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RESEARCH PAPER

Silver ions disrupt K^+ homeostasis and cellular integrity in intact barley (*Hordeum vulgare* L.) roots

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Abstract

The heavy metals silver, gold, and mercury can strongly inhibit aquaporin-mediated water flow across plant cell membranes, but critical examinations of their side effects are rare. Here, the short-lived radiotracer ^{42}K is used to demonstrate that these metals, especially silver, profoundly change potassium homeostasis in roots of intact barley (*Hordeum vulgare* L.) plants, by altering unidirectional K^+ fluxes. Doses as low as 5 μM $AgNO_3$ rapidly reduced K^+ influx to 5% that of controls, and brought about pronounced and immediate increases in K^+ efflux, while higher doses of Au^{3+} and Hg^{2+} were required to produce similar responses. Reduced influx and enhanced efflux of K^+ resulted in a net loss of >40% of root tissue K^+ during a 15 min application of 500 μM $AgNO_3$, comprising the entire cytosolic potassium pool and about a third of the vacuolar pool. Silver also brought about major losses of UV-absorbing compounds, total electrolytes, and NH_4^+. Co-application, with silver, of the channel blockers Cs^+, TEA^+, or Ca^{2+}, did not affect the enhanced efflux, ruling out the involvement of outwardly rectifying ion channels. Taken together with an examination of propidium iodide staining under confocal microscopy, the results indicate that silver ions affect K^+ homeostasis by directly inhibiting K^+ influx at lower concentrations, and indirectly inhibiting K^+ influx and enhancing K^+ efflux, via membrane destruction, at higher concentrations. Ni^{2+}, Cd^{2+}, and Pb^{2+}, three heavy metals not generally known to affect aquaporins, did not enhance K^+ efflux or cause propidium iodide incorporation. The study reveals strong and previously unknown effects of major aquaporin inhibitors and recommends caution in their application.

Key words: Aquaporins, barley (*Hordeum vulgare* L.), heavy metals, ion transport, membrane integrity.

Introduction

Aquaporins (AQPs) comprise a diverse, recently discovered group of membrane-bound proteins that facilitate the movement of water, and of small, usually uncharged molecules such as urea, glycerol, carbon dioxide, and ammonia, across the membranes of living cells (Maurel *et al.*, 2008; Hove and Bhave, 2011). In plants and other organisms, AQP function has been studied extensively by use of mercury (Hg^{2+})-containing compounds, which inhibit water flux at both the tissue and cellular level (Preston *et al.*, 1992; Maurel *et al.*, 1993). More recently, it has been shown that silver (Ag^+) and gold (Au^{3+}) ions can block the flow of water through AQPs even more effectively than mercury, albeit by a different mechanism (Niemietz and Tyerman, 2002; see also Supplementary Table S1 avialable at *JXB* online). Silver is also used widely as an inhibitor in the study of ethylene biosynthesis (Beyer, 1976; see also Supplementary Table S1).

As with the use of any inhibitory treatment, it is crucial to determine the specificity of the inhibition. The toxic nature of mercury is legendary (Patra and Sharma, 2000; Eisler, 2006), and Santoni *et al.* (2000) cautioned that its disruption of cellular metabolism and solute homeostasis could cause confounding side effects such as the down-regulation of AQP activity, or the collapse of water potential gradients across cell membranes. These authors concluded that mercury could, nevertheless, be reasonably applied in water transport studies, provided that low concentrations (<100 μM) are used, and that control

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experiments are conducted to indicate that perturbations to cellular functions, such as ion transport, are minimal (see also Meharg and Jardine, 2003). Similarly, Zhang and Tyerman (1999) suggested that high concentrations of mercury (300 μM) could have non-specific, detrimental effects on cell membranes, and that lower concentrations should therefore be employed in AQP studies.

In practice, however, such control experiments can be difficult, and are rarely conducted. In addition, as can be seen in Supplementary Table S1 at *JXB* online, which shows the result of a literature survey on the use of mercury, silver, and gold as AQP inhibitors (and, in the case of silver, also as an inhibitor of ethylene biosynthesis), applied concentrations of several hundred micromolar, or even several millimolar, are frequently used, well in excess of the (somewhat arbitrary, as we shall see) limit suggested by Santoni *et al.* (2000).

In the present authors' own laboratory, AQP function has recently been investigated due to a long-standing interest in the mechanisms of N uptake in plant cells in the context of the hypothesis that low-affinity NH_3/NH_4^+ transport into root cells is mediated by AQPs. While there is growing evidence for such mediation in heterologous expression systems (Jahn *et al.*, 2004; Holm *et al.*, 2005; Loque *et al.*, 2005; Hove and Bhave, 2011), a convincing *in planta* demonstration is still lacking. The initial hypothesis was supported by the strong reduction, by mercury and silver, of ^{13}N-labelled NH_3/NH_4^+ influx into barley roots, but, surprisingly, was then contradicted by a potent silver-induced stimulation of ^{13}N efflux.

These somewhat paradoxical findings led to a new hypothesis, that the effects of silver, and possibly other heavy metal inhibitors, on tracer fluxes extend beyond a simple inhibition of AQP activity. In the present study, this hypothesis was tested by examination of the effects of Hg^{2+}, Ag^+, and Au^{3+} on unidirectional fluxes (root influx and efflux) of K^+, an ion not transported to a significant extent by AQPs (Agre *et al.*, 1997). As will be shown, the results led to a new research question: is membrane integrity disrupted in roots treated with AQP inhibitors? This possibility, similar to one previously put forward to explain sodium-stimulated K^+ efflux (Britto *et al.*, 2010), was investigated here using a wide variety of methods, including measurements of tracer fluxes, release of UV-absorbing compounds and electrolytes, tissue ion content, and confocal microscopy.

Materials and methods

Plant culture

Seeds of barley (*Hordeum vulgare* L. cv. 'Metcalfe') were surface-sterilized for 15 min in 1% sodium hypochlorite and germinated under acid-washed sand for 3 d before placement in 12.0 l hydroponic vessels containing modified Johnson's nutrient solutions. All solutions were composed of 0.5 mM NaH_2PO_4, 0.25 mM $MgSO_4$, 25 μM H_3BO_3, 20 μM FeEDTA, 6.25 μM $CaCl_2$, 2 μM $ZnSO_4$, 0.5 μM $MnSO_4$, 0.5 μM $CuSO_4$, and 0.125 μM Na_2MoO_4 (pH adjusted to 6.3–6.5 using 1 M NaOH), with two variations. For experiments examining K^+ fluxes and accumulation, membrane integrity by means of confocal imagery, and release of electrolytes or UV-absorbing compounds, the solution also contained 0.05 mM K_2SO_4 and 0.5 mM $Ca(NO_3)_2$, while solution for plants used in measuring NH_3/NH_4^+ fluxes and accumulation, and also root hydraulic conductivity, contained 5 mM $(NH_4)_2SO_4$, 0.2 mM $CaSO_4$, and 11.25 μM K_2SO_4, to maximize internal NH_3/NH_4^+ pools (Britto *et al.*, 2001; Szczerba *et al.*, 2008). Solutions were completely exchanged on days 5 and 6 following germination, to ensure that plants remained at a nutritional steady state, for experimentation on day 7. Plants were grown in climate-controlled, 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 ~70%.

