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Cadmium impairs ion homeostasis by altering K^+ and Ca^{2+} channel activities in rice root hair cells

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ABSTRACT

Cadmium (Cd^{2+}) interferes with the uptake, transport and utilization of several macro- and micronutrients, which accounts, at least in part, for Cd^{2+} toxicity in plants. However, the mechanisms underlying Cd^{2+} interference of ionic homeostasis is not understood. Using biophysical techniques including membrane potential measurements, scanning ion-selective electrode technique for non-invasive ion flux assays and patch clamp, we monitored the effect of Cd^{2+} on calcium (Ca^{2+}) and potassium (K^+) transport in root hair cells of rice. Our results showed that K^+ and Ca^{2+} contents in both roots and shoots were significantly reduced when treated with exogenous Cd^{2+}. Further studies revealed that three cellular processes may be affected by Cd^{2+}, leading to changes in ionic homeostasis. First, Cd^{2+}-induced depolarization of the membrane potential was observed in root hair cells, attenuating the driving force for cation uptake. Second, the inward conductance of Ca^{2+} and K^+ was partially blocked by Cd^{2+}, decreasing uptake of K^+ and Ca^{2+}. Third, the outward K^+ conductance was Cd^{2+}-inducible, decreasing the net content of K^+ in roots. These results provide direct evidence that Cd^{2+} impairs uptake of Ca^{2+} and K^+, thereby disturbing ion homeostasis in plants.

Key-words: cadmium toxicity; K^+ and Ca^{2+} transport; patch clamp; rice; ~~SIET~~.

INTRODUCTION

In higher plants, cadmium (Cd^{2+}) is a non-essential element that interferes with plant growth and development. Although the toxic effects of Cd^{2+} in plants have been documented by a growing number of reports, the mechanisms underlying Cd^{2+} toxicity are not well understood. The morphological toxic effects of Cd^{2+} include leaf rolling and chlorosis, prevented seed germination and root elongation (Fan *et al*. 2011) and reduced growth in both in stems and in roots (Aidid & Okamoto 1993). Cd^{2+} interferes with several physiological processes including stomatal opening and transpiration and photosynthesis (Barcelo & Poschenrieder 1990; Costa & Morel 1994; Sanitá di Toppi & Gabbrielli, 1999). The light-harvesting complex II (Krupa 1988) and the photosystems II and I (Siedlecka & Baszynsky 1993; Siedlecka & Krupa 1996) can be damaged by high levels of Cd^{2+}. Furthermore, Cd^{2+} inhibits mitochondrial oxidative phosphorylation and causes increased activity of antioxidant enzymes (Zhang *et al*. 2005; Verma *et al*. 2008). Cd^{2+} also reduced the absorption of nitrate and its transport from roots to shoots, and may cause oxidative stress (Hernandez, Carpena-Ruiz & Garate 1996).

Potassium and calcium are the very important inorganic macronutrients of the living cell. Excessive Cd^{2+} in the medium has been shown to interfere with the uptake, transport and utilization of several macro- and micronutrients, even though the underlying mechanisms remain highly controversial. Since the photosynthesis mainly relies and directly depends on the root mineral and water uptake, any change in root transport would control leaf functions, thereby influencing the productivity. The contents of polyvalent cations are affected by Cd^{2+}, possibly through competition for binding sites of proteins or transporters (Gussarson *et al*. 1996), In the hydroponic condition, Cd^{2+} exposure significantly reduced N, accumulation in rice roots and shoots irrespective N forms (Hasson, Zhang & Zhu 2008). A decrease of calcium content was observed when Cd^{2+} treatments were applied to birch and pea leaves (Gussarson *et al*. 1996; Sandalio *et al*. 2001; Rodríguez-Serrano *et al*. 2009). Using the vibrating electrode technique, a clear inhibition of calcium influx into *Arabidopsis* root hairs was demonstrated recently (Fan *et al*. 2011). However, an increase of calcium content was also reported in *Mesembryanthemum crystallinum* (Ghnaya *et al*. 2005). Such discrepancy was also found when studying Cd^{2+} effect on potassium nutrition. The K^+ uptake into oat root segments was inhibited to 80% of control after 30 min in the presence of 1 mM Cd^{2+} (Keck 1978). However, an increase in K^+ concentration in oat roots was reported by another group (Cie'cko, Kalembasa & Wyszkowski 2004). Same controversy was also found in maize, yellow lupine, radish, pea and *M. crystallinum* (Carpena-Ruiz & Gárate 1996; Cie'cko *et al*. 2004; Ghnaya *et al*. 2005). These inconsistent results are probably due to differences in plant species, organs and/or assay methods used in various studies. Such studies make it difficult to interpret and a model plant and consistent assay system are therefore critically needed to decipher the true effects and underlying mechanism of Cd^{2+} toxicity on mineral nutrition in plants.

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Rice is an important crop worldwide and is considered to be a model plant among monocots for biological research because of its small genome size (Goff *et al*. 2002). Studies on Cd^{2+} uptake and toxicity in rice plants are important, by which Cd^{2+} content in rice grains will be reduced to avoid accumulation of toxic Cd^{2+} in human. In regions where rice is the staple food source, rice grains have been the major source of Cd^{2+} intake in human. To reduce the Cd^{2+} deposition in rice grain, studies have focused on absorption, transport and accumulation of Cd^{2+} in rice (Tanaka *et al*. 2007; Fujimaki *et al*. 2010; Tezuka *et al*. 2010; Ishikawa *et al*. 2010; Kashiwagi *et al*. 2009; Uraguchi *et al*. 2009). It is also reported that there is a complicated relationship between the absorption and accumulation of Cd^{2+} and status of micromineral nutrients (Fe, Zn, Mn, Cu and Mg) in rice plants (Rubio *et al*. 1994; Liu *et al*. 2003). However, little is known about how Cd^{2+} exposure influences the uptake, transport and homeostasis of macronutrients such as K^+ and Ca^{2+} in rice plants.

In this study, we chose rice cultivar 'Nipponbare' as an experimental material, and investigated the effects of Cd^{2+} application on the uptake of calcium and potassium. We isolated protoplasts from root hairs of rice seedlings, and detected the whole-cell calcium and potassium currents across plasma membrane. The results showed that Cd^{2+} exposure disturbed K^+ and Ca^{2+} homeostasis by altering ion channel activities in the root cells.

