New Phytologist

Research

^{42}K analysis of sodium-induced potassium efflux in barley: mechanism and relevance to salt tolerance

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Received: *28 October 2009*
Accepted: *28 November 2009*

New Phytologist (2010) **186**: 373–384
doi: 10.1111/j.1469-8137.2009.03169.x

Key words: barley (*Hordeum vulgare*), efflux, ion channels, membrane integrity, potassium transport, salt stress.

Summary

- Stimulation of potassium (K^+) efflux by sodium (Na^+) has been the subject of much recent attention, and its mechanism has been attributed to the activities of specific classes of ion channels.
- The short-lived radiotracer $^{42}K^+$ was used to test this attribution, via unidirectional K^+-flux analysis at the root plasma membrane of intact barley (*Hordeum vulgare*), in response to NaCl, KCl, NH_4Cl and mannitol, and to channel inhibitors.
- Unidirectional K^+ efflux was strongly stimulated by NaCl, and K^+ influx strongly suppressed. Both effects were ameliorated by elevated calcium (Ca^{2+}). As well, K^+ efflux was strongly stimulated by KCl, NH_4Cl and mannitol , and NaCl also stimulated $^{13}NH_4^+$ efflux. The Na^+-stimulated K^+ efflux was insensitive to cesium (Cs^+) and pH 4.2, weakly sensitive to the K^+-channel blocker tetraethylammonium (TEA^+) and quinine, and moderately sensitive to zinc (Zn^{2+}) and lanthanum (La^{3+}).
- We conclude that the stimulated efflux is: specific neither to Na^+ as effector nor K^+ as target; composed of fluxes from both cytosol and vacuole; mediated neither by outwardly-rectifying K^+ channels nor nonselective cation channels; attributable, alternatively, to membrane disintegration brought about by ionic and osmotic components; of limited long-term significance, unlike the suppression of K^+ influx by Na^+, which is a greater threat to K^+ homeostasis under salt stress.

Introduction

Salinity, particularly in the form of dissolved NaCl, is a widespread environmental problem, affecting nearly a billion hectares of land on earth, including > 20% of irrigated agricultural areas (Munns, 2005; Ottow *et al.*, 2005). One of the most commonly observed consequences of NaCl stress on glycophytic plants is a reduction in the tissue content of essential nutrient ions, notably potassium (K^+) (Helal & Mengel, 1979; Fricke *et al.*, 1996). This reduction can be caused by the inhibition, by sodium (Na^+), of K^+ influx into the cell (Kochian *et al.*, 1985; Kronzucker *et al.*, 2006, 2008), but another potentially important cause is the stimulation, by Na^+, of K^+ efflux from the cell. This enhanced efflux has been observed many times, both by direct observation of Na^+-stimulated K^+ release from plant tissues (Nassery, 1975, 1979; Wainwright, 1980; Lynch & Läuchli, 1984; Cramer *et al.*, 1985) and algal cells (Katsuhara & Tazawa, 1986), and more indirectly through conductivity analysis of electrolyte release (Lutts *et al.*, 1996; Kaya *et al.*, 2002; Tuna *et al.*, 2007). The agronomic importance of Na^+-stimulated K^+ release from plant cells is suggested by the inverse relationship between the extent of release and the salt tolerance of a species or cultivar, which may prove to be a valuable basis for crop screening (Nassery, 1979; Chen *et al.*, 2005; but see also Picchioni *et al.*, 1991; cf. Kinraide, 1999).

The mechanism(s) underlying this loss are poorly understood, but a substantial amount of recent intracellular and extracellular electrophysiological work (e.g. Shabala *et al.*, 2006) has led to a proposal that the phenomenon occurs through a combination of ion-channel activities and changes in the electrical potential gradient across the plasma membrane. To briefly summarize this view, a Na^+ challenge in the external medium is thought to cause roots to take up large quantities of the ion via nonselective cation channels (NSCCs), resulting in a strong electrical depolarization at the plasma membrane. Consequently, the role of K^+ in maintaining the cell's electrical potential across the plasma membrane comes into play, and voltage-regulated,

outwardly-rectifying K^+ channels (and/or outwardly-directed NSCCs) are theorized to open, resulting in K^+ release from the cell (Shabala *et al.*, 2006).

In the present study, we have conducted the first detailed examination of Na^+-stimulated K^+ efflux by use of radiotracers. The principal advantage of this method lies in its ability to identify unidirectional fluxes, in contrast to other methods (e.g. vibrating microelectrodes or chemical analyses) which can only be used to determine net fluxes (Britto & Kronzucker, 2003; see Discussion). Here, we have used tracers and channel-modifying chemical agents to test the proposal outlined above, against an alternative hypothesis that osmotic and membrane-disintegrating effects constitute the underlying mechanism of accelerated K^+ release. In addition, we have investigated short- and long-term effects of Na^+ on the unidirectional fluxes of K^+, and on tissue ion content, as well as the ionic specificity of the efflux-stimulation effect.

Materials and Methods

Plant culture

For all experiments, seeds of barley (*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 vessels containing aerated hydroponic growth medium (modified ¼-strength Johnson's solution, pH 6.3–6.5) for an additional 4 d. The solution was modified to provide three levels of calcium (Ca^{2+}, as $CaCl_2$): 0.1 mM, 1 mM, and 10 mM. Nitrogen (N) and K sources were NH_4NO_3 (0.5 mM) and K_2SO_4 (0.75 mM), except for plants in which NH_4^+ fluxes were measured; these plants were provided with $(NH_4)_2SO_4$ (5 mM) and K_2SO_4 (0.05 mM), to maximize internal NH_4^+ pools (Britto *et al.*, 2001; Szczerba *et al.*, 2008). Unless plants were grown under the steady-state condition of 160 mM NaCl, NaCl was not added to solutions until the time of experiment (day 7). Otherwise, plants were grown with 160 mM NaCl for steady-state measurements. Solutions were exchanged every 2 d to prevent nutrient depletion. 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 *c.* 70%.

Flux analysis

Details of each flux-measurement protocol are given in the following sections. The general features of protocols are as follows: replicates consisted of bundles of five 1-wk-old intact plants (except for those grown under 160 mM NaCl with low and intermediate Ca^{2+}; in this case, 15 plants were bundled because of low biomass), held together at the shoot base by a plastic collar. Plant bundling was prepared 1 d before experimentation. Roots of intact plants were loaded in complete nutrient solutions containing either the radiotracer ^{42}K ($t_{1/2}$ = 12.36 h; as K_2CO_3), provided by McMaster University Nuclear Reactor, in Hamilton (ON, Canada), or the radiotracer ^{13}N ($t_{1/2}$ = 9.98 min; as $^{13}NH_4^+$), provided by the CAMH cyclotron facility (University of Toronto, ON, Canada). 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 (Packard Instrument Co., Meriden, CT, USA)). For comparison charts of $^{42}K^+$ efflux, the specific activities of all replicates were normalized to 2×10^5 cpm μmol^{-1}.