Flux analyses

Unidirectional tracer influx and efflux rates were measured in replicate units of 3–5 seedlings, bundled together at the shoot base by a plastic collar 0.5 cm in height. Roots were labelled in nutrient solutions identical to growth solution but containing either ^{42}K ($t_{1/2}$=12.36 h), received as $^{42}K_2CO_3$ from the McMaster University Nuclear Reactor (Hamilton, ON, Canada), or ^{13}N ($t_{1/2}$=9.98 min), received as $^{13}NH_4^+$ from the CAMH cyclotron facility (University of Toronto, ON, Canada). Radioactivity in plant organs and efflux eluates 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).

Efflux experiments: Changes in tracer efflux over time were monitored using previously described protocols (Britto *et al.*, 2010; Coskun *et al.*, 2010). In brief, after a 1 h labelling period in nutrient solution, identical to growth solution, except that it contained radiotracer (^{42}K or ^{13}N), seedling bundles were secured inside glass efflux funnels and roots were eluted of radioactivity with successive 13-ml aliquots of non-radioactive growth solution. The first 22 eluates (15.5 min into the desorption series) were identical to growth solution, while the last 14 (over the remaining 14 min) were composed of growth solution plus the chemical treatment of interest (Coskun *et al.*, 2010). The heavy metal AQP inhibitors used in this study were Ag^+ (as $AgNO_3$), Au^{3+} (as $HAuCl_4$), and Hg^{2+} (as $HgCl_2$), all provided at 5, 50, and 500 μM. A small set of experiments used silver sulphadiazine (silver 4-amino-*N*-2-pyrimidinylbenzenesulphonamide, provided at 5 μM in 0.5% (v/v) dimethylsulphoxide (DMSO; Niemietz and Tyerman, 2002). The organic AQP inhibitors acetazolamide and zonisamide (at 10 μM in 0.1% (v/v) DMSO; Yool *et al.*, 2010), and the AQP stimulator forskolin [at 10 μM in 0.1% (v/v) DMSO (Maurel *et al.*, 1995; Yool *et al.*, 1996)] were also tested. Control experiments showed no effect of either 0.1% or 0.5% (v/v) DMSO on K^+ fluxes (not shown). In addition, the heavy metals Cd^{2+} (as $CdCl_2$), Ni^{2+} (as $NiSO_4$), and Pb^{2+} [as $Pb(NO_3)_2$], known to not generally inhibit AQPs (Niemietz and Tyerman, 2002; see Zelenina *et al.*, 2003; Verdoucq *et al.*, 2008) were tested (all at 500 μM). Efflux experiments investigating hypothesized channel activity during silver application involved the co-application, with silver, of the ion channel blockers $CaSO_4$ (5 mM), TEA-NO_3 (10 mM), or Cs_2SO_4 (5 mM). All solutions were mixed with a fine stream of air bubbles. Immediately following elution, roots were detached from shoots and spun in a low-speed centrifuge for 30 s, to remove surface water before weighing. Eluates and plant organs were placed in a gamma counter (see above) to measure radioactivity. 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}.

Influx experiments: Short-term ^{42}K and ^{13}N labelling was performed as described in detail elsewhere (Balkos *et al.*, 2010; Coskun *et al.*, 2010). In brief, roots of bundled seedlings were pre-equilibrated for 5 min in a solution either identical to growth solution (control), or in a growth solution supplemented with a chemical treatment (see below). Roots were then immersed for 5 min in a solution identical to pre-equilibration solution but containing the radiotracer of interest (^{42}K or ^{13}N). Following labelling, bundles were transferred to non-radioactive growth solution for 5 s to reduce tracer carryover, and further desorbed for 5 min in fresh growth medium before plant organs were separated and counted for gamma radiation (see above).

Tissue analyses

K^+ content: Tissue K^+ content was determined by methods previously described for steady-state and non-steady-state conditions (Britto *et al.*, 2010). In brief, after 15 min incubation in normal growth medium (control) or supplemented with 500 μM Ag^+, roots of bundled seedlings were desorbed in 10 mM $CaSO_4$ for 5 min to release extracellular K^+, then separated from shoots and spun in a low-speed centrifuge for 30 s to remove surface water before weighing. Roots were oven-dried at 85–90 °C for 3 d, pulverized using a VWR VDI12 homogenizer (VWR International, Mississauga, ON, Canada), and digested for an additional 3 d in 30% HNO_3. The K^+ concentration of the digested tissues was quantified using a dual-channel flame photometer (Model 2655-10; Cole-Parmer Instrument Company, Anjou, QC, Canada).

NH_4^+ content: Tissue NH_4^+ content was determined by the *o*-phthalaldehyde (OPA) method as previously described (Szczerba *et al.*, 2008), under conditions described above for K^+ content measurement. Following desorption in 10 mM $CaSO_4$, separation of organs, and weighing, root and shoot tissue (~0.5 g) were pulverized under liquid N_2 using a mortar and pestle, and NH_4^+ was extracted by addition of 6 ml of formic acid (10 mM) (Husted *et al.*, 2000). Subsamples (1 ml) of the homogenate were centrifuged at 16 000 *g* at 2 °C for 10 min. Supernatants were transferred into 2 ml polypropylene tubes affixed with 0.45 μm nylon filters (Costar, Corning Inc., Lowell, MA, USA) and centrifuged at 5000 *g* at 2 °C for 5 min. The resulting supernatant was analysed using the OPA method to determine NH_4^+ content (Goyal *et al.*, 1988). OPA reagent was prepared 1 d before use and was composed of 100 mM KH_2PO_4, 100 mM K_2HPO_4, 3.75 mM OPA, and 2 mM 2-mercaptoethanol. Prior to the addition of 2-mercaptoethanol, the solution was adjusted to pH 7 with 1 M NaOH and filtered through grade-2 Whatman filter paper. A 10 μl aliquot of tissue extract was added to 3 ml of OPA reagent, and the colour was allowed to develop in the dark for 30 min at room temperature. Sample absorbance was measured at 410 nm using a spectrophotometer (Hewlett Packard 8453, Agilent Technologies Canada Inc., Mississauga, ON, Canada).

Relative leakage analyses

Electrical conductivity: Electrolyte leakage, as a measure of membrane intactness, was assessed as described by Tuna *et al.* (2007), but adapted here for excised roots. After a 15 min incubation in either normal growth solution (control; see above) or growth solution plus 500 μM $AgNO_3$, roots (~150 mg) of intact seedlings were excised and rinsed three times with deionized water to remove surface-adhering electrolytes and damaged cells from the cut surface. Root segments were placed in 20 ml vials containing 10 ml of deionized water and incubated at 25 °C for 24 h in a rotary shaker. Subsequently, electrical conductivity (EC) of the bathing medium was measured (CON510 Bench Conductivity/TDS meter, Oakton Instruments, Vernon Hills, IL, USA). After samples were autoclaved at 120 °C for 20 min, the final electrical conductivity of the bathing medium was measured following equilibration at 25 °C. The relative leakage ratio was determined as the quotient of the EC before and after autoclaving.