MATERIALS AND METHODS

Plant materials and growth conditions

Rice (*Oryza sativa* cv. 'Nipponbare') seeds were surface sterilized with 75% alcohol for 15 min and germinated as described previously (Zhao & Qin 2005). The 4-day-old seedlings were transplanted into plastic pots containing mesh-washed perlite. The plants were grown in one-half-strength Hoagland solution (renewed every two days) at a photon flux density of 900 to 1000 μmol m^{-2} s^{-1} with 11/13 h day/night cycle at 28 °C/25 °C in the phytotron. Plants at the four-leaf stage (about 30 d after germination) were treated with $CdCl_2$ except control as described previously (Perfus-Barbeoch *et al*. 2002). After 7 d treatment with $CdCl_2$, the rice plants were harvested to detect Ca^{2+} and K^+ contents. For electrophysiological measurements (ion flux and membrane potential recordings), 5-day-old rice seedlings were grown hydroponically as described earlier.

For patch-clamp experiments, ~~scanning ion-selective electrode technique (SIET)~~ and membrane potential measurements, seeds of rice were surface sterilized in an aqueous solution of 1% NaClO for 5 min. The seeds were then washed several times with filter-sterilized water and placed on wet filter paper in Petri dishes that had been exposed to ultraviolet light. The Petri dishes were sealed with parafilm and stored in the dark for 3 d at 28 °C, by which the germinated seeds had roots approximately 3 to 4 cm long with visible root hair growth.

Relative chlorophyll content determination

After 7 d treatment with $CdCl_2$ as described earlier, chlorophyll content was measured on the second leaf of each plant. Chlorophyll content (estimated as a chlorophyll content index, or CCI) was measured using a Minolta Chlorophyll Meter SPAD-502 (Konica Minolta, Tokyo, Japan) according to manufacturer's instruction.

Ca^{2+} and K^+ determination

The roots and shoots of the plants were rinsed with deionized water three times and then dried at 80 °C to a constant weight after filtration with Whatman paper. A total of 0.1 g dry powder samples were then extracted with 2 mL HCl and HClO at 50 °C 48 h to release the cations and centrifuged at 10 000 g for 10 min. The resulting supernatants of the extracts were diluted and Ca^{2+} and K^+ were determined using an ICP-MS (ELAN 9000, PE).

~~SIET~~ for ion flux analysis

Root hair tissues were left immersed in the base solution (0.5 mM KCl, 0.1 mM $CaCl_2$, pH 5.6 unbuffered). To create a stable condition, several little stone panes were used to fix the materials in a measuring chamber of volume approximately 5 mL. Then the chamber was mounted on the micromanipulator, and the electrodes were positioned closely to the tip of a selected root hair and left to equilibrate. Net fluxes of Ca^{2+}, K^+ and H^+ were measured using ~~SIET~~ (the ~~SIET system~~ BIO-001A; ~~Younger USA Sci. & Tech. Corp.; Applicable Electronics Inc.; and ScienceWares Inc.;~~ Sun *et al*. 2009). The concentration gradients of the target ions were measured by moving the ion-selective microelectrode between two positions close to the plant material in a preset excursion (20 μm in our experiment) at a programmable frequency of 0.18 Hz. The ~~SIET~~ can measure ionic fluxes down to picomolar levels but must be measured slowly at approximately 1 to 2 s per point. This is mainly due to the mechanical disturbance of gradient by the electrode movement, although the time constant of the liquid ion exchanger (LIX) electrodes is also a factor. The ionic gradient is accurately measured due to the following reasons: (1) while conducting the experiment, the electrode stays in one position for a certain time (from 0.8 to a few seconds) to let the gradient recover before it takes a reading of that position; (2) the time the electrode spends in one position is also needed for the electrode to get stabilized to give a reliable measurement. In fact, the time spent on electrode stabilization is longer than the time for gradient recovery, which is one of the key factors determining the efficiency of electrode (Kunkel *et al*. 2006). The electrode is stepped from one position to another in a predefined sampling routine while also being scanned with the three-dimensional microstepper motor manipulator (CMC-4).

Pre-pulled and silanized glass micropipettes (2–4 μm aperture, XYPG120-2; ~~Xuyue Sci. and Tech. Co., Ltd, Beijing, China~~) were first filled with a backfilling solution (Ca^{2+}:

100 mM $CaCl_2$; K^+: 100 mM KCl; H: 40 mM KH_2PO_4 and 15 mM NaCl, pH 7.0) to a length of approximately 1 cm from the tip. Then the micropipettes were front filled with approximately 15 μm columns of selective liquid ion-exchange cocktails (LIX; Ca^{2+}: Fluka 21048; K^+: Fluka 60398; H: Fluka 95293, Gallen, Switzerland). Before detecting, all the electrodes were measured for ion selectivity. An Ag/AgCl wire electrode holder (XYEH01-1; ~~Xuyue Sci. and Tech. Co., Ltd~~) was inserted in the back of the electrode to make electrical contact with the electrolyte solution. DRIREF-2 (Word Precision Instruments, Sarasota, FL, USA) was used as the reference electrode. Ion-selective electrodes of the following target ions were calibrated prior to flux measurements: (1) Ca^{2+}: 0.05 mM, 0.1 mM, 1.0 mM (Ca^{2+} concentration was usually 0.1 mM in the measuring buffer for root samples); (2) K^+: 0.1 mM, 0.5 mM, 2.0 mM (K^+ concentration was usually 0.5 mM in the measuring buffer for root samples); (3) H^+: pH 5.5, 6.0, 6.5 (pH of the measuring buffer was usually adjusted to 6.0 for root samples).

Only electrodes with Nernstian slopes >50 mV/decade (>25 mV/decade for Ca^{2+} electrode) were used in our study. Ion flux was calculated by Fick's law of diffusion:

$$J = -D(dc/dx)$$

where J represents the ion flux in the x direction, dc/dx is the ion concentration gradient, and D is the ion diffusion constant in particular medium. The steady fluxes were measured for 10–20 min to make sure that a steady-state condition was reached. Then Cd^{2+} treatment was given, and transient Ca^{2+}, K^+ and H^+ kinetics were measured for another 30–40 min. Flux data were obtained with the ASET software, which is part of the ~~SIET system.~~ They were finally converted into specific ion fluxes (pmol $cm^{-2}\,s^{-1}$), using MageFlux, developed by Xu-Yue company (http://xuyue.net/mageflux).