Compartmental analysis for K^+ fluxes and pool sizes Compartmental analysis by tracer efflux was used to measure subcellular fluxes and compartmental concentrations of K^+, based upon a three-compartment model of surface film, cell wall, and cytosol as revealed by short-term labeling (briefly described here; for details see Pierce & Higinbotham, 1970; Walker & Pitman, 1976; Memon *et al.*, 1985; Lee & Clarkson, 1986; Siddiqi *et al.*, 1991; Kronzucker *et al.*, 1995, 2003). Labeling of plants via the roots took place for 1 h in radioactive nutrient solutions, which were chemically identical to growth solutions. Labeled seedlings were attached to plastic efflux funnels, and roots were eluted of radioactivity with a series of 13-ml aliquots of nonradioactive desorption solutions (identical to growth solutions in the steady-state runs; see below). The desorption series for K^+ fluxes was timed as follows, from first to final eluate: 15 s (four times), 20 s (three times), 30 s (twice), 40 s (once), 50 s (once), 1 min (23 times), 1.5 min (three times), 2 min (three times), 3 min (three times), 4 min (twice), and 5 min (once), for a total of 1 h of elution. Nonsteady-state experiments contained additional solutes (see the Results section for specific treatments) in the final 23 or 24 vials (applied at elution time t = 15.5 or 16.5 min).

Linear regression of the function $\log_e \Phi_{co(t)^*} = \log_e \Phi_{co(i)^*} - kt$, in which $\Phi_{co(t)^*}$ is tracer efflux at elution time t, $\Phi_{co(i)^*}$ is the initial tracer efflux, and k is the rate constant describing the exponential decline in tracer efflux, obtained from the slope of the rate of tracer release from the slowest-exchanging, cytosolic, compartment (Kronzucker *et al.*, 2003) was used to resolve unidirectional influx and efflux of K^+, net flux, and the size and turnover rate of the cytosolic K^+ pool. Unidirectional K^+ efflux was determined from $\Phi_{co(i)^*}$, divided by the specific activity of the cytosol (S_{cyt}) at the end of the labeling period; S_{cyt} was estimated by using external specific activity (S_o), labeling time t, and the rate constant k, which are related in the exponential rise func-

tion $S_{cyt} = S_o(1-e^{-kt})$ (Walker & Pitman, 1976). Net K^+ flux was found using total plant ^{42}K retention after desorption, and unidirectional K^+ influx was calculated from the sum of net flux and influx. Cytosolic $[K^+]$ ($[K^+]_{cyt}$) was determined using the flux turnover equation, $[K^+]_{cyt} = \Omega \Phi_{oc}/k$, where Ω is a proportionality constant correcting for the cytosolic volume being *c.* 5% of total tissue (Lee & Clarkson, 1986; Siddiqi *et al.*, 1991)

NH_4^+ efflux The NH_4^+ efflux experiments followed a protocol identical to the previous one with a few exceptions related to the much faster radioactive decay rate (9.98 min vs 12.36 h). The roots of intact plants were loaded for 30 min instead of 1 h. The desorption series for nitrogen fluxes was timed as follows: 15 s (four times), 20 s (three times), 30 s (twice), 40 s (once), 50 s (once), 1 min (five times), 1.25 min (once), 1.5 min (once), 1.75 min (once) and 2.0 min (eight times) for a total elution period of 30 min. Desorption solutions were identical to the growth solution for the first 17 vials, but 160 mM NaCl was added to the last nine vials (applied at $t = 13$ min). No steady-state experiments were carried out for NH_4^+ efflux and, thus, compartmental analysis was not undertaken with this procedure.

Short-term K^+ influx Short-term labeling with $^{42}K^+$ was used to study the effects of exogenously applied NaCl and Ca^{2+}on K^+ influx, under steady-state and nonsteady-state conditions (Szczerba *et al.*, 2008). For steady-state measurements, seedlings were grown as described earlier, but with 160 mM $[Na^+]_{ext}$ and either 0.1 or 1 mM Ca^{2+}. Bundles of seedlings were pre-equilibrated for 5 min in growth solution, then roots were immersed in labeling solution (identical to the growth solution, except that it contained $^{42}K^+$) for 5 min. Plants were then transferred to nonradioactive solution for 5 s to reduce tracer carry-over to the desorption solution, and finally desorbed for 5 min in fresh nutrient solution. All solutions were chemically identical to the growth medium. Nonsteady-state experiments were conducted in the same way, with some exceptions. Plants were grown with 1.5 mM $[K^+]_{ext}$, and 0.1 mM $[Ca^{2+}]_{ext}$, and the pre-equilibration, labeling, and desorption solutions were all different from the growth medium in that they contained 160 mM NaCl and one of three external $[Ca^{2+}]$ provisions (0.1, 1, or 10 mM).

Tissue analyses

K^+ content To measure tissue K^+ content of steady-state plants, roots of a bundle of 5 1-wk-old barley seedlings were first desorbed in 10 mM $CaSO_4$ for 5 min, to release extracellular K^+. Shoots and roots were then separated and weighed. Tissue was then oven-dried for 3 d at 85–90°C, and then reweighed. The dried tissue was pulverized, then digested with 30% HNO_3 for an additional 3 d. The K^+ concentrations in tissue digests were determined using a single-channel flame photometer (Digital Flame Analyzer model 2655-00; Cole-Parmer, Anjou, QC, Canada). Nonsteady-state plants were analysed for tissue K^+ content in a similar manner, except that the seedlings were subjected to salt stress for various periods of time (see the Results section) before analysis.

Tissue NH_4^+ content To measure tissue NH_4^+ content, barley seedlings were harvested and desorbed as described earlier. Roots were excised and weighed, then transferred to polyethylene plastic vials and frozen in liquid N_2 for storage at −80°C. Approximately 0.5 g of root tissue was homogenized under liquid N_2 using a mortar and pestle, followed by the addition of 6 ml of formic acid (10 mM) for NH_4^+ extraction. Subsamples (2 ml) of the homogenate were centrifuged at 17 000 × ***g*** at 2°C for 25 min then transferred to 2 ml polypropylene tubes. The resulting supernatant was analysed using the indophenol colorimetric (Berthelot) method to determine tissue NH_4^+ content, as described in detail elsewhere (Solorzano, 1969; Husted *et al.*, 2000). Briefly, three solutions were combined with 1.6 ml of tissue extract: 200 μl of 11 mM phenol in 95% (v : v) ethanol; 200 μl of 1.7 mM sodium nitroprusside (prepared weekly); and 500 μl of solution containing 100 ml of 0.68 M trisodium citrate in 0.25 M NaOH with 25 ml of commercial strength (11%) sodium hypochlorite. The color was allowed to develop for 60 min at room temperature (25°C) in the dark, and sample absorbance was measured at 640 nm).