UV absorbance spectrophotometry: Another gauge of membrane stability involved measuring the release of UV-absorbing substances from excised roots (Redman *et al.*, 1986). Roots were treated as described above but absorbance (at 280 nm, A_{280}) of the bathing medium was measured spectrophotometrically (see above). The relative leakage ratio was determined as the quotient of A_{280} before and after autoclaving.

Confocal microscopy

Following a 15 min treatment with the chemical of interest, lateral root segments of intact seedlings were sectioned 0.5–1 cm from the apex and incubated with 25 μg ml^{-1} propidium iodide with 0.1% Silwet to counterstain the cell wall and nuclei of ruptured cells (Oh *et al.*, 2010). Propidium iodide was excited at 488 nm and fluorescence was detected at ⩾ 585 nm (red channel) using a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany).

Root water potential, Ψ_R

Water potential of control roots or roots treated with either 500 μM $AgNO_3$ or $HAuCl_4$ were measured by means of the pressure chamber (Model 1000 Pressure Chamber Instrument, PMS Instrument Co., Albany, OR, USA) (Scholander *et al.*, 1965). Following a 15 min incubation in the chemical treatment of interest, seedlings were detopped at the hypocotyl region (~1–2 cm above the seed) and the root system was placed into the pressure chamber, allowing ~0.5 cm of the stem to protrude from the chamber. Root water potential (in MPa) was determined by applying N_2 gas pressure to the root system until the meniscus reached the cut surface.

K^+ release quantification

In efflux experiments, silver stimulated radiotracer release from roots over the course of its application (see Results section). To express this release in terms of moles of traced ions, it was essential to estimate the specific activity (SA) of the tracer pool captured in efflux aliquots. This was done by first determining cytosolic SA following the 1 h labelling period, using the exponential rise function $SA_t = SA_o\ (1-e^{-kt})$, where SA_o represents the external specific activity of the loading solution, t the labelling time, and k the rate constant representing the slope of the semi-logarithmic regression line of the slowest exchanging (cytosolic) phase, determined in control experiments under steady-state conditions (see Memon *et al.*, 1985; Lee and Clarkson, 1986; Siddiqi *et al.*, 1991; Kronzucker *et al.*, 1995, 2003). Vacuolar labelling during this period was negligible, given the much longer half-times of exchange across the tonoplast (12–20 h; Memon *et al.*, 1985). Cytosolic SA, however, had to be corrected for $^{42}K^+$ lost during the first 15.5 min of efflux desorption (i.e. prior to silver application), using the exponential decay function $SA_{t'} = SA_t\ e^{-kt'}$, or by substitution $SA_{t'} = SA_o\ (1-e^{-kt})\ e^{-kt'}$, where t'=15.5 min. Thus, to quantify the K^+ released [in μmol g (root FW)$^{-1}$] after silver application, the radioactivity released (in cpm) during this time was summed, divided by $SA_{t'}$, and corrected for root FW. For comparison purposes, this protocol was conducted for each individual treatment on efflux (Fig. 3, internal legend).

Statistics

In efflux experiments, each bundle of five seedlings was considered a single replicate, and each treatment was replicated a minimum of three times. Traces from experiments involving chemical treatments (following application at 15.5 min) were compared with

control traces by quantifying the areas under the efflux curves following treatment, as specified above. Areas under efflux curves in individual treatments and controls were compared by use of Student's t-test (P <0.05). For influx experiments, each bundle of three seedlings was considered a single replicate, and each treatment was replicated a minimum of four times. Significant differences between treatments and controls were determined either by Student's t-tests (Fig. 1) or by analysis of variance

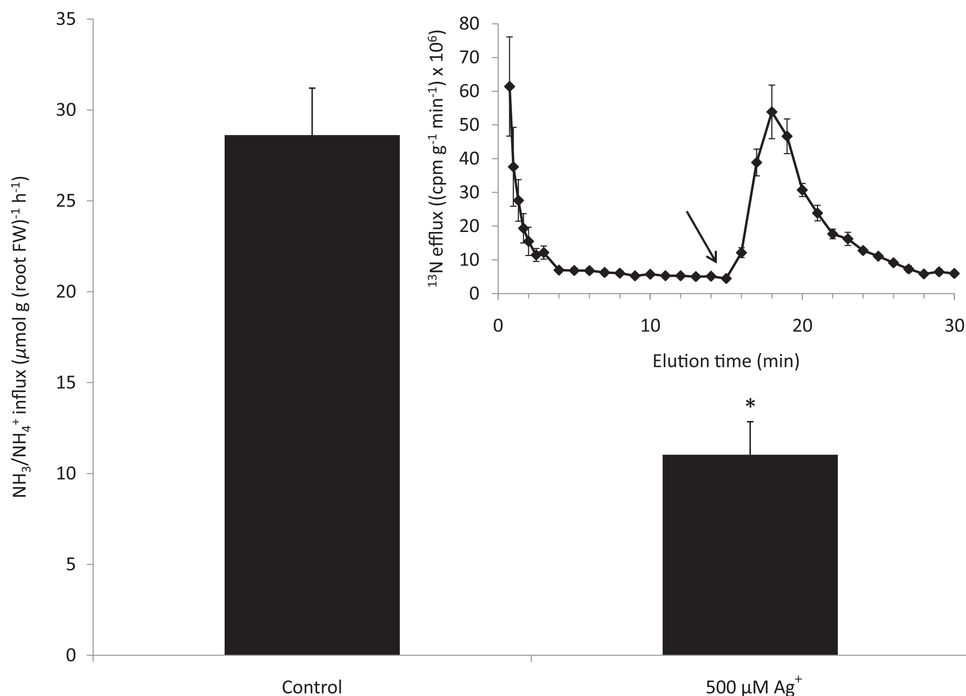

Fig. 1. Response of NH_3/NH_4^+ influx into roots of intact barley (*Hordeum vulgare* L.) seedlings to 10 min incubation in 500 µM Ag^+. Inset: response of $^{13}NH_3/^{13}NH_4^+$ efflux from roots of intact barley seedlings to sudden provision (at elution time=15 min, see arrow) of 500 µM Ag^+. Asterisk represents a significantly different mean from control (t-test, P <0.05). Each treatment represents the mean of three replicates. Error bars indicate ±SEM.