Membrane potential measurement

Ag/AgCl microelectrodes filled with KCl (Maathuis & Sanders 1993) with a tip diameter of about 0.5 μm were used to measure Cd^{2+}-induced kinetics of membrane potential in root epidermal cells. Intact 3-day-old germinated rice seed was mounted in a measuring chamber by puncturing the root through a pair of parallel holes raised on the bottom. After 50–60 min equilibration, membrane potential was recorded when it was regaining stabilization following cell penetration (Shabala *et al*. 2010). Then, a $CdCl_2$ stock solution was added, and the changes of membrane potential were recorded. $CdCl_2$ was washed away with bath solution containing 0.1 mM KCl and 0.1 mM $CaCl_2$ in the end of the experiment to observe the recovery of membrane potential from $CdCl_2$ treatment.

Root hair protoplast preparation

Protoplasts were isolated using a protocol modified from our previous procedure (Zhao *et al*. 2007). Whole intact seedlings with roots were placed in 5 mL of enzyme solution in water bath shaker (100 rpm) at 30 °C for 45 min. The enzyme solution contained (in mM) 10 KCl, 2 MES/KOH, 1 $CaCl_2$, pH 5.7, and 1.5% (w/v) cellulase (Yakult Honsha, Tokyo, Japan), 0.1% (w/v) pectolyase Y23 (Yakult Honsha) and 0.1% bovine serum albumin. The osmolality of the enzyme solution was adjusted to 650 mOsm with D-sorbitol. After enzyme treatment, the seedlings were pulled out and the digest solution was filtered using 50 μm nylon mesh and centrifuged at 100 g for 10 min. The pellet was resuspended in 8 mL of ice-cold bath solutions and centrifuged again. Large protoplasts (about 15 to 20 μm in diameter) with visible cytoplasmic streaming were selected for patch clamping.

Patch-clamp recordings

Patch-clamp pipettes were pulled from glass capillaries on a puller (P-97, Sutter Instrument, Novato, CA, USA) and fire polished to a tip resistance of 10 to approximately 15 MΩ. Whole-cell currents were measured using patch-clamp technique with an amplifier (Multiclamp 700 B, Axon). Digital low-pass Bessel filtering of whole-cell currents elicited by positive-going voltage pulses was performed at a cut-off frequency of 2.9 kHz. Data were analysed with the software Clampex and Clampfit (version 10.0).

Standard pipette solutions for K and Ca conductance measurements contained (concentrations in mM): for K^+ currents, 90 K-gluconate, 10 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 1.4 ethylene glycol tetraacetic acid (EGTA), 1.5 Mg-ATP, 2 HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) adjusted to pH 7.2 with Tris and 550 mOsm using D-sorbitol; for Ca^{2+} currents, as described previously (Pei *et al*. 2000; Zhang, Fan & Wu 2007), 10 $BaCl_2$, 2 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, and 10 HEPES/Tris, pH 7.1, and the osmolality was adjusted to 500 mOsm with D-sorbitol. For analysing the possible influence of Ba^{2+} in pipette solution on the Ca^{2+} conductance, Ba^{2+} pipette solution was replaced with standard K^+ pipette solution, in which free Ca^{2+} adjusted to 100 nM. Bath solution consisted of (in mM): for K^+ currents, 30 K-gluconate, 2 KCl, 10 HEPES, 2 $MgCl_2$, 2 $CaCl_2$ (adjusted to pH 5.8 with MES and 500 mOsm using D-sorbitol); for Ca^{2+} currents, 50 $BaCl_2$, 10 MES/Tris, pH 5.6, and the osmolality was adjusted to 500 mOsm with D-sorbitol. Considering the variations in the cell surface area, some final whole-cell currents were expressed as current density (μA/cm^2). $CdCl_2$ and some specific ion channel blockers were applied to bath solution as mentioned in the figure legends.

RESULTS

Whole-plant toxicity of Cd^{2+} and ICP test

Under hydroponic condition, a wide range of Cd^{2+} concentrations (from 0.001 mM to 5 mM) in the solutions have been reported previously (Rout, Samantaray & Das 2000; Hsu & Kao 2004; Tanaka *et al*. 2007; Hasson *et al*. 2008; Rodríguez-Serrano *et al*. 2009), and the exogenous Cd^{2+} is

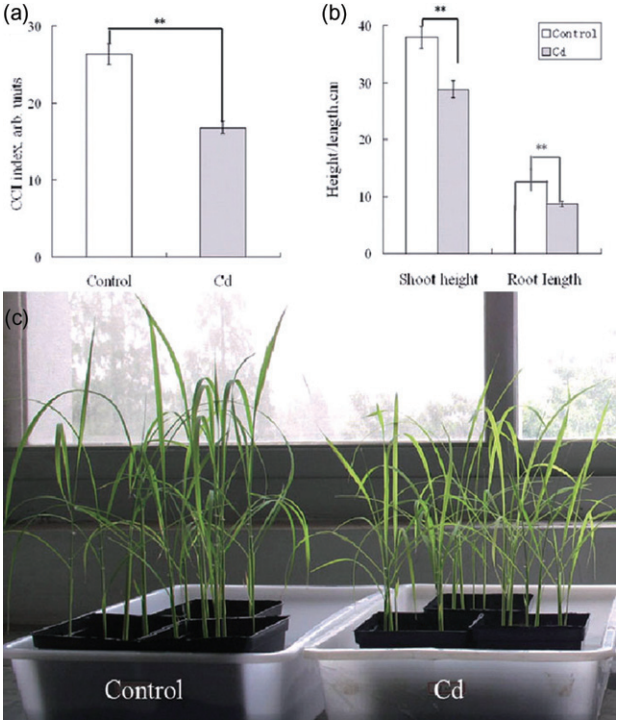

Figure 1. Effects of exogenous application of cadmium at 0.05 mM on growth and chlorophyll content of rice. (a) Chlorophyll content index (CCI) measured from intact leaves. (b) Effect of Cd on shoot height and root length. Open bars, root length; closed bars, shoot height. (c) Symptom of Cd toxicity in rice – chlorosis. ** indicate the difference at $P < 0.01$ by Student's *t*-test. Data represent mean ± SD.

exceptionally toxic even at a low concentration of 0.001 mM (Hasson *et al*. 2008). In this study, we added a medium concentration of 0.05 mM Cd^{2+} in the hydroponic culture and determined its effect on the growth of rice seedlings by measuring several parameters including chlorophyll content, K^+ and Ca^{2+} contents, root length and seedling height. The chlorophyll content was significantly decreased by exogenous Cd^{2+} (Fig. 1a), and the seedlings showed an obvious symptom of Cd^{2+} toxicity – chlorosis (Fig. 1c). Furthermore, the presence of Cd^{2+} in the culture solution caused a strong inhibition of plant growth (Fig. 1b). In addition, the Ca^{2+} contents in roots and shoots were significantly decreased by the application of 0.05 mM $CdCl_2$ ($P < 0.01$) (Fig. 2a). The root K^+ content was also significantly decreased by $CdCl_2$, although the shoot K^+ content was not changed significantly (Fig. 2b). These results indicated that 0.05 mM $CdCl_2$ is sufficient to induce an obvious Cd^{2+} toxicity in rice seedlings. Therefore, we chose 0.05 mM Cd^{2+} to investigate its effects on ion uptake in rice seedling roots in the following experiments.