Results

Fig. 1 shows the changing rate of $^{42}K^+$ efflux (Lee & Clarkson, 1986) from labeled roots of intact barley seedlings, before and following the imposition of NaCl treatments. A substantial, concentration-dependent stimulation of $^{42}K^+$-labeled efflux was observed in response to three levels of salt stress (40, 80 and 160 mM NaCl) that were imposed midway through the experiment, once the cytosolic phase of efflux was well established (Fig. 1a; Kronzucker *et al.*, 1995, 2003). After *c.* 45 min, the stimulation of $^{42}K^+$ efflux responded roughly linearly to the NaCl concentration. Increased concentrations of $CaCl_2$, applied at the time of salt stress, strongly reduced the stimulation of K^+ efflux (Fig. 1b), with 10 mM Ca^{2+} lowering K^+ efflux to control (unstressed) levels within as little as 20 min. By contrast, in the absence of NaCl stress, a 100-fold variation in Ca^{2+}supply ($[Ca^{2+}]_{ext}$) had no discernable effect on K^+ efflux (Fig. 1b).

In contrast to the elevated efflux in Fig. 1 (but acting upon the net flux in the same way), a strong, rapid inhibition of K^+ influx was seen in the presence of 160 mM Na^+,

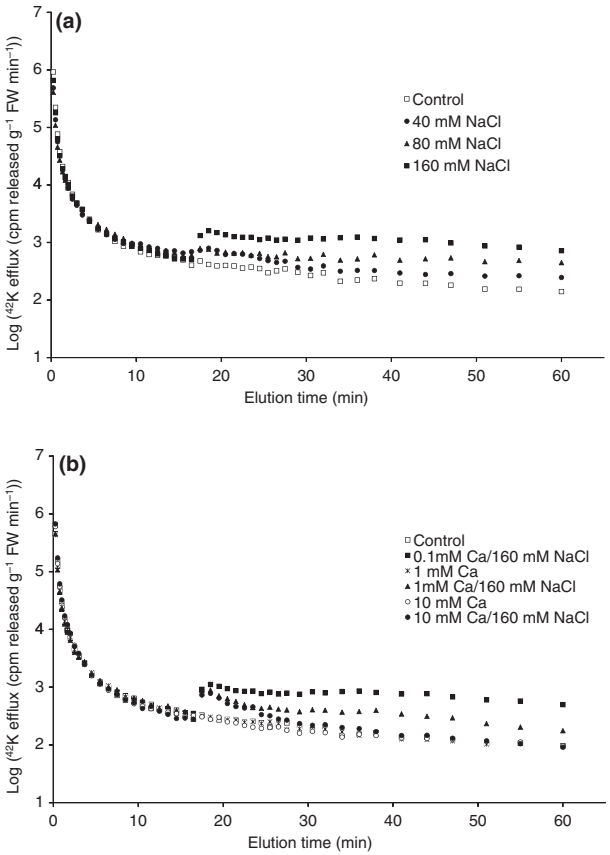

Fig. 1 Response of $^{42}K^+$ efflux from roots of intact barley seedlings to sudden provision (at elution time = 15.5 min) of (a) NaCl alone, (b) Ca^{2+}alone, and NaCl with Ca^{2+}.

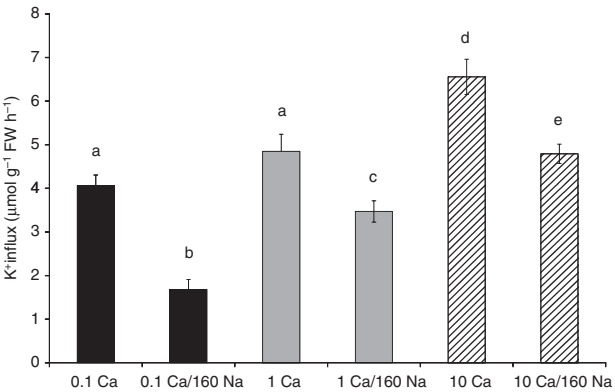

Fig. 2 Short-term (5 min) $^{42}K^+$ influx measurements into roots of intact barley seedlings, in response to Na^+ challenge and changes in Ca^{2+}provision. Plants were grown on 0.1 mM $Ca^{2+}$and 1.5 mM K^+, in the absence of NaCl. Letters indicate significantly different groups ($P \leq 0.05$); error bars indicate ± SE of the mean.

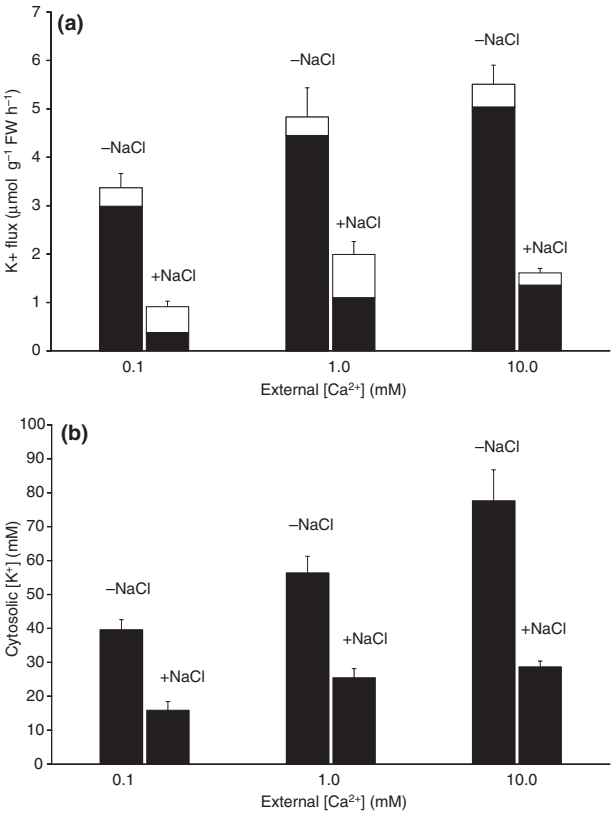

Fig. 3 (a) Steady-state K^+ flux (open bars, efflux; closed bars, net flux) and (b) cytosolic pool sizes, in roots of intact barley seedlings, grown with 1.5 mM K^+ with or without 160 mM NaCl, and at various levels of Ca^{2+}. Error bars indicate ± SE of the mean (of influx, in (a)).

as determined by short-term influx measurements (Fig. 2). This effect was also substantially reduced by increasing $[Ca^{2+}]_{ext}$. However, the inhibition pattern was complicated by the observation that increased Ca^{2+}also stimulated K^+ influx in the absence of NaCl. Nevertheless, the per cent suppression of K^+ influx by NaCl tended to decrease as $[Ca^{2+}]_{ext}$ was increased from 0.1 to 1 to 10 mM (by 59%, 28%, and 27%, respectively), and 10 mM $[Ca^{2+}]_{ext}$ (with 160 mM NaCl) restored K^+ influx to control levels (i.e. 0.1 mM $[Ca^{2+}]_{ext}$ without NaCl treatment).