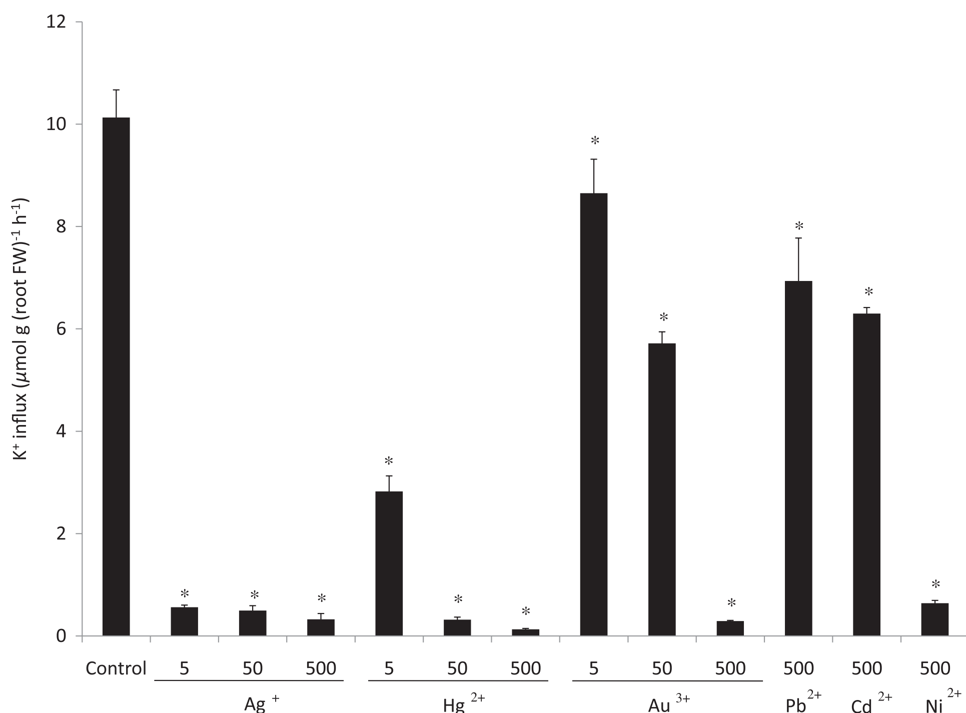

Fig. 2. Response of K^+ influx into roots of intact barley (*Hordeum vulgare* L.) seedlings to 10 min incubation in Ag^+, Hg^{2+}, and Au^{3+} (at 5, 50, and 500 µM), and Pb^{2+}, Cd^{2+}, and Ni^{2+} (at 500 µM). Asterisks represent significantly different means from control (one-way ANOVA with Dunnett's post-test). Each treatment represents the mean of 4–7 replicates. Error bars indicate ±SEM.

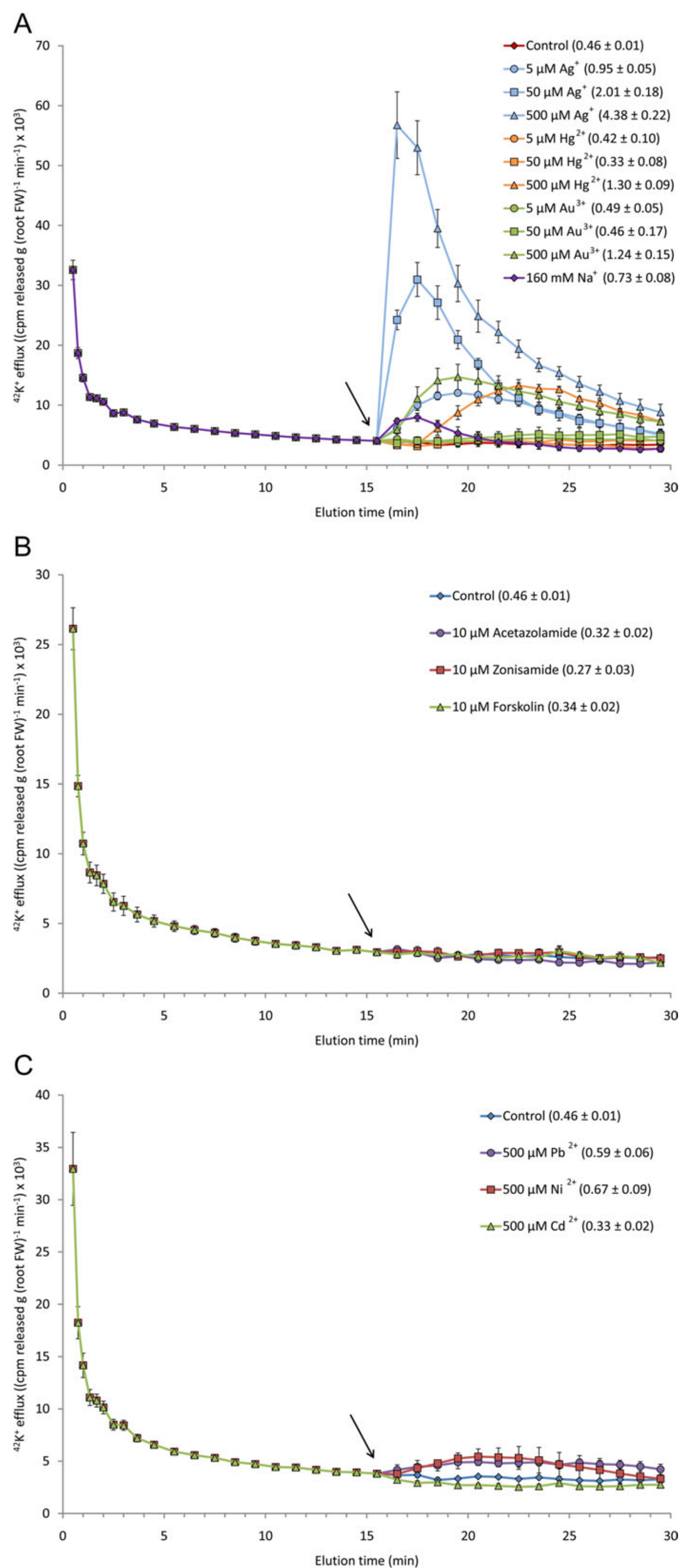

Fig. 3. Response of $^{42}K^+$ efflux from roots of intact barley (*Hordeum vulgare* L.) seedlings to sudden provision (see arrow) of (a) Ag^+, Hg^{2+}, and Au^{3+} (at 5, 50, and 500 μM), and 160 mM Na^+; (b) 10 μM acetazolamide, zonisamide, and forskolin.; (c) 500 μM Pb^{2+}, Cd^{2+}, and Ni^{2+}. In the internal legend, the numbers in parentheses indicate the amount of K^+ released during a specific treatment (t=16.5–29.5 min), in μmol g (root FW)$^{-1}$. Each plot represents the mean of 3–8 replicates. Error bars indicate ±SEM.

(ANOVA) followed by Dunnett's Multiple Comparison test (Fig. 2). For tissue content analyses, each bundle of five seedlings was considered a single replicate, and for relative leakage assays, a single seedling was considered as one replicate. Treatments for both tests were replicated a minimum of six times. Student's *t*-tests were performed to determine significantly different means.