Cd^{2+}-induced ion flux kinetics from rice root hairs

To further understand how Cd^{2+} exposure influences the potassium and calcium levels in rice seedlings, we measured net Ca^{2+} and K^+ flux responses in rice root hairs (Fig. 3a) using the ~~SIET~~. Adding 0.05 mM $CdCl_2$ to the bath resulted in a remarkable increase in Ca^{2+} and K^+ efflux to the vicinity of root hairs, accompanied by a substantial decrease in net

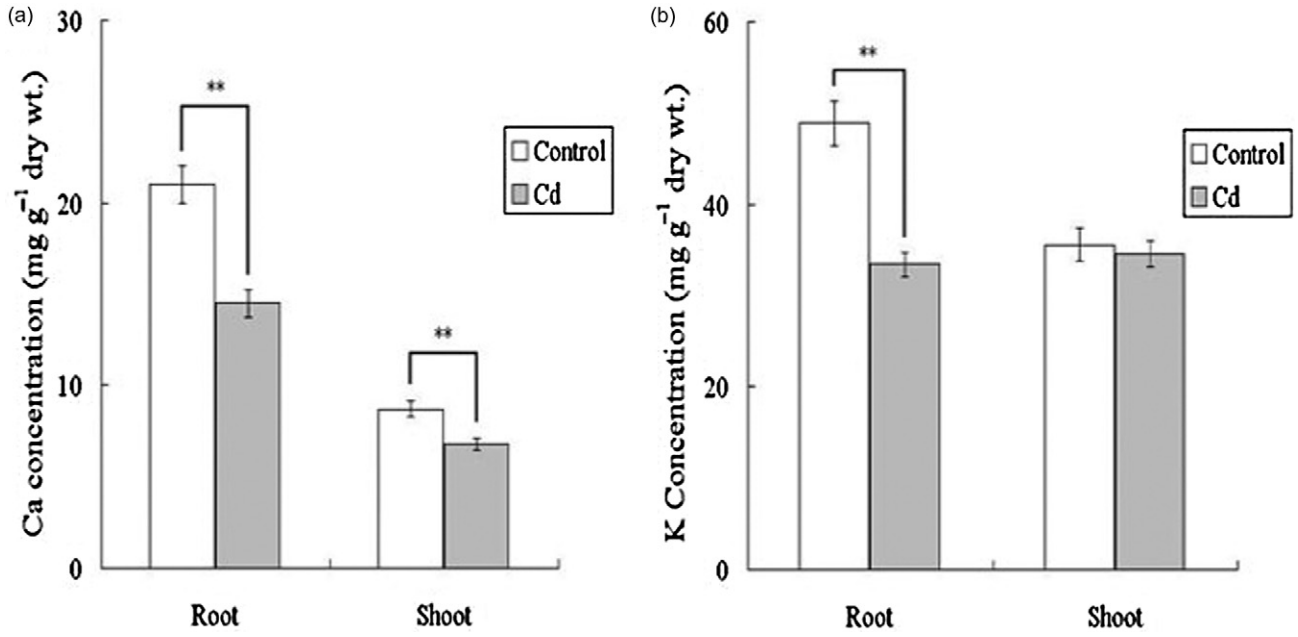

Figure 2. Effects of exogenous application of cadmium on Ca^{2+} and K^+ distribution in rice plants. (a) Ca^{2+} contents in roots and shoots of rice seedlings treated with 0.05 mM $CdCl_2$. (b) K^+ contents in roots and shoots of rice seedlings treated with 0.05 mM $CdCl_2$. ** indicate the difference at $P < 0.01$ by Student's *t* test. Data represent mean ± SD.