Steady-state unidirectional flux measurements were made, by use of compartmental analysis, on plants grown for 4 d on 160 mM NaCl. These experiments showed that the effects of Na^+ on K^+ fluxes are long-lasting (Fig. 3a), with plants taking up K^+ at rates even lower than seen with short-term NaCl treatment (Fig. 2). When $[Ca^{2+}]_{ext}$ was low (0.1 mM), K^+ influx was approximately one-third of that in plants grown under no salt stress (Fig. 3a). These absolute and relative rates were confirmed by direct, short-term influx measurements (not shown). Unidirectional K^+ efflux also remained elevated under long-term NaCl provision, except at the highest $[Ca^{2+}]_{ext}$. The enhanced K^+ influx seen in the short term with 10 mM $[Ca^{2+}]_{ext}$ under salinity, relative to the salt-free, low-Ca^{2+}controls (Fig. 2), however, was not found under steady-state conditions, but the net flux of K^+ was slightly improved by increasing $[Ca^{2+}]_{ext}$ from 0.1 mM to 1 or 10 mM (Fig. 3a).

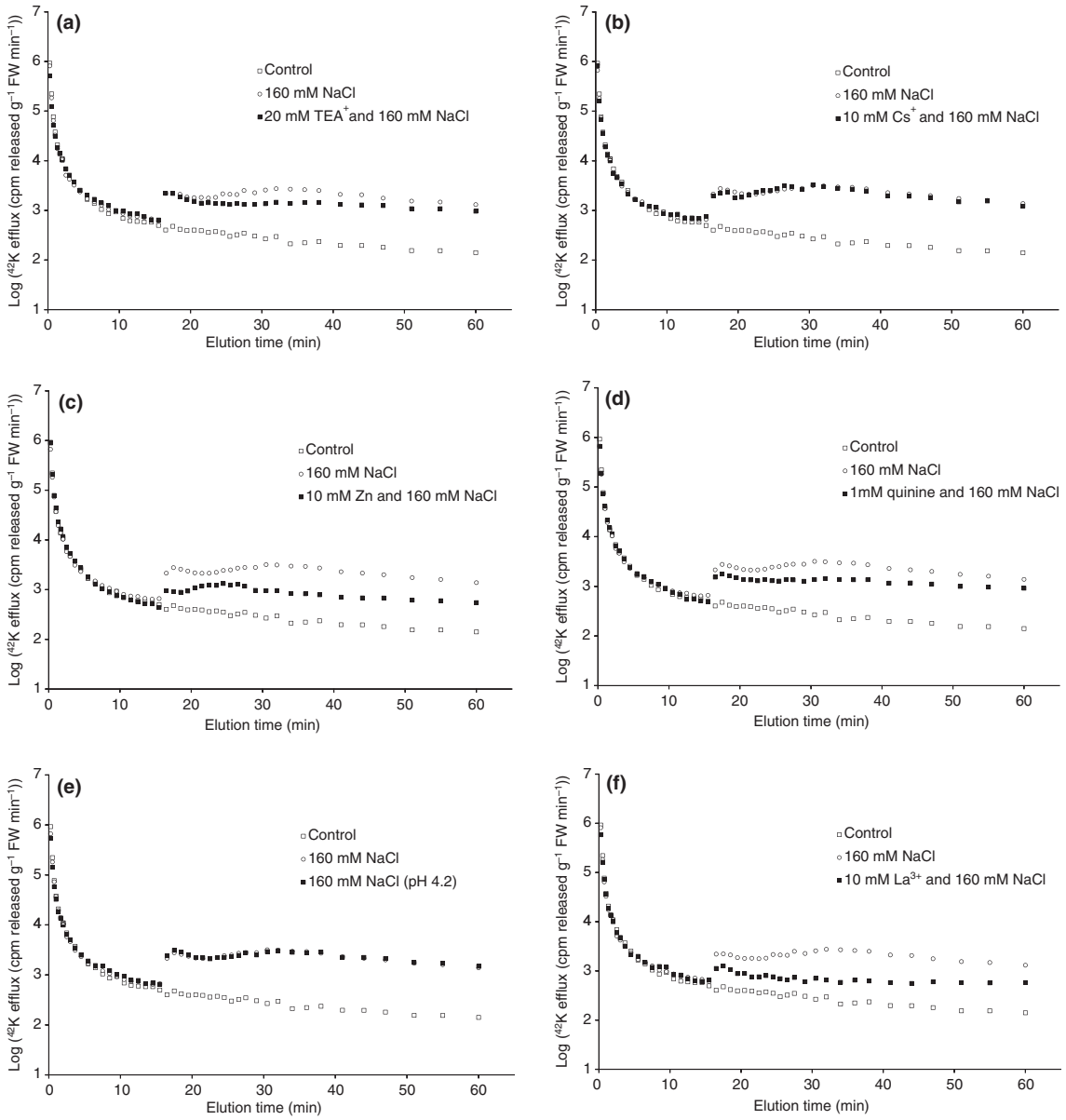

Fig. 4 Changes in $^{42}K^+$ efflux from roots of intact barley seedlings (labeled at 1.5 mM external $[K^+]$), in response to NaCl alone, or in combination with a range of channel inhibitors, applied at concentrations shown in individual graphs. NaCl and inhibitors (when present) were applied at t = 15.5 min from the start of elution. See text for details of each treatment.

In parallel with the changes in influx, the steady-state cytosolic concentrations of K^+ ($[K^+]_{cyt}$) were also suppressed by NaCl stress (Fig. 3b). These concentrations, determined by compartmental analysis, are in excellent agreement with a host of other methods (Kronzucker *et al.*, 2003), as is the suppressive effect Na^+ (Kronzucker *et al.*, 2008). Increasing $[Ca^{2+}]_{ext}$ from 0.1 mM to 1 mM brought about a slight increase in $[K^+]_{cyt}$. In the absence of NaCl, the influx, net flux, and cytosolic pools of K^+ all increased with increasing Ca^{2+}supply (Fig. 3a,b).

We tested a range of channel inhibitors on K^+ efflux, applied at the time that NaCl stress was imposed (Fig. 4). Of these, only the NSCC blocker zinc (Zn^{2+}, Fig. 4c) and the NSCC and K^+-channel blocker lanthanum (La^{3+}, Fig. 4f) substantially reduced the Na^+-stimulation of K^+ efflux, although at 10 mM neither of these agents was as effective as Ca^{2+}, which completely suppressed the stimulation within 20 min (Fig. 1b). Application of the K^+-channel blocker tetraethylammonium (TEA^+, Fig. 4a) and the NSCC blocker quinine (Fig. 4d) brought about slight reductions of the stimulated efflux, while the K^+ channel blocker cesium (Cs^+, Fig. 4b) was completely ineffective in changing the pattern of K^+ loss. In addition, increasing the external $[H^+]$ to a pH of 4.2, known to inhibit NSCCs

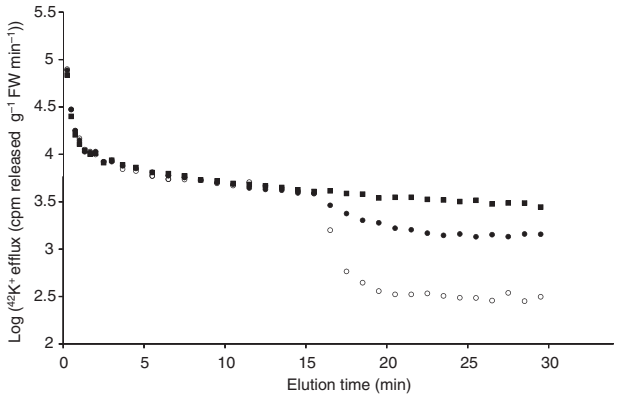

Fig. 5 Response of $^{42}K^+$ efflux from roots of intact barley seedlings to the K^+-channel blockers tetraethylammonium (TEA^+) and Cs^+. Closed squares, control; closed circles, 10 mM TEA^+; open circles, 10 mM Cs^+. External [K^+] was 0.1 mM to establish conditions for a passive outward K^+ flux.

