K^+]_{ext}$ (Fig. 3a,b). Thus, our data indicate that channel-mediated K^+ efflux is likely to be inoperative under the high-K^+ conditions. This is consistent with the conclusion drawn from patch-clamping studies that show KOR channels to be gated closed, via sensing of both external $[K^+]$ and membrane voltage, at membrane potentials more positive than the Nernst potential for K^+ (Roberts & Tester, 1995; Dreyer & Blatt, 2009; Gajdanowicz *et al.*, 2009).

Under the higher-K^+ growth regime, the resistance of $^{42}K^+$ efflux traces to multiple inhibitors raises the question of how to interpret the observed $^{42}K^+$ release. To test the hypothesis that these traces represent $^{42}K^+$ driven from the cell via secondary active transporters (e.g. K^+/H^+ antiporters) (Szczerba *et al.*, 2006), as has been proposed for members of the CHX family (Pardo *et al.*, 2006), we decreased the external $[H^+]$ by almost three orders of magnitude and applied the metabolic disruptors cyanide and vanadate. However, none of these treatments could decrease the flux (Fig. 5, inset). In addition, the role of CHX proteins in K^+ transport is unclear, with one recent report indicating that at least one member of this family (the *Arabidopsis* AtCHX13) plays a role in K^+ acquisition, rather than in its loss (Zhao *et al.*, 2008). Furthermore, while membrane pumps catalysing ATP-driven, monovalent cation efflux from the cell have been found in organisms such as yeast (Benito *et al.*, 2002) and bryophytes (Benito & Rodriguez-Navarro, 2003), there is no evidence that they exist in higher plants. Thus, our study suggests that the ostensibly channel-mediated efflux system observed at 0.1 mM $[K^+]_{ext}$ is not likely to be operating at 1.0 mM, nor are K^+-efflux pumps. Rather, the evidence suggests that the $^{42}K^+$ released under these conditions crosses no membrane. As such, this observation is reminiscent of the phenomenon of apoplastic bypass flow, which has been documented in the case of sodium transport in cereals (Oertli, 1968; Flowers *et al.*, 1991; Gong *et al.*, 2006; Malagoli *et al.*, 2008; Krishnamurthy *et al.*, 2009). If the observed $^{42}K^+$ traces at 1.0 mM $[K^+]_{ext}$ are indeed the result of extracellular K^+ fluxes, this raises an important issue about cellular flux analysis by use of tracers: it is not always possible to distinguish components of the measured flux across the plasma membrane from those that occur outside the cell, as discussed by Cheeseman (1986). It is of further interest that, in Table 1, the calculated chemical efflux of K^+ at 1.0 mM $[K^+]_{ext}$ is less than one-third that of the efflux calculated at 0.1 mM $[K^+]_{ext}$, an unexpected outcome given that, over broader concentration ranges, efflux tends to rise (Britto & Kronzucker, 2006) for most ions, including potassium (Szczerba *et al.*, 2006). This provides further evidence that a different phenomenon is occurring at 1.0 mM $[K^+]_{ext}$, which is reflective of the cessation of physiological efflux across the plasma membrane and the observation of efflux from the apoplast. It should also be pointed out that, even in the 0.1 mM $[K^+]_{ext}$ condition, a small proportion of the observed efflux may also be apoplastic. However, this contribution will amount to no more than 10% of the relatively low efflux seen at 1.0 mM, assuming linearity of this component with respect to $[K^+]_{ext}$. Nevertheless, this additional efflux component may dominate tracer release kinetics at higher concentrations (e.g. those used in studies of salinity stress), wherein efflux becomes a sizeable proportion of the total flux (Britto & Kronzucker, 2006; Malagoli *et al.*, 2008). It should be pointed out that other possibilities, such as vesicular transport, cannot be ruled out (Peiter *et al.*, 2007; Britto & Kronzucker, 2009). Further research will be required to explore this possibility.