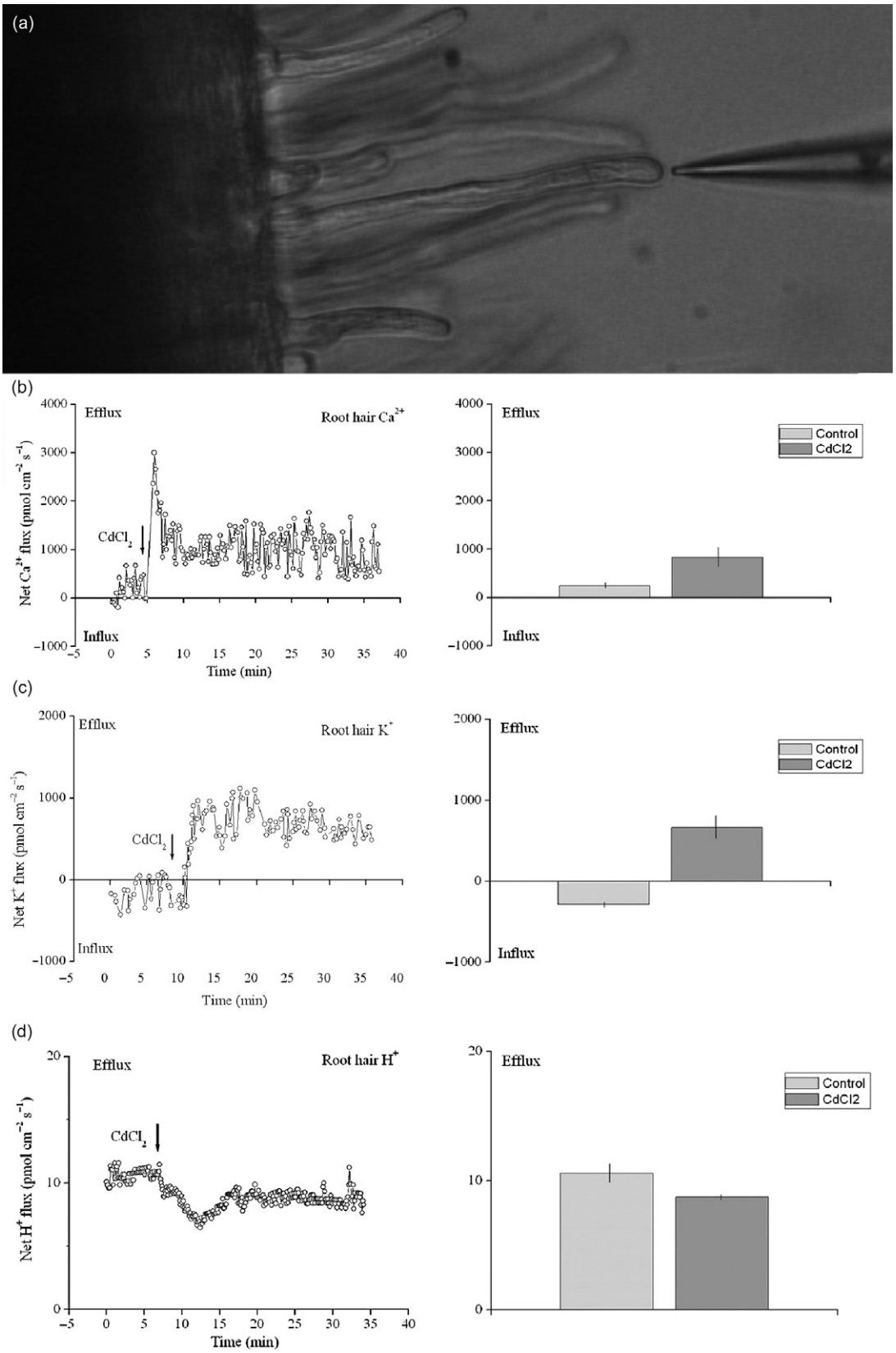

Figure 3. Effects of cadmium on kinetics of net Ca^{2+} and K^+ fluxes in rice root hairs. (a) Rice root hair tissue. (b) Effects of 0.05 mM cadmium on kinetics of net Ca^{2+} fluxes in rice root hair ($n = 15$). (c) Effects of 0.05 mM cadmium on kinetics of net K^+ fluxes in rice root hair ($n = 15$). (d) Effects of 0.05 mM cadmium on kinetics of net H^+ fluxes in rice root hair ($n = 15$). The sign convention to be used is 'efflux positive'.

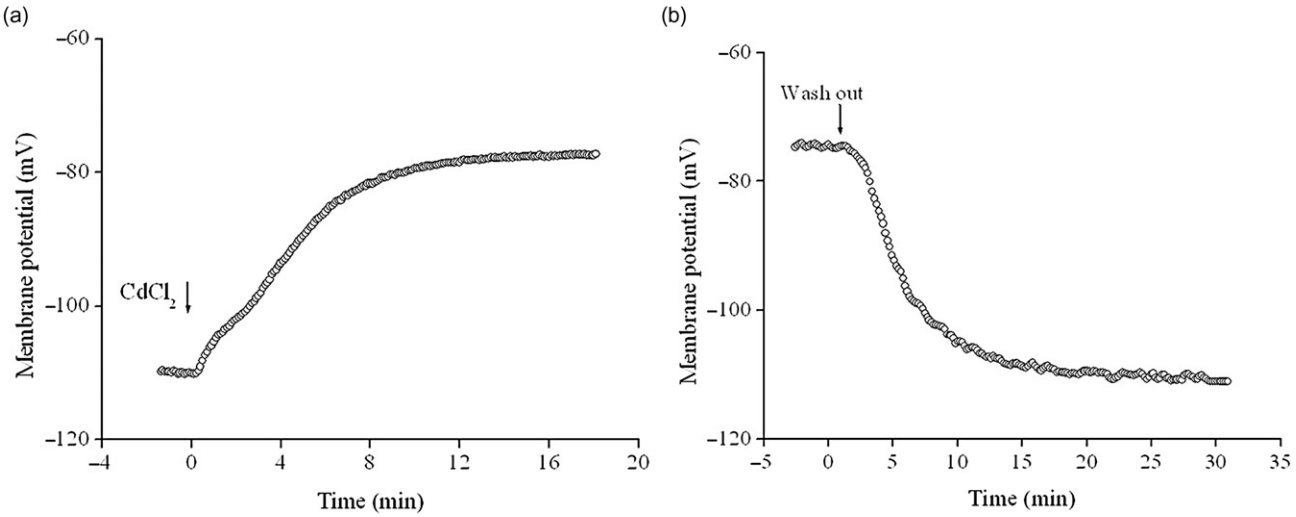

Figure 4. $CdCl_2$-induced changes in membrane potential (*Em*) of rice epidermal cells. (a) Transient *Em* kinetics of rice epidermal cell in response to 0.05 mM $CdCl_2$ treatment. One (of 16) typical example is shown. (b) The depolarization could be reversed after washing the root hairs with bath solution ($n = 16$).

H^+ efflux (Fig. 3d). As shown in Fig. 3b,c, the Ca^{2+} and K^+ efflux reached peak levels after few seconds of $CdCl_2$ exposure, and remained significantly ($P < 0.05$, $n = 15$) higher than the efflux without Cd^{2+} treatment, on average, a sevenfold and an 11-fold increase from the control respectively. The Ca^{2+} efflux caused by Cd^{2+} application in our SIET experiment probably was also correlated with ion exchange in the cell wall based on Donnan system (Arif & Newman 1993; Shabala & Newman 2000). To verify if the observed Ca^{2+} efflux originated from the Cd^{2+}/Ca^{2+} exchange in the cell wall, or from the activity of plasma membrane Ca^{2+} transporters, the root hair was pretreated with 0.1 mM Gd^{3+}, a known inhibitor of plasma membrane Ca^{2+} channels. After 20 min of Gd^{3+} pretreatment, blocking the Ca^{2+} channels with Gd^{3+} had a large effect on the Cd^{2+}-induced Ca^{2+} efflux, on average, an 8.5-fold increase from the control (without Gd^{2+} pretreatment and Cd^{2+} treatment) (Supporting Information Fig. S2). Based on these data, we can clearly conclude that part of the net Ca^{2+} efflux measured from Cd^{2+}-treated rice root hairs originated from the activity of plasma membrane Ca^{2+} transporters, and part from the cell wall.

Cd^{2+}-induced changes in the membrane potential of rice root epidermal cells

To address the underlying mechanisms responsible for the observed Cd^{2+}-induced ion efflux from rice root hair cells, we measured the membrane potential. At steady state, in a bath solution containing 0.1 mM KCl and 0.1 mM $CaCl_2$, membrane potential values were in the range of –110 to –130 mV in root epidermal cells (–118 ± 7 mV, $n = 15$). Addition of 0.05 mM $CdCl_2$ in the bath solution depolarized the *Em* (membrane potential) gradually (Fig. 4a). This depolarization lasted for 10 min until the *Em* values became stable around –70 mV ($n = 16$, $P < 0.001$). The Cd^{2+}-induced depolarization in root epidermal cells could be reversed after washing the root hairs with bath solution ($n = 16$, $P < 0.001$).