(see the Discussion section) had no discernable effect (Fig. 4e).

The effects of the K^+-channel-blocking agents TEA^+ and Cs^+ on the steady-state efflux of K^+ were also examined under low-K^+ (0.1 mM) conditions, in the absence of salt stress (Fig. 5). These experiments were conducted to demonstrate the efficacy of the channel blockers, under conditions where K^+ efflux is known to be passive (Kochian & Lucas, 1993; Maathuis & Sanders, 1993, 1996; Szczerba *et al.*, 2006). Both agents were found to substantially inhibit K^+ efflux immediately upon their application at 10 mM, unlike elevated Ca^{2+}which had no such effect (Fig. 1b).

Fig. 6 shows that NH_4^+, mannitol and K^+ itself can stimulate K^+ efflux. In particular, the K^+ efflux pattern observed after KCl application was virtually identical to that observed after NaCl application, and K^+ efflux responded to KCl in a concentration-dependent manner (Fig. 6a). The 160 mM NH_4Cl treatment was nearly as effective as 160 mM NaCl (Fig. 6b), while an iso-osmotic concentration of mannitol (320 mM) transiently stimulated K^+ efflux to a similar extent before approximating control levels after 45 min of treatment (Fig. 6c). Application of 160 mM mannitol also brought about a mild, short-lived, stimulation of K^+ efflux (Fig. 6c).

Tissue K^+ analysis (Fig. 7a) shows that the unidirectional efflux stimulated by NaCl was a net efflux, entailing rapid and massive loss of potassium from the root over the first 2 h (at a rate of *c.* 25 μmol g^{-1} FW h^{-1}), with a lesser depletion from the shoot (*c.* 12 μmol g^{-1} FW h^{-1}). Application of 1 mM Ca^{2+}curtailed the NaCl-induced loss of K^+ from roots by *c.* 50% over 24 h (not shown). After the first 2 h of NaCl treatment, net K^+ loss from both organs was substantially reduced (Fig. 7a), but plants after 4 d of NaCl treatment had even lower K^+ status, particularly in the root,

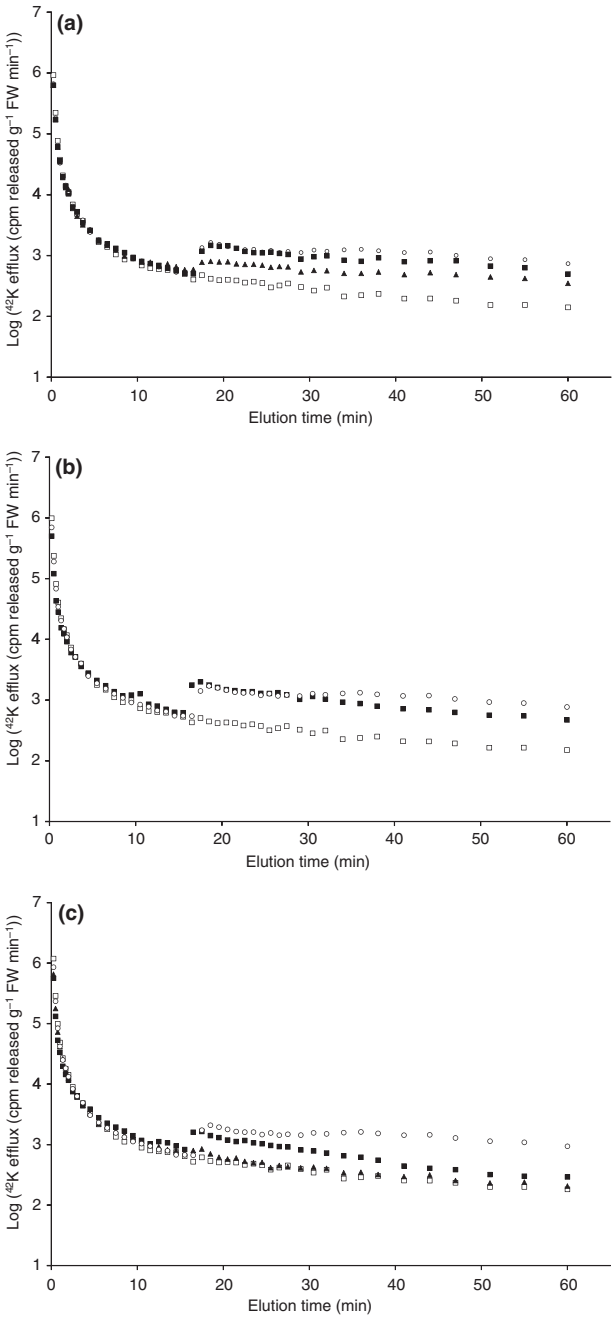

Fig. 6 Changes in $^{42}K^+$ efflux from roots of intact barley seedlings (labeled at 1.5 mM external [K^+]), in response to (a) KCl, (b) NH_4Cl, (c) mannitol. For comparison, NaCl-enhanced efflux is overlaid on each plot.

which had only 5% as much K^+ per gram compared with control roots (Fig. 7b).