It has long been known that inorganic nitrogen source strongly influences the uptake of K^+ by plant roots, particularly in the high-affinity range, over which K^+ uptake is inhibited by NH_4^+ (Vale *et al.*, 1988a; Santa-María *et al.*, 2000; Britto & Kronzucker, 2002; Szczerba *et al.*, 2008). In addition, it is known that the co-presence of NO_3^- and K^+ enhances the uptake of both ions (Blevins *et al.*, 1974; Vale *et al.*, 1988b). However, the impact of different N sources on root K^+ efflux has been unexplored, apart from a few studies conducted under steady-state conditions (Rygiewicz & Bledsoe, 1986; Kronzucker *et al.*, 2003; Szczerba *et al.*, 2006). In the present study, we found that changing N source and strength during tracer elution from $^{42}K^+$-labelled barley roots had pronounced effects on the pattern of efflux (Fig. 3). In particular, the addition of 10 mM NH_4^+ alone, or 10 mM NH_4NO_3, accelerated $^{42}K^+$ efflux at both 0.1 mM $[K^+]_{ext}$ and 1.0 mM $[K^+]_{ext}$. Indeed, these were the only treatments that affected efflux at 1.0 mM. These results suggest, along with the effects brought about by the co-provision, with NH_4^+, of Cs^+, TEA^+ (Fig. 3a,b) and elevated K^+ and Rb^+ (not shown), that the introduction of high NH_4^+ to high-K^+ plants triggers the activity of a physiologically responsive, channel-mediated efflux. The incomplete inhibition of NH_4^+-enhanced $^{42}K^+$ efflux by these agents, relative to the NH_4^+-free conditions (Fig. 1), might be explained by the opening,

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by NH_4^+, of additional channels with different pharmacological profiles, but which are silent under NH_4^+-free conditions; this idea warrants further investigation.

The simplest explanation for the general NH_4^+-stimulated elevation of $^{42}K^+$ efflux, under both low-K^+ and high-K^+ conditions, is the depolarization of the plasma membrane of root cells, which is known to occur when ammonium is applied (Ullrich *et al.*, 1984; Ayling, 1993; Wang *et al.*, 1994; Nocito *et al.*, 2002). This would shift the thermodynamic condition in favour of passive K^+ efflux in the case of the high-K^+ plants, and enhance the already favourable conditions driving K^+ efflux in the low-K^+ plants. In support of this idea is the data in Fig. 5, in which vanadate and cyanide, both known to rapidly depolarize the plasma membrane (Lew, 1991), enhanced the efflux of $^{42}K^+$, while treatment with bicarbonate, which hyperpolarizes the membrane (Poole, 1969), reduced $^{42}K^+$ efflux. These effects were most pronounced in low-K^+ conditions, but were also seen in the mild stimulation of efflux by vanadate and cyanide in high-K^+ conditions. The state of membrane electrical polarization as an explanation for changes in K^+ efflux was also the explanation put forward by Nocito *et al.* (2002), who showed that the cations Rb^+, Cs^+ and NH_4^+ all penetrated into cells of excised maize roots, causing depolarization and inducing K^+ efflux, while Li^+ and Na^+ neither penetrated nor depolarized cells, nor induced K^+ efflux. This attractive explanation, however, may be incomplete, as elevated nitrate in the present study reduced $^{42}K^+$ efflux in low-K^+ plants, despite the fact that nitrate application also results in transient membrane depolarization, because of its symport mechanism with an excess of H^+ ions (Glass *et al.*, 1992).

The stimulation of K^+ efflux by high NH_4^+, and its partial stimulation by NH_4NO_3, were mirrored in the repression of K^+ influx by these same N sources (Fig. 4). Thus, the homeostasis of K^+ in barley plants, in terms of both its acquisition and loss, appears to be optimized by NO_3^- provision and compromised, in two important ways, by the presence of NH_4^+, which greatly stimulates efflux of K^+ and inhibits its influx. This pattern is consistent with the sensitivity of plant species such as barley to NH_4^+ toxicity (Britto & Kronzucker, 2002). Indeed, the potency of NH_4^+ to accelerate K^+ efflux is greater than that of Na^+, which, while being an important cause of stress in barley, had no effect on K^+ efflux when applied at the same concentration as NH_4^+ (10 mM). By contrast, the threshold for a stimulatory effect of Na^+ was > 25 mM. These differential effects warrant further investigation, particularly with regard to their agronomic consequences.

We have demonstrated here that radiotracer analysis can reveal subtle and complex aspects of K^+ transport in intact plants, and may thus help refine the search for molecular mechanisms underlying this process. The study provides a framework of characteristics, including pharmacological and nutritional profiles, by which discoveries made at other levels of organization (e.g. molecular) might be gauged. In addition, our study provides new physiological evidence for the classic two-system model of K^+ acquisition, and enables a grasp of the elusive efflux component of this acquisition process. Lastly, we have shown that, in addition to the pronounced effects that nitrogen source has on the influx of K^+, K^+ efflux plays an important role in cellular and whole-root K^+ utilization efficiency under changing nitrogen sources.

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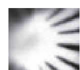

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