Results

Figure 1 shows measurements of unidirectional ^{13}N-radiolabelled NH_3/NH_4^+ influx into intact barley seedlings, with and without a 10 min treatment of 500 μM $AgNO_3$. Silver application reduced ^{13}N influx to less than half that of control, while, in the opposite direction of transport, it brought about an immediate and substantial stimulation of ^{13}N efflux from pre-labelled roots (Fig. 1, inset). Similar, if even more pronounced, effects of $AgNO_3$ were observed when tracing unidirectional potassium influx using $^{42}K^+$, with concentrations as low as 5 μM reducing influx by 95% (Fig. 2). A small subset of experiments involving 5 μM silver sulphadiazine also showed a dramatic suppression of K^+ influx (Supplementary Fig. S1 at *JXB* online). In addition, treatment with Au^{3+} and Hg^{2+}, two other heavy metals known to inhibit AQPs (Niemietz and Tyerman, 2002), essentially abolished K^+ influx at 500 μM, while lower concentrations also showed significant reductions in influx, particularly with mercury (Fig. 2). In contrast, Cd^{2+} and Pb^{2+}, heavy metals described as non-AQP inhibitors (Niemietz and Tyerman, 2002), brought about only a slight inhibition of K^+ influx, far less than Ag^+, Au^{3+}, and Hg^{2+}, (Fig. 2). However, Ni^{2+}, though also not identified as an AQP inhibitor (Niemietz and Tyerman, 2002), did significantly reduce K^+ influx to a similar extent as Ag^+, Au^{3+}, and Hg^{2+} (Fig. 2). As with ^{13}N, stimulation of $^{42}K^+$ efflux was also observed. Application of 500 μM Ag^+ was the most powerful stimulatory treatment, followed by 50 μM Ag^+, while 500 μM Au^{3+} and Hg^{2+} stimulated K^+ release to an extent similar to 5 μM Ag^+ (Fig. 3a). $^{42}K^+$ efflux also showed an immediate and substantial acceleration due to 5 μM silver sulphadiazine, an identical response to that of equimolar $AgNO_3$ (Supplementary Fig. S1, inset). The dose dependence of $AgNO_3$ revealed an ~2-fold increase in tracer released with a 10-fold increase in applied silver concentration (Fig. 3a, internal legend). Interestingly, K^+ efflux stimulation by 160 mM Na^+, a well-studied phenomenon (Nassery, 1979; Shabala *et al.*, 2006; Britto *et al.*, 2010), was modest compared with these treatments, with tracer release falling below that seen with 5 μM silver. Applications of Au^{3+} and Hg^{2+}, at lower concentrations (5 μM or 50 μM), did not result in $^{42}K^+$ efflux stimulation, nor did the application of the organic AQP modulators acetazolamide, zonisamide, or forskolin (all at 10 μM) (Fig. 3b). Applications of 500 μM Pb^{2+}, Cd^{2+}, and Ni^{2+} produced little change to the efflux of K^+ compared with control (Fig. 3c).

To address the hypothesis that the observed stimulation of efflux was mediated by K^+ channels or non-selective cation channels (NSCCs), the K^+ channel blocking agents

Cs^+ and TEA^+ (both at 10 mM), and the NSCC blocker Ca^{2+} (at 5 mM) were co-applied with 500 μM Ag^+ (Fig. 4). None of these channel blockers significantly attenuated the stimulation of efflux observed with 500 μM Ag^+ (P <0.05). Moreover, no reduction by 10 mM Cs^+ of the stimulated efflux induced by 5 μM Ag^+ was observed, whereas when 5 μM Au^{3+} and Hg^{2+} were co-applied with 10 mM Cs^+, a depression of efflux below control values was observed (not shown). We should note that Cs^+ was applied as the chloride salt here to avoid precipitation (similarly, sulphate salts were used in the case of Ag^+ application).

To test the alternative hypothesis that the effects of silver, gold, and mercury on ion fluxes are related to membrane integrity, two independent assays of membrane leakiness were conducted. Table 1 shows the relative leakage ratio of control and silver-treated roots, as determined by measuring either electrical conductivity or UV absorbance of the bathing medium, before and after autoclaving the roots. Both assays showed that a 15 min incubation of intact roots in 500 μM Ag^+ significantly increased the relative leakiness of cellular membranes, suggesting that this treatment caused membrane damage. It should be noted that the measurements were taken after silver treatment was terminated; that is, after substantial loss of cellular materials had already occurred. Therefore, these standard protocols underestimated the difference between treatment and control (Table 1).

Changes in ionic content of roots following silver application were also measured, by photometric analyses of tissues (Table 1). Root K^+ content decreased by 43% and NH_4^+ content decreased by 69% after a 15 min application of 500 μM Ag^+, further suggesting that extensive membrane damage had taken place. When plants exposed to 500 μM Ag^+ for 15 min were returned to normal growth solution for 1 week, root growth cessation and shoot growth suppression was observed over the following 6 d (not shown), showing that silver brought about irreversible damage.

For comparison with total K^+ loss from tissue, the tracer released following 500 μM Ag^+ treatment in $^{42}K^+$ efflux

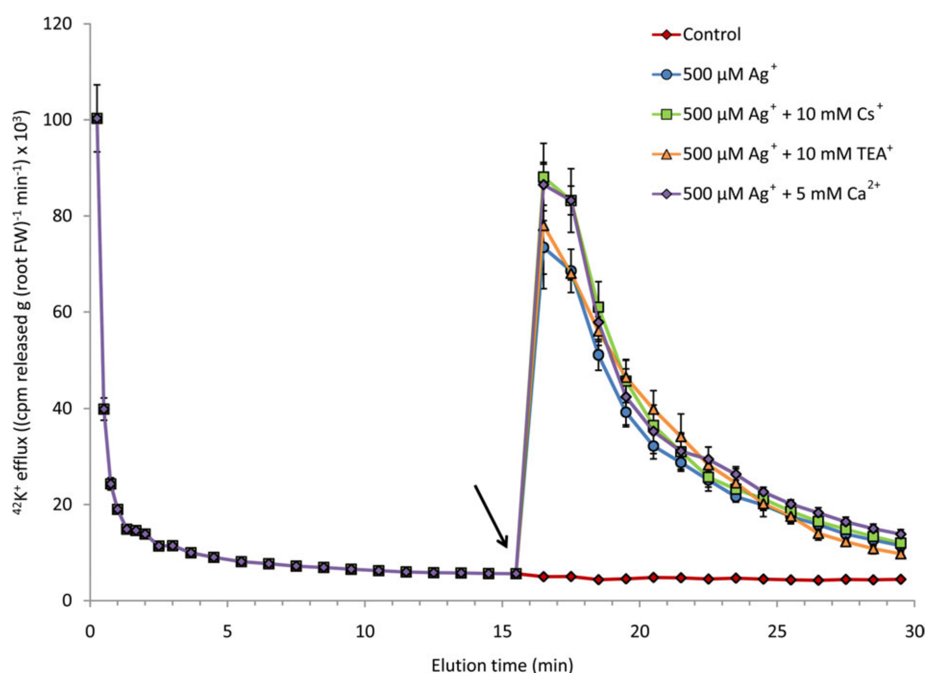

Fig. 4. Response of $^{42}K^+$ efflux from roots of intact barley (*Hordeum vulgare* L.) seedlings to sudden provision (see arrow) of 500 μM Ag^+ in combination with the channel inhibitors Cs^+ (10 mM, as Cs_2SO_4), TEA^+ (10 mM, as $TEA\text{-}NO_3$), and Ca^{2+} (5 mM, as $CaSO_4$). Each plot represents the mean of 3–8 replicates. Error bars indicate ±SEM.

Table 1. Effects of Ag^+ on the relative leakage ratio (RLR), and K^+ and NH_4^+ contents, of roots from 7-day-old barley (*Hordeum vulgare* L.) seedlings

Errors indicate ±SEM of 6–12 replicates. Asterisks indicate significant differences from control (t-test, P <0.05).