Effects of extracellular Cd^{2+} on Ca^{2+}-permeable channel activities in root hair cells

The ionic contents and fluxes across plasma membrane are largely controlled by membrane transporters; some of which are ion channels. To further address whether Cd^{2+}-induced inhibition of K^+ and Ca^{2+} uptake is caused by altered ion channel activities, we applied patch-clamp technique to investigate Ca^{2+} and K^+ conductances in root hair cells. Thus, we prepared protoplasts from rice root hairs (Fig. 5) and recorded the currents in whole-cell configuration in the presence or absence of Cd^{2+} in the bath solution.

To analyse Cd^{2+}-inhibited Ca^{2+} influx conductance, we used Ba^{2+} ions, which are permeant to Ca^{2+} channels, in the pipette and bath solutions. The inwardly directed Ca^{2+} conductances were detected under two types of voltage clamp protocols using voltage steps and ramps before and after Cd^{2+} addition. In voltage step recording model, the membrane potential was a series of hyperpolarizing steps of 2 s duration from a holding potential of 10 mV (i.e. close to the resting potential of the spheroplasts in the patch-clamping solutions). Under control condition (without Cd^{2+} in bath solution), a large inwardly directed hyperpolarization-activated Ca^{2+} channel (HACC) conductance could be detected in all protoplasts derived from rice root hair cells 30–40 min after establishing the whole-cell configuration (Fig. 6a). After addition of 0.05 mM $CdCl_2$ in bath solution, inward Ca^{2+} current was inhibited dramatically as compared with control without Cd^{2+} (Fig. 6b,c). On average,

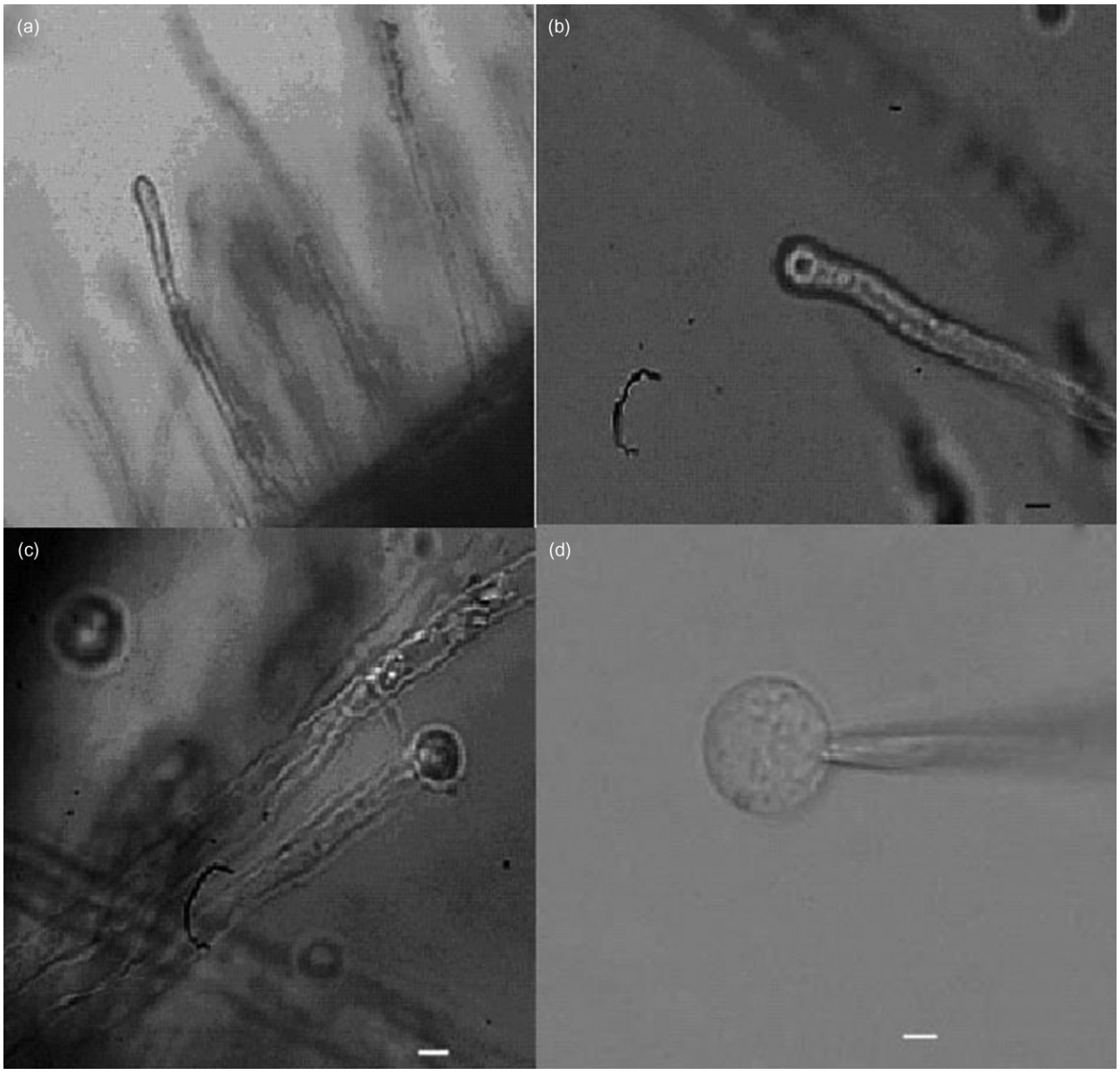

Figure 5. Isolation of rice root hair protoplasts. (a) Intact and healthy rice root hairs. (b) Swelling root hair tip after 30 min of digestion in enzyme solution. (c) root hair cell protoplasts round up after 45 min of digestion in enzyme solution. (d) Giga ohm seal was obtained with an isolated root hair protoplast. Scar bar is 20 μm in b and c, 10 μm in d.