Using the short-lived radiotracer ^{13}N, we found that NaCl provision could also immediately stimulate NH_4^+ efflux (Fig. 8) in plants grown on 10 mM NH_4^+. This enhancement was less than what was seen with K^+ efflux. In parallel, tissue NH_4^+ analysis showed that longer-term

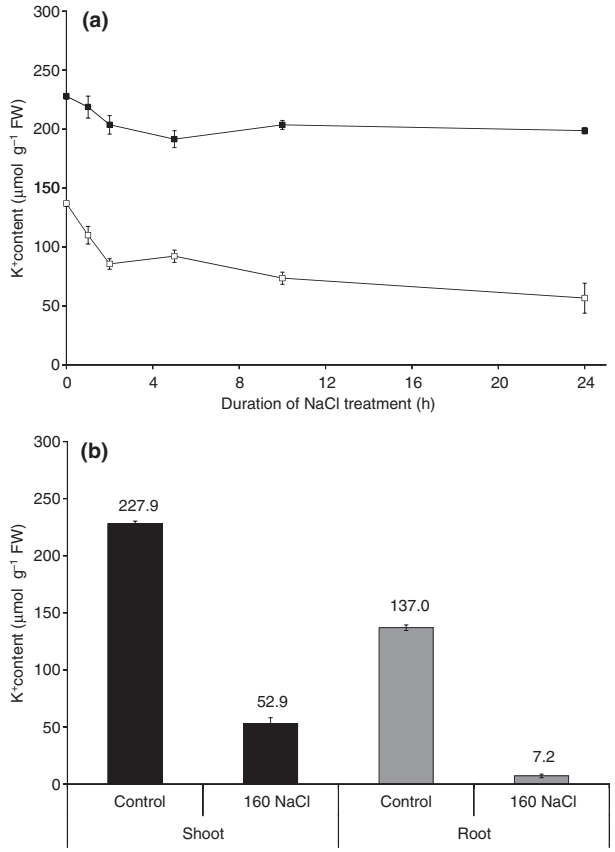

Fig. 7 Tissue K^+ content, as influenced by elevated external Na^+, over (a) 24 h (barley shoot, closed squares; root, open squares), (b) in the steady state, after 4 d of growth on high NaCl), error bars indicate ± SE of the mean.

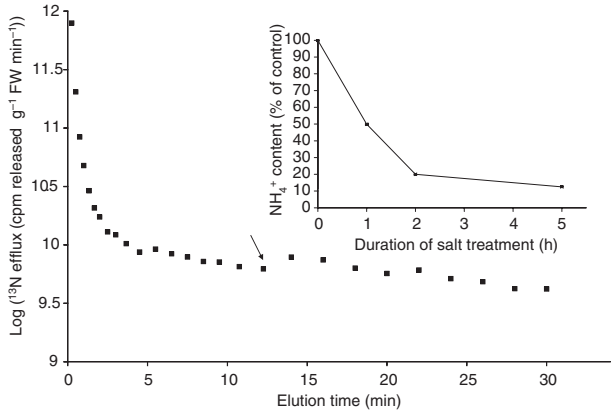

Fig. 8 Response of ^{13}N efflux from roots of intact barley seedlings to sudden provision of 160 mM NaCl (arrow). Inset: changes in root tissue NH_4^+ following salt treatment. Standard errors were within 5% of the mean.

NaCl application brought about almost complete NH_4^+ loss from root tissue, at a rate of 1–1.5 µmol g^{-1} FW h^{-1} over the first 2 h (Fig. 8, inset). The pattern in the decline of tissue NH_4^+ resembled that of tissue K^+ decline in that the majority of loss occurred within 2 h.

Discussion

The present demonstration is one of the few detailed studies that use radiotracers in the context of Na^+-stimulated K^+ efflux. In addition to being the only available means by which unidirectional fluxes can be quantified, the use of tracers offers several other advantages over net flux measurement by use of extracellular microelectrodes, the leading method by which this phenomenon is currently under investigation. First, it provides a comprehensive view of ion fluxes for the whole root, rather than individual microscopic zones that can vary substantially in their transport characteristics (Garnett *et al.*, 2001; Vallejo *et al.*, 2005). This is important if one seeks to gauge the impact of Na^+-stimulated K^+ release upon the K economy of the whole plant, and, consequently, its performance in the field. Second, tracer analysis as presented here entails no problem relating to ion selectivity, unlike with the use of electrode cocktails (Cuin *et al.*, 1999; Britto & Kronzucker, 2003). Third, it allows for very sensitive measurements to be made even in the presence of high concentrations of the traced ion. With the use of microelectrodes or more traditional depletion experiments, this background interference issue often requires that the external concentration of the ion of interest is lowered well below that provided during growth (Shabala *et al.*, 2006). Thus, the starting condition for measurement is often an aberrant one, entailing net nutrient loss from the plant, before any experimental treatment (Shabala *et al.*, 2005, 2006; Sun *et al.*, 2009). In addition, extracellular microelectrodes sometimes yield unexplained anomalies, such as a large and sustained net efflux of Na^+, paradoxically suggesting that the plant cumulatively releases more Na^+ than it takes up (e.g. Fig. 5 in Shabala *et al.*, 2006). The consequences of this net efflux on membrane electrical polarization also require consideration.

In the present study, unidirectional K^+ efflux (Fig. 1a) and influx (Fig. 2) responded immediately to the imposition of salt stress, an enhancement and a diminishment, respectively, that were both strongly attenuated by increased external Ca^{2+} (Figs 1b,2). Steady-state K^+ influx and cytosolic K^+ concentrations averaged throughout the root, were also reduced by salt stress, and, again, Ca^{2+}ameliorated these effects (Fig. 3). Together, these data show that Na^+ disrupts K^+ homeostasis by increased loss, reduced uptake, and reduced cytosolic pools, of K^+, and that improved Ca^{2+}supply can significantly counteract all three effects, underscoring the crucial role of Ca^{2+}in protecting plants from salt stress (Marschner, 1995; Cramer *et al.*, 1985; Rengel, 1992).

How does Ca^{2+}prevent massive K^+ release from the cell under salinity stress? Shabala *et al.* (2006) have argued, on

the basis of intra- and extra-cellular microelectrode measurements, that K^+ loss in *Arabidopsis* root and mesophyll tissue is initially brought about by a large, depolarizing inward flux of Na^+ across the plasma membrane. This triggers depolarization-activated channels (DAPCs) and/or NSCCs which are proposed to mediate the observed enhancement of K^+ efflux. Elevated $Ca^{2+}$is proposed to counteract this process via its channel-blocking characteristics, inhibiting the influx of Na^+ through NSCCs, or the efflux of K^+ through DAPCs/NSCCs, or both.

While many reports using patch-clamp methodology have indeed shown that Na^+ currents into the cell can be blocked by elevated Ca^{2+}, these blocks are usually only partial (Roberts & Tester, 1997; Tyerman *et al.*, 1997; Davenport & Tester, 2000; Demidchik & Tester, 2002). In addition, radiotracer measurements have shown that, in many instances, Ca^{2+}addition does not abolish unidirectional Na^+ influx but permits a substantial Ca^{2+}-independent flux to proceed (Epstein, 1961; Rains & Epstein, 1967; Jacoby & Hanson, 1985; Cramer *et al.*, 1985, 1987, 1989; Davenport *et al.*, 1997; Essah *et al.*, 2003). In our previous work, Ca^{2+}provision had no effect whatsoever on Na^+ influx (Malagoli *et al.*, 2008); similarly, Cramer *et al.* (1987) concluded that Ca^{2+}had little or no influence on the low-affinity Na^+ transport system in cotton seedlings, which catalysed the majority of the influx. In a study on *Arabidopsis*, elevated $Ca^{2+}$was observed to increase Na^+ influx when it had been partially inhibited by other agents (Essah *et al.*, 2003). Lastly, in a recent review (Zhang *et al.*, 2009), it was pointed out that, in most soils, $Ca^{2+}$is sufficiently high as to make the Ca^{2+}-inhibitable Na^+ flux (i.e. through NSCCs) largely irrelevant to most field conditions, including, in particular, saline soils (Zidan *et al.*, 1991; Garciadeblas *et al.*, 2003). This would lessen the agronomic importance of its protective effects.