Treatment	EC (μS)		A_{280} (AU)		Root K^+ content (μmol g^{-1} FW)	Root NH_4^+ content (μmol g^{-1} FW)
	Initial	Final	Initial	Final		
Control	75.74±4.48 RLR=0.36±0.02	221.65±20.72	0.10±0.02 RLR=0.17±0.04	0.49±0.04	37.16±1.75	63.54±2.28
500 μM $AgNO_3$	21.20±0.56 RLR=0.62±0.02*	34.4±1.08	0.12±0.01 RLR=0.61±0.03*	0.20±0.01	21.30±2.54*	19.89±0.89*

experiments was summed and expressed in terms of micromoles. Analysis showed that treated roots lost a minimum of 4.38 μmol g^{-1} K^+ over a 15 min period (Fig. 5; note that these curves were truncated slightly prematurely; a small set of experiments was conducted wherein elution was extended for an additional 30 min, resulting in an additional 25% of tracer loss).

Figure 6 directly illustrates the membrane damage brought about by 5 μM and 500 μM Ag^+ (Fig. 6B and C, respectively), 500 μM Hg^{2+} (Fig. 6D), and 500 μM Au^{3+} (Fig. 6E), by means of propidium iodide staining visualized with confocal microscopy. Compared with control (Fig. 6A), where only cell walls were stained, all regions of the root tip showed nuclear staining (and, by inference, membrane disruption) when intact seedlings were incubated for 15 min with the AQP inhibitor. In contrast, roots treated for 15 min with the heavy metals Cd^{2+}, Ni^{2+}, and Pb^{2+} (all at 500 μM) (Fig. 6F–H, respectively) lack nuclear staining, indicating lack of membrane damage. Root tips treated with 160 mM NaCl for the same duration also showed no nuclear staining (Fig. 6I).

Pressure chamber measurements were conducted to test the effect of Ag^+ and Au^{3+} on the water potential of intact seedling roots. Supplementary Fig. S2 at *JXB* online shows that a 15 min treatment with 500 μM Ag^+ or Au^{3+} significantly impeded water flow in roots of experimental plants.

Discussion

In 2002, Niemietz and Tyerman demonstrated that silver, gold, and mercury ions reduced osmotically induced shrinking of human erythrocytes and of vesicles composed of root plasma membrane of beet or peribacteroid membrane from soybean. Silver was particularly effective in this regard, followed by gold and then mercury. Because the osmotic shrinking response of cells and vesicles is directly linked to water permeability, the authors concluded that silver is a potent AQP inhibitor, much more so than mercury, which has been, and continues to be, widely used as a blocker in AQP studies (see Table S1). This discovery may help explain why silver is effective as an antimicrobial agent (Kim *et al.*, 2008) and in the preservation of cut flowers (Beyer, 1976; Lü *et al.*, 2010).

In the present study, it was shown that when Ag^+, Au^{3+}, and Hg^{2+} are applied to roots of intact barley plants, pronounced effects on transmembrane ion fluxes and membrane integrity occur in addition to the reduction in water flux. Initially, it was observed that Ag^+ stimulated the efflux of ^{13}N-labelled NH_3/NH_4^+ from roots (Fig. 1, inset), but follow-up experiments indicated that the effect is more general, with Ag^+ also increasing efflux of labelled potassium, even at concentrations as low as 5 μM Ag^+ (Fig. 3a). In addition, 500 μM Au^{3+} and Hg^{2+} also stimulated K^+

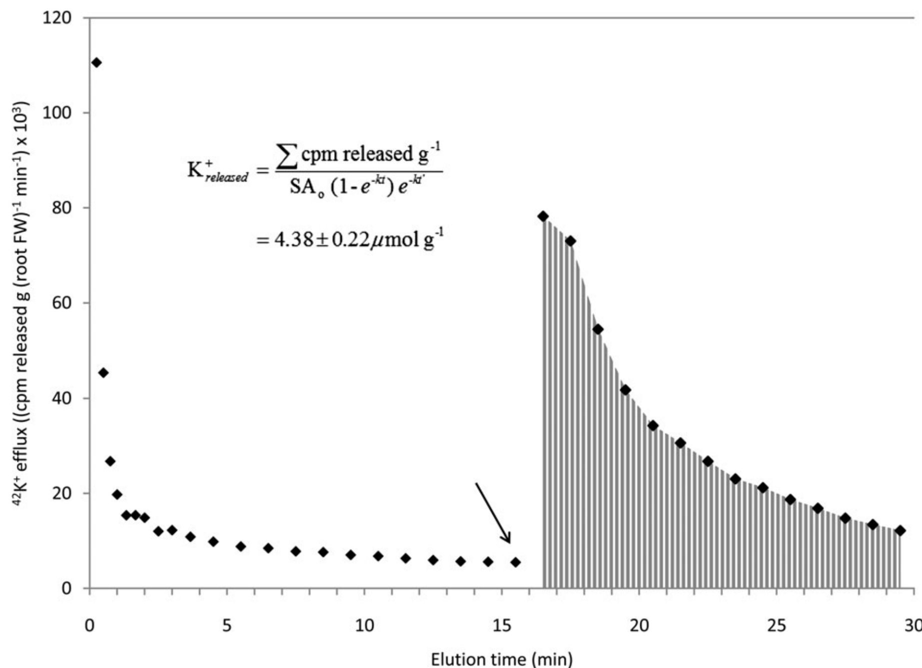

Fig. 5. Illustration of the integration technique employed to quantify total K^+ released during treatment with 500 μM Ag^+. As indicated in the equation, K^+ released is determined by the summation of counts released per gram during Ag^+ treatment (shaded area), divided by the corrected specific activity of the cytosol, where SA_o represents the specific activity of the loading solution, k the rate constant representing the slope of the semi-logarithmic regression line of the slowest exchanging (cytosolic) phase, t the loading time (60 min), and t' the time between the start of the elution series and the beginning of the treatment (15.5 min). Based on eight replicates, 4.38±0.22 μmol K^+ g^{-1} was released from seedlings treated with 500 μM Ag^+ for 15 min. Note that this graph is prematurely truncated; however, a few experiments involving longer term (45 min) treatment and elution were conducted, and showed that ~25% additional K^+ was available for release.