the current densities at –180 mV were decreased from –17.4 ± 2.5 to –8.1 ± 1.5 μA/cm^2 in response to 0.05 mM $CdCl_2$ ($P < 0.01$) (Fig. 6c), a 53% decrease from the control. To investigate the ionic selectivity of Ca^{2+} conductances, we analysed tail current (Fig. 6d). The reversal potential of inward current ranged from 0 to 20 mV (mean ± SE = +11 ± 2 mV, $n = 8$). This was close to the equilibrium potential for Ba^{2+} ($E_{Ba^{2+}} = 20$ mV), and far from the Cl^- equilibrium potential ($E_{Cl^-} = -41.3$ mV), showing that the Ca^{2+} conductances we recorded are carried predominantly by Ba^{2+}. In voltage ramp recording model, the membrane potential was slowly ramped from –180 mV to 80 mV in 26 s after a 1 s holding pulse at –180 mV to activate the Ca^{2+} conductance. The inhibition of 0.05 mM $CdCl_2$ application could also be found under voltage ramps (Fig. 6e). On average, the current densities at –180 mV were decreased from –14.1 ± 1.5 to –6.1 ± 0.9 μA/cm^2 in response to 0.05 mM $CdCl_2$ ($P < 0.01$) (Fig. 6e,g), a 64% decrease from the control. We applied rapid voltage ramps with a fivefold bath-cytosol $BaCl_2$ gradient to identify the ionic selectivity of Ca^{2+} conductances (Fig. 6f). Under voltage ramp protocol, the mean ± SE reversal potential (E_{rev}) of +14 ± 3 mV ($n = 16$) was very close to the equilibrium potential for Ba^{2+} ($E_{Ba^{2+}} = 20$ mV), and far from the Cl^-

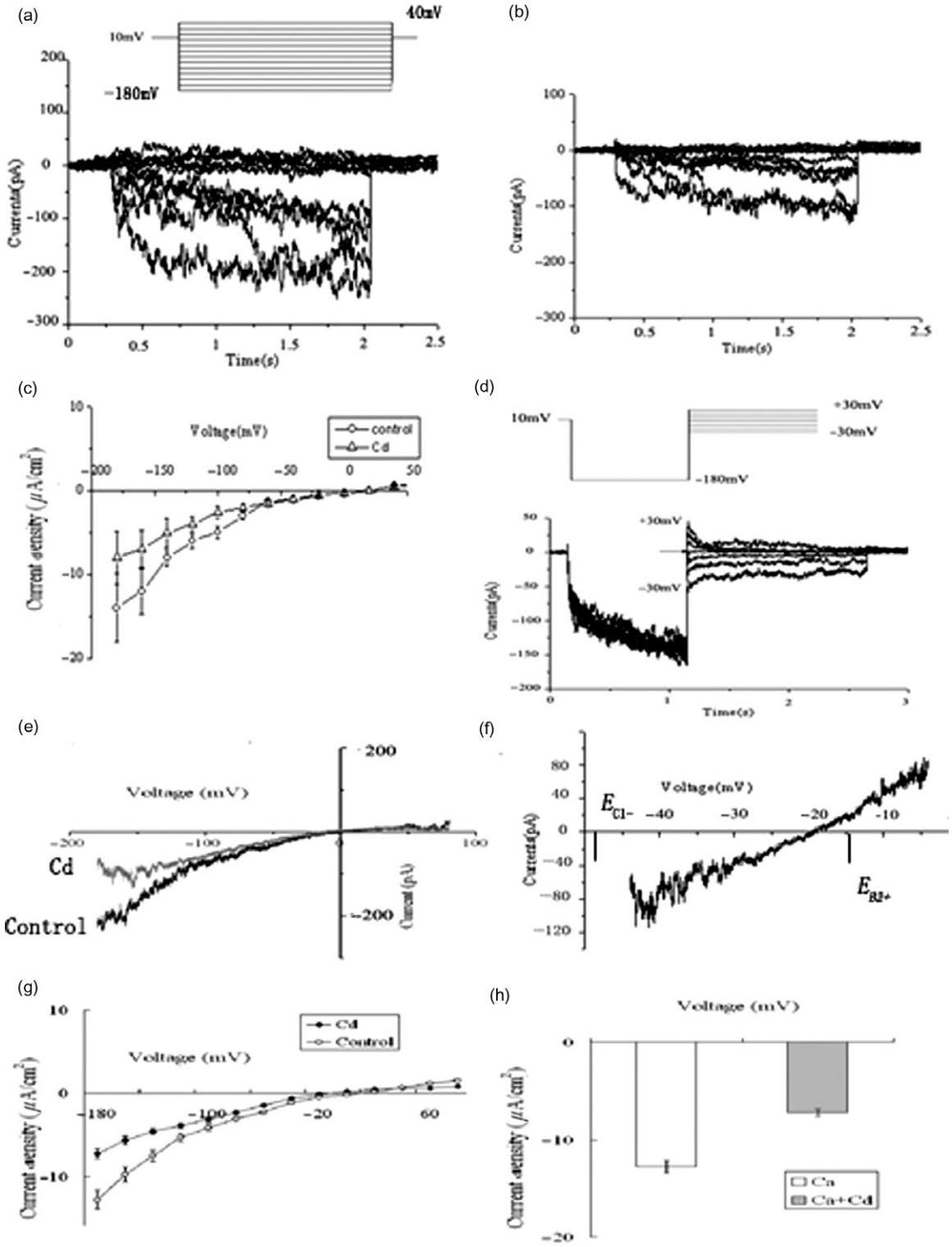

Figure 6. Effects of extracellular cadmium on the Ca^{2+} currents in rice root hair cells. (a) Whole-cell recording of inward Ca^{2+} current (capacitance = 5 pF). (b) The same cell as in (a) was treated with 0.05 mM $CdCl_2$ in the bath solution for 30 min. The current was recorded by a 0.5 s prepulse at 10 mV followed by voltage steps of 40 to –180 mV (in 20 mV decrements, 1.5 s duration), followed by a 0.5 s deactivation at 10 mV. (c) Current–voltage relationship from cells as in (a, b) (n = 19 cells). (d) Evidence for Ca^{2+} current in the inward currents by tail current analysis; the arrow marks current reversal. (e) Whole-cell recording of Ca^{2+} current under voltage ramp protocol. The current was recorded by performing slow ramps of potential (from –180 to 80 mV, 10 mV s^{-1}) after a 1 s holding pulse at –180 mV to activate the hyperpolarization-activated Ca^{2+} currents. (f) Whole-cell current recorded during a rapid voltage ramp (–30 to +40 mV; 50 mV s^{-1}). E_{Cl^-},E_{Ba}, Cl^- and Ba^{2+} equilibrium potentials, respectively. (g) Current–voltage relationship from cells as in (e) (n = 27 cells). (h) Whole-cell Ca^{2+} current densities at –180 mV before and 30 min after treatment with 0.05 mM $CdCl_2$. The data present mean ± SD.

equilibrium potential ($E_{Cl^-} = -41.3$ mV), again, showing that the Ca^{2+} conductances we recorded are carried by Ba^{2+}.