The blockade of K^+ efflux by extracellular $Ca^{2+}$is also not well established in the electrophysiological literature, from which examples can be readily drawn of Ca^{2+}-independent K^+ flux from the cell (Vogelzang & Prins, 1994; Roberts & Tester, 1995; White & Lemtiri-Chlieh, 1995). The lack of a strong effect of Ca^{2+}on K^+ efflux in a whole-root context is also apparent in the present study, both in the short term (Fig. 1b), in which a 100-fold variation in Ca^{2+}showed no change in K^+ efflux when NaCl was absent, and under steady-state conditions (Fig. 3a). Moreover, under NaCl stress, our tracer efflux plots still show substantial $^{42}K^+$ release at elevated Ca^{2+}, which in no case was reduced below control levels (Fig. 1).

Taken together, the present results, and the precedents cited, cast doubt on the recent proposal (Shabala *et al.*, 2006) that Na^+-stimulated K^+ efflux is essentially a channel-mediated process. At the very least, it does not appear to be a universal explanation for an apparently ubiquitous phenomenon. In addition, crucial to the channel mediation of these fluxes is a rapid depolarization of the plasma-membrane electrical potential by Na^+, which, however, is not always observed (Bowling & Ansari, 1971, 1972; Cheeseman, 1982; Nocito *et al.*, 2002).

Other results obtained in the present study are also at odds with this interpretation. $^{42}K^+$ experiments conducted with a range of channel inhibitors show that they have rather limited effects on salt-stimulated K^+ efflux (Fig. 4). In particular, the K^+-channel blockers TEA^+, Cs^+, and the NSCC blocker Zn^{2+}, changed the pattern of efflux very little, whereas the nonselective cation channel blocker quinine, the broad spectrum blocker La^{3+}, as well as low external pH (4.2), which has been shown to reduce NSCC-catalysed Na^+ fluxes (Demidchik & Tester, 2002), also had little effect. By contrast, in the absence of salt stress, and under conditions where K^+ efflux is passive and probably channel-mediated (0.1 mM $[K^+]_{ext}$), Cs^+ caused a pronounced inhibition of steady-state K^+ efflux, as did TEA^+, but to a lesser extent (Fig. 5). This indicates that, with our experimental system, we can indeed measure such effects, where present. The relative effectiveness seen with these channel-blocking agents in the absence of NaCl is in agreement with a large body of electrophysiological studies (for a review see White & Broadley, 2000).

An alternative explanation for the phenomenon was provided by Cramer *et al.* (1985), who interpreted the stimulation of K^+ efflux by sodium as an outcome of the displacement of $Ca^{2+}$from the plasma membrane, resulting in a loss of structural integrity of the membrane and an increase in its leakiness (Frota & O'Leary, 1973; Lynch *et al.*, 1987; Kinraide, 1999; Rengel, 1992; for evidence of strong competition between Na^+ and $Ca^{2+}$for binding to the cell wall see Stassart *et al.*, 1981). This interpretation explains why the efflux-acceleration effect can be ameliorated by increased Ca^{2+}provision, and is supported by extensive research on the critical involvement of Ca^{2+}in membrane stability and permeability (Marinos, 1962; Gary-Bobo, 1970; Clarkson, 1974; Mansour, 1997; Hepler, 2005; Rengel, 1992; Van Steveninck, 1965). Indeed, such effects of Ca^{2+}can be observed even in simple synthetic membranes of cephalin or lecithin, free of proteinaceous transporters (Gary-Bobo, 1970; Levine *et al.*, 1973). The 'classical' explanation of the role of Ca^{2+}in preventing or reducing Na^+-stimulated K^+ efflux by increasing membrane stability may also help explain the effects of $Zn^{2+}$and La^{3+}shown in Fig. 4(c,f). Several studies have shown that these ions can mimic $Ca^{2+}$with respect to its membrane-stabilizing characteristics, including improving the membrane's ability to restrict K^+ loss (Poovaiah & Leopold, 1976; Pinton *et al.*, 1993; Cakmak & Marschner, 1988). In one study on membrane permeability effects of polyvalent cations, it was concluded that La^{3+}can indeed be more effective than Ca^{2+}in preventing membrane leakiness to solutes (Poovaiah & Leopold, 1976).

In the present work, the stress counteracted by $Ca^{2+}$and other polyvalent cations is clearly not ion-specific. As shown in Fig. 6, both NH_4^+ and K^+ itself can produce enhancements of unidirectional K^+ efflux that are, at least initially, indistinguishable from the effect produced by equimolar Na^+. Subsequent small deviations from the Na^+-induced efflux trace may reflect a stronger displacement of $Ca^{2+}$for binding sites by Na^+, relative to the other ions (Stassart *et al.*, 1981). Particularly interesting is the stimulation by external K^+ of its own efflux from the cell (Fig. 6a); in this situation, even a dramatic depolarization of the membrane by K^+ influx is highly unlikely to shift the electrochemical potential gradient in favour of passive K^+ efflux; given a typical cytosolic [K^+] of *c.* 100 mM and external [K^+] of 80 or 160 mM (Kochian & Lucas, 1993; Maathuis & Sanders, 1993; Walker *et al.*, 1996; Fig. 3b). This result is further evidence to support the idea that the stimulated efflux of K^+ is not primarily channel-mediated, as this would require an outwardly directed gradient to sustain a net efflux, but can instead be attributed to disruptions in membrane integrity.

The observation that mannitol induces K^+ efflux (Fig. 6c) also strongly suggests that osmotic stresses are at least partially responsible for the observed enhancements of K^+ efflux. While this finding contradicts that of Shabala *et al.* (2006), who, it should be noted, used mannitol at concentrations hypo-osmotic to comparative Na^+ treatments, it is in agreement with many other studies showing increased K^+ efflux, or decreased K^+ retention, upon application of nonionic osmolytes (Sutcliffe, 1954; Greenway *et al.*, 1968; Dessimoni Pinto & Flowers, 1970; Smith *et al.*, 1973; Nassery, 1975, 1979; Cramer *et al.*, 1985). However, in the present study, mannitol was not as effective as NaCl in sustaining the stimulated K^+ efflux (as also seen by Nassery, 1975, 1979), indicating that there may be both osmotic and ionic components to the stimulatory stress, just as there are both osmotic and ionic components responsible for salt injury to plants (Munns & Tester, 2008). The osmotic component of the efflux-stimulating effect is likely to be related to membrane disintegrity caused by osmotically driven water loss from the cell. Indeed, Sutcliffe (1954) found that K^+ loss from osmotically stressed beetroot discs only occurred once the osmotic potential of the medium was more negative than that of the tissue (i.e. once 'incipient plasmolysis' had been achieved). From this point of view, the slight protection against K^+ loss afforded by treatment with TEA^+ in the present study, and the more pronounced effect of TEA^+ found by Shabala *et al.* (2006), might be explained by its blockage of aquaporins (Detmers *et al.*, 2006) and a subsequent reduction of cellular dehydration – an alternative to the explanation that TEA^+ may block channel-meditated K^+ efflux (Shabala *et al.*, 2006). It is instructive in this context to examine Fig. 5, which shows that K^+-channel inhibition by TEA^+ is only partial (also White & Broadley, 2000), compared with the effect of Cs^+ which, nevertheless, had no effect on the Na^+-stimulated efflux.