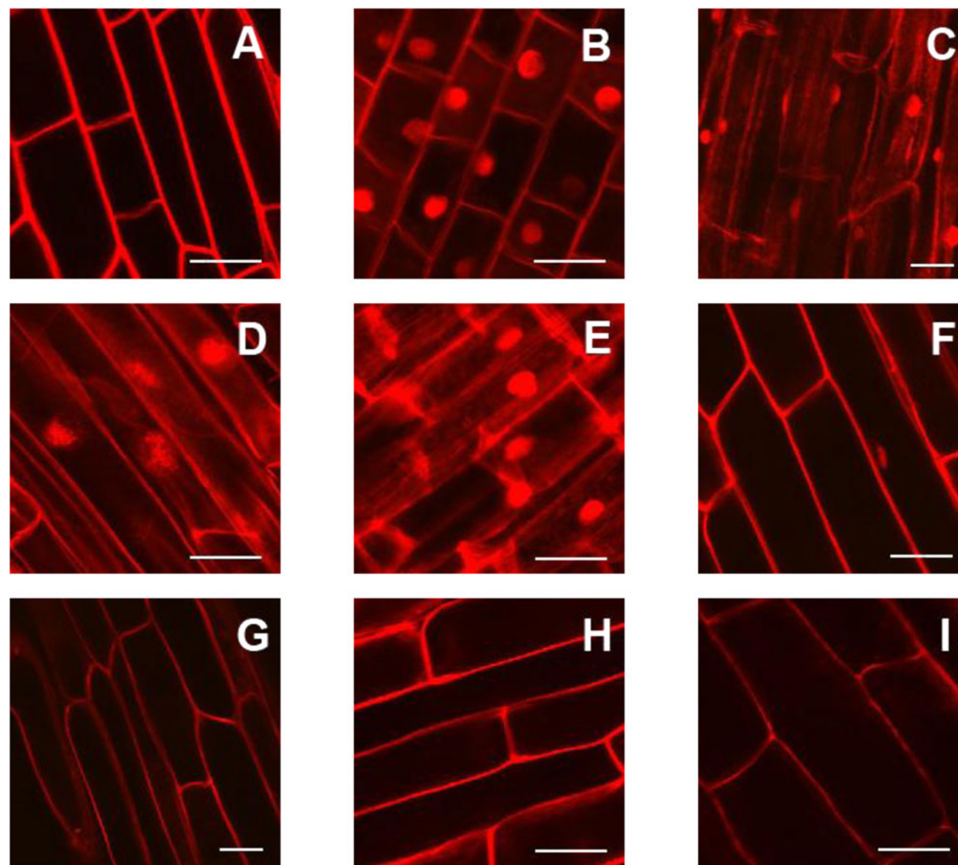

Fig. 6. Confocal micrographs showing propidium iodide staining of the cell wall and nuclei of damaged cells from lateral root tips of intact barley (*Hordeum vulgare* L.) seedlings treated for 15 min in (A) control, (B) 5 μM Ag^+, (C) 500 μM Ag^+, (D) 500 μM Hg^{2+}, (E) 500 μM Au^{3+}, (F) 500 μM Cd^{2+}, (G) 500 μM Ni^{2+}, (H) 500 μM Pb^{2+}, and (I) 160 mM Na^+. Scale bars represent 20 μm.

release, but the same metals applied at lower concentrations (5 μM and 50 μM) showed no effect, in contrast to Ag^+ (Fig. 3a). Because the K^+ channel blockers TEA^+ and Cs^+, and the NSCC blocker Ca^{2+}, failed to reduce the stimulation of K^+ efflux by 500 μM Ag^+ (Fig. 4), and similar results were seen with 5 μM Ag^+ plus Cs^+ (not shown), the possibility was ruled out that this effect is due to increased activity of ion channels in root cell membranes, an explanation put forward for the NaCl-stimulated efflux of potassium in the work of Shabala *et al.* (2006) (see also Iwabuchi *et al.*, 2008; but cf. Britto *et al.*, 2010). On the other hand, when 10 mM Cs^+ was co-applied with 5 μM Au^{3+} or Hg^{2+}, an inhibition of efflux was observed (not shown), similar to what was seen in a previous study, in which 10 mM Cs^+ suppressed steady-state K^+ efflux in the same cultivar of barley and at the same external $[K^+]$ (Coskun *et al.*, 2010). These observations suggest that membranes are intact, and outward rectifying channels are functional under these conditions, unlike at high (500 μM) concentrations of Au^{3+} and Hg^{2+} and all tested concentrations of Ag^+.

In contrast to the present finding, MacRobbie (2006) suggested that one component of stimulated K^+ release from vacuoles of guard cells, as measured by $^{86}Rb^+$ efflux, is stimulated by the AQP inhibitors mercury and silver, and mediated by ion channels. However, membrane intactness was not determined in that study, and the present results may offer an alternative explanation for the stimulation. It should also be noted that thresholds and degrees of sensitivity to inhibitors do vary among experimental systems. This is apparent from Supplemetary Table S1 at *JXB* online, which shows that Ag^+, Au^{3+}, and Hg^{2+} do not inhibit water flow in all cases. In addition, some studies using the cell pressure probe have indicated that cellular turgor is maintained in the presence of these metals, which would not be possible were significant membrane damage to have occurred (Zhang and Tyerman, 1999; Tazawa *et al.*, 2001; Hukin *et al.*, 2002; Bramley *et al.*, 2009).

Based on additional experimentation showing silver-induced losses of electrolytes and UV-absorbing compounds from barley roots (Table 1), as well as confocal microscopy analysis showing membrane damage resulting from 500 μM Au^{3+} and Hg^{2+}, and all tested Ag^+ concentrations (Fig. 6B–E), the present study strongly suggests that the stimulation of ion efflux due to these treatments is a direct result of membrane destruction. The suppression of ion influx (Figs 1, 2) can also be partly explained in this way; cells with a compromised membrane barrier and high efflux cannot be expected to retain tracer to the same extent as cells with intact membranes. It is interesting to note that the potency of the 500 μM Ag^+ treatment was so great that co-application of 5 mM Ca^{2+} failed to protect membranes, as

is sometimes observed with more benign treatments such as NaCl (Cramer *et al.*, 1985; Britto *et al.*, 2010). In contrast, the inhibition of influx observed under low (5 μM and 50 μM) Au^{3+} and Hg^{2+} (Fig. 2) cannot be explained by accelerated efflux or membrane damage, since no efflux stimulation was seen (Fig. 3a). These observations point toward a direct inhibition of K^+ influx pathways brought about by these lower concentrations of Au^{3+} and Hg^{2+}, possibly via effects on cellular energetics (e.g. Martínez-Ballesta *et al.*, 2003). In contrast to the heavy metal AQP inhibitors, the organic AQP inhibitors acetazolamide and zonisamide, and the stimulator forskolin, showed no effect on K^+ efflux in barley roots (Fig. 3b), suggesting that the stimulatory effects observed are specific to heavy metal inhibitors. Further experimentation will need to be conducted to explore these novel findings. Moreover, the heavy metals used here that did not modify water relations in Niemietz and Tyerman (2002), namely nickel, cadmium, and lead, also had minimal effects on ion efflux in barley roots (Fig. 3c), and did not appear to cause membrane damage as determined by confocal microscopy (Fig. 6F–H). Curiously, however, K^+ influx was moderately inhibited by Cd^{2+} and Pb^{2+} and strongly inhibited by Ni^{2+} (Fig. 2); reasons for this are presently unclear, but may also be related to cellular energetics. The data demonstrate the clear distinction between heavy metals that block AQPs and those that do not, strongly corroborating the findings of Niemietz and Tyerman (2002). The data also provide an alternative interpretation of the recent (and unexplained) observation that silver nitrate (⩽10 μM) stimulates the release of indole-3-acetic acid from roots of *Arabidopsis thaliana*, independently of its effect on ethylene signalling (Strader *et al.*, 2009).