Clearly, this is non-physiological condition with the Ba^{2+} in pipette solution, because Ba^{2+} is very similar to Ca^{2+} and may affect Ca^{2+}-mediated signalling/regulatory pathways, and therefore may change transmembrane currents. The aim of this study is to detect the effect of Cd^{2+} application on Ca^{2+} influx conductance. To exclude the possible artificial influence of Ba^{2+} on the Cd^{2+}- inhibited Ca^{2+} influx conductance, we replaced Ba^{2+} pipette solution with the standard potassium pipette solution, in which free Ca^{2+} adjusted to 100 nM, and replaced Ba^{2+} in the bath with Ca^{2+}. The results showed that the magnitude discrepancy of Ca^{2+} conductances was not significant between two different ionic bath and pipette solutions, and the inhibitory effect of Cd^{2+} was comparable (data not shown). By applying rapid voltage ramps, with 100 nM free Ca^{2+} in pipette solution, the mean ± SE reversal potential (E_{rev}) was shifted towards the estimated equilibrium potential of Ca^{2+} ($E_{Ca} = 167$ mV) from +14 ± 3 mV ($n = 16$) to +62 ± 5 mV ($n = 9$) as compared with Ba^{2+} in pipette solution, indicating Ca^{2+} permeation of this hyperpolarization-activated channel. Despite almost the same inhibitory effect of Cd^{2+} could be detected in two different ionic pipette solutions, since Ba^{2+} pipette solution is non-physiological condition, the conclusion we got here still can only be speculated on; further experimentation would be necessary to dismiss this hypothesis irrefutably.

Moreover, the inhibitory effect of Cd^{2+}on the inwardly directed Ca^{2+} current in the root hair cells was reversible by washout with bath solution (data not shown). To investigate effects of Cd^{2+} at different concentrations on inward Ca^{2+} conductance, we added a series of concentrations of Cd^{2+} in bath solution ranging from 1 μM to 50 μM. The results showed that the inhibition of exogenous Cd^{2+} on inward Ca^{2+} conductance was in a dose-dependent manner when the Cd^{2+} concentration was lower than 10 μM (Fig. 7). Above 10 μM, the response was not changed significantly with the increase of Cd^{2+} concentration (Fig. 7).

To further confirm the characteristics of the recorded inward conductances, specific Ca^{2+} and K^+ channel inhibitors were added in bath solution. Gd^{3+} was widely used as non-selective cation channel blocker (Davenport & Tester 2000; Demidchik & Maathuis 2007), and verapamil was reported as an antagonist of Ca^{2+} channels (Demidchik *et al*. 2002; Kurusu *et al*. 2005). As shown in Fig. 8, the inward Ca^{2+} currents were dramatically blocked by 0.1 mM Gd^{3+} and partially blocked by 0.02 mM verapamil. To exclude the possibility that the inward Ca^{2+} currents we observed here were conducted by K^+, a specific blocker of K^+-selective channels tetraethylammonium chloride (TEA^+) was used. The result showed that treatment of 10 mM TEA^+ had no effect on the Ca^{2+} conductances (Fig. 8). To further exclude the possibility that Cl^- efflux might contribute the conductances we recorded here, a potent blocker of anion channels NPPB (5-nitro-2,3-phenylpropylaminobenzoic acid) was used in this study.

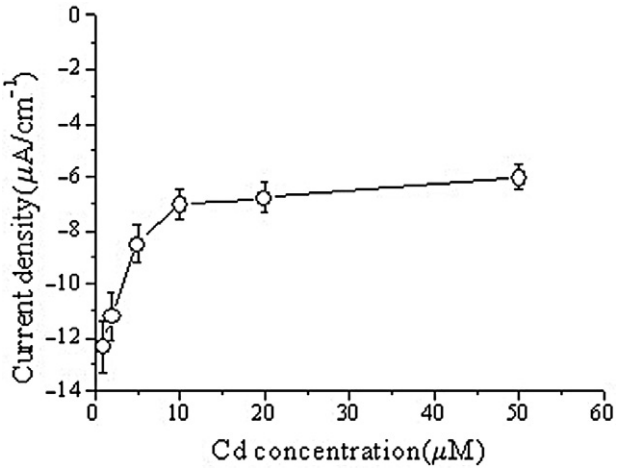

Figure 7. The current density of inward Ca^{2+} currents at –180 mV in response to different concentrations of Cd^{2+} ($n = 11$). The data present mean ± SD.

The result showed that 10 μM NPPB had no significant effect on the Ca^{2+} conductances (Fig. 8).

Effects of extracellular Cd^{2+} on K^+ channel activities in root hair cells

For recording K^+ currents, a different mode with –52 mV holding potential was applied to the protoplasts. During the recording, the membrane potential was stepped from –190 mV to 90 mV with 20 mV increments. Under the control condition (without Cd^{2+} in bath solution), Fig. 9a shows a typical time course of inwardly directed K^+ currents recorded from root hair cell protoplast. Addition of 0.05 mM $CdCl_2$ brought a significant decrease in the inward K^+ currents (Fig. 9b). After 30 min of treatment of Cd^{2+}, a large outward K^+ current was induced significantly by Cd^{2+}. Figure 9c summarized the current–voltage (I–V) relationship in the presence or absence of 0.05 mM Cd^{2+} in the bath solution. At –190 mV, 0.05 mM extracellular Cd^{2+} reduced the whole-cell inward K^+ current density from –12.1 ± 1.5 to –6.6 ± 0.7 μA/cm^2, a 43% decrease from the control. However, when membrane potential was clamped at 90 mV, Cd^{2+} addition induced a 9.4-fold increase in the outward K^+ current densities as compared to the control without Cd^{2+} (from 0.8 ± 0.3 to 7.7 ± 1.2 μA/cm^2, $P < 0.01$, $n = 21$) (Fig. 9d).

To further analyse the characteristics of Cd^{2+}-induced outward K^+ conductance, a time and Cd^{2+} concentration dependence of outward K^+ currents was recorded. Figure 10a shows that under control condition (without Cd^{2+} in bath solution), the rice root hair protoplasts lack outward K^+ conductance over the entire course of recording of 2 h. However, after addition of 0.05 mM $CdCl_2$ in bath solution, a dramatic outward K^+ conductance was stimulated after 30 min of exposure and the magnitude was not changed significantly in the