Superimposed upon the osmotic stress appears to be an ionic stress which sustains the enhancement of K^+ efflux above that brought about by mannitol. As discussed earlier, this may be caused by the loss of Ca^{2+}associated with the plasma membrane by ion exchange with elevated amounts of external cations, which leads to greater compromise of membrane integrity. However, it must be reiterated that these ionic effects are not specific to Na^+, but can be brought about by NH_4^+ or K^+ itself (Fig. 6; Okamura & Wada, 1984). The efflux of K^+ is not the only process that is affected: we have found that NaCl provision also accelerates the efflux of NH_4^+, as traced by the short-lived radioisotope ^{13}N (Fig. 8). This increase, however, was not as pronounced as the efflux of K^+, possibly because NH_4^+ efflux under these conditions (10 mM external [NH_4^+]) is known to already be extremely high in barley roots, nearly equalling the high values of NH_4^+ influx in a futile transport cycle (Britto *et al.*, 2001). When examined over a longer time-scale, and at the level of tissue ion content, it can be seen that NH_4^+ is readily lost from the root (Fig. 8, inset), in a pattern resembling that of K^+ loss (Fig. 7a). This suggests that, in addition to accelerating NH_4^+ efflux, Na^+ may suppress the influx of NH_4^+. The losses of NH_4^+ and K^+ are in agreement with other studies showing that NaCl treatment enhances the release of a wide range of materials from the plant cell, including chloride (Sun *et al.*, 2009), ureides (Mansour, 1995), surface proteins (Maas *et al.*, 1979) and UV-absorbing compounds, including nucleotides, phenylpropanoids and flavonoids (Rauser & Hanson, 1966; Leopold & Willing, 1984; Redmann *et al.*, 1986; Picchioni *et al.*, 1991). Moreover, a similarly wide range of materials has also been shown to be released from plant cells in response to nonionic osmotica or drought stresses (Greenway *et al.*, 1968; Resnik & Flowers, 1971; Krishnamani *et al.*, 1984). In some of these studies, additional Ca^{2+}provision was shown to moderate these diverse losses (Rauser & Hanson, 1966; Leopold & Willing, 1984; Picchioni *et al.*, 1991; Mansour, 1995). In summary, the likelihood is low that all of these simultaneously occurring fluxes are mediated by ion channels; a more parsimonious explanation is that a generic, calcium-relieved disruption in membrane integrity is brought about by osmotic and ionic components of salt stress.

An interesting methodological consequence of the disruption of membrane integrity by NaCl is that membrane transporters may no longer dictate ion fluxes into and out of the cell over this time-scale, and a very rapid, futile cycle that bypasses the membrane could result. Thus, the reduced K^+ influx observed in response to sudden NaCl provision (Fig. 2) may be greatly underestimated owing to simultaneous leakage from the cell, and the measured flux would then represent a reduced net accumulation of K^+. The

observation that K^+ efflux under steady-state salinity is much reduced compared with that seen upon sudden NaCl application, however, suggests that significant recovery in membrane integrity occurs in the long term, which can be at least in part explained by changes in lipid composition (López-Pérez *et al.*, 2009).

In addition to examining the immediate effects of Na^+ on the release of K^+ from the cell, and with the agronomic significance of K^+ homeostasis in mind, it was of considerable interest to investigate Na^+–K^+ interactions in the longer term. Fig. 7(a) shows the loss of K^+ from roots and shoots over 24 h following salt treatment, and indicates that, within the first 2 h, root K^+ loss is *c.* 25 μmol g^{-1} FW h^{-1}. Given that cytosolic [K^+] is *c.* 100 mM or less (Fig. 3b; Walker *et al.*, 1996), which translates into *c.* 5 μmol cytosolic K^+ per gram tissue (assuming that tissue is isopycnic with water and cytosolic volume is 5% of tissue volume) an efflux of 25 μmol K^+ g^{-1} FW h^{-1} would deplete the cytosolic pool within 12 min, and would rapidly begin to draw upon vacuolar resources. Thus, changes in permeability at the plasma membrane, regardless of mechanism, are evidently accompanied by changes in tonoplast permeability (a situation likely to occur in the mobilization of NH_4^+ also demonstrated here; Fig. 8, inset). The mechanism(s) underlying this mobilization process are clearly important, and require investigation.

It is evident from Fig. 7(b) that in the steady state, tissue K^+ values are even more severely affected by Na^+ stress than over the first 24 h of stress. Steady-state tracer analyses of K^+ fluxes in the inward and outward directions (Fig. 3a) show that K^+ efflux increased with long-term Na^+ provision, except at the highest Ca^{2+}supply, and K^+ influx, as well as the ratio of influx to efflux, decreased in all cases. It is noteworthy that the absolute decline in K^+ influx was substantially greater than the absolute increase in K^+ efflux, which suggests that, of the two, K^+ influx is the more important component in the disruption of cellular K^+ homeostasis by Na^+, and as such might be a more accurate predictor of Na^+ tolerance among cultivars or species (Nassery, 1979; Chen *et al.*, 2005). This is underscored by observations that Na^+-stimulated K^+ efflux is all but eliminated by high external [Ca^{2+}], both in the short term (Fig. 1b; Shabala *et al.*, 2006) and in the steady state (Fig. 3a), while the apparent influx of K^+ remains suppressed by Na^+ over short and long time-scales (Figs 2,3a). Given that soluble soil Ca^{2+}tends to be at similarly high levels in saline soils (typically 15 mM; Zidan *et al.*, 1991), K^+ efflux may thus not play a broadly significant role in K^+ homeostasis in the short or long run. Nevertheless, examination of the relative size of the Na^+-stimulated K^+ efflux, regardless of mechanism, may yet be of diagnostic value with respect to a plant's ability to withstand sudden osmotic and ionic stresses and may, thus, provide some insight into inherent salt stress tolerance among cultivars.

Acknowledgements

We thank M. Butler at the McMaster University Nuclear Reactor and the Centre for Addiction and Mental Health (CAMH) cyclotron team, University of Toronto, for providing radiotracers. Funding for this work was provided by the University of Toronto, the Natural Sciences and Engineering Research Council of Canada (NSERC), the Canada Research Chair (CRC) program, and the Canadian Foundation for Innovation (CFI).

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