As in the study of Niemietz and Tyerman (2002), the effect of Ag^+ was the most potent, followed distantly by Au^{3+} and Hg^+ (Fig. 3a). Indeed, when the dose dependence of Ag^+ was investigated, it was found that a 5 μM application had an effect approximately equal to that seen at 500 μM Hg^{2+} and Au^{3+}. This Ag^+ concentration was as effective whether applied as $AgNO_3$ or silver sulphadiazine, a result in contrast to the finding of Niemietz and Tyerman (2002), who showed a greater effect of silver sulphadiazine on water flow (Supplementary Fig. S1 at *JXB* online). Interestingly, the stimulation of K^+ efflux by high provision of NaCl, an effect that has been extensively investigated by numerous groups (e.g. Nassery, 1979; Shabala *et al.*, 2006; Britto *et al.*, 2010), was less pronounced, and less protracted, than that of any of the heavy metal AQP inhibitors examined here. It had previously been proposed (Britto *et al.*, 2010) that NaCl-induced K^+ efflux was also a result of membrane disintegrity, but, in the present study, confocal micrographs of roots stained with propidium iodide did not reveal such damage (Fig. 6I). This may indicate that the destructive effect of NaCl on membranes is below the threshold for entry of this large fluorescent molecule into the cell, a suggestion consistent with the relatively small stimulation of K^+ efflux by NaCl, compared with the heavy metal AQP inhibitors, despite an orders-of-magnitude greater applied concentration. Another possibility is that membrane disintegrity when NaCl is applied may be rapidly reversible through re-annealing of the lipid bilayer, as suggested by the observation of rapid recovery from an initial wilting response to NaCl (not shown). This could conceivably occur through the regulated response of AQPs to osmotic shock, a response not possible in the presence of AQP inhibitors.

It was of further interest to quantify the ionic release from roots treated with silver. Analysis showed that 500 μM Ag^+ application brought about the release of ~5 μmol of K^+ per gram of root (Fig. 3a, internal legend; and Fig. 5). If all the $^{42}K^+$ released were to have originated in the cytosol of root cells, and if all the cytosolic K^+ were released after silver application, this value would correspond to an approximate cytosolic $[K^+]$ of 100 mM, considering that cytosolic volume is ~5% of tissue volume (Lee and Clarkson, 1986), and 1 g of root has a volume of ~1 ml. This amount is remarkably close to cytosolic $[K^+]$ values measured in a large number of studies (for a review, see Britto and Kronzucker, 2008) using techniques as diverse as non-aqueous fractionation (Speer and Kaiser, 1991), longitudinal ion profiling (Jeschke and Stelter, 1976), X-ray microanalysis (Flowers and Hajibagheri, 2001), fluorescing dyes (Halperin and Lynch, 2003), ion-selective intracellular microelectrode analysis (Walker *et al.*, 1996), and compartmental analysis by tracer efflux (Pitman and Saddler, 1967; Kronzucker *et al.*, 2003). Considering that labelling times in the present study were restricted to 60 min, and that vacuolar half-times of exchange for K^+ are in the order of 12–20 h (Memon *et al.*, 1985), contributions from the vacuole to the tracer-release spikes should be negligible (Britto and Kronzucker, 2001). Nevertheless, substantial loss from the vacuole is also sustained during a 15 min application of 500 μM silver, as demonstrated by tissue analysis using flame photometry, which showed that ~40% of total root K^+ is lost (Table 1). Because of these considerations of labelling kinetics and tissue accumulation, therefore, the loss of cytosolic K^+ appears to be essentially complete, while the majority of vacuolar K^+ is retained. Given the importance of K^+ as a major plant nutrient, such a massive loss may be sufficient to explain the cessation of growth in roots treated for 15 min (see above), and the eventual death of the plant when treated for 1 week (not shown). However, the concomitant loss of other intracellular components and the major membrane damage incurred by silver application are additional effects that probably contribute to the lethality of silver ions.

An important fundamental question remains. What causes the observed phenomena? Are the deleterious effects of heavy metal AQP inhibitors on potassium homeostasis and root cell membranes of intact barley plants related purely to their blockage of water flux, or are there other, unrelated, physiological effects caused by these agents? Certainly, the observed inhibition of K^+ influx by low concentrations of Hg^{2+} and Au^{3+} and the concomitant lack of effect on efflux suggest the latter. Moreover, the lack of effect by the organic AQP modulators acetazolamide, zonisamide, and forskolin point towards a phenomenon more specific to heavy metal blockers, rather than

AQP modulators in general. It is interesting to note that the osmotic shrinking assays conducted by Niemietz and Tyerman (2002) did not appear to involve membrane damage in vesicles or erythrocytes, nor did Schreurs and Rosenberg (1982) find evidence that silver induces cell damage or reduces cell viability in *Escherichia coli* cells, even though they observed stimulated efflux of accumulated phosphate, proline, glutamine, mannitol, and succinate. Similarly, Asharani *et al.* (2010) demonstrated that, while silver nanoparticles caused lysis and deterioration of human erythrocytes, silver ions alone showed no such effect. Moreover, it is well known that silver nitrate stimulates callus proliferation, shoot regeneration, and somatic embryogenesis in tissue cultures of many plant species, via its inhibition of ethylene action (Al-Khayri and Al-Bahrany, 2001, and references therein). An open question is whether the elevated efflux and membrane destruction demonstrated here is linked to the scale and complex multicellular nature of the intact, transpiring barley plant, as compared with embryonic, single-celled or subcellular systems, such as callus cultures, *E. coli*, erythrocytes, or membrane vesicles, in which transpiration is limited or absent. Certainly, further experimentation is required to address these issues.

Finally, a note of caution is warranted regarding the use of heavy metal AQP inhibitors, greatly extending the caveats of Santoni *et al.* (2000) (see also Strader *et al.*, 2009). The present investigation into the stimulated efflux observed upon application of heavy metal AQP inhibitors stemmed from the examination of an entirely different problem: that of NH_3 penetration into cells via AQPs. Only after the focus was shifted to questions related to membrane transport and integrity, as well as K^+ homeostasis, did the severe side effects become apparent. These include membrane destruction brought about by high (500 μM) concentrations of Au^{3+} and Hg^{2+}, and a wide range (5, 50, and 500 μM) of Ag^+ concentrations, rendering the cell incapable of retaining critical cellular constituents. Moreover, it was demonstrated that low (5 μM and 50 μM) concentrations of Au^{3+} and Hg^{2+} directly inhibit K^+ influx, without compromising cellular integrity. However, inhibition of K^+ uptake will have wide-ranging effects on numerous aspects of plant function (Britto and Kronzucker, 2008), and, because K^+ is the most abundant cellular osmoticum, blockage of its uptake may in itself change water gradients in the longer term, confounding the use of heavy metal AQP inhibitors in the study of plant water relations. The present study points to the need for a more discriminating use of inhibitors as a tool for physiological examination, particularly with heavy metal AQP inhibitors *in planta*.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Response of K^+ influx into roots of intact barley (*Hordeum vulgare* L.) seedlings to 10 min incubation in 5 μM $AgNO_3$ and Ag-sulphadiazine. Inset: response of $^{42}K^+$ efflux from roots of intact barley (*Hordeum vulgare* L.) seedlings to sudden provision (see arrow) of 5 μM $AgNO_3$ and Ag-sulphadiazine. Asterisks represent significantly different means from control (one-way ANOVA with Dunnett's post-test). Each plot represents the mean of 3–7 replicates. Error bars indicate ±SEM.

Figure S2. Effect of 500 μM $HAuCl_4$ and $AgNO_3$ on the water potential in barley roots. Each plot represents the mean of 5–19 replicates. Error bars indicate ±SEM.

Table S1. Survey of published studies using mercury, silver, and gold to examine aquaporin function, water flow, or ethylene biosynthesis (asterisks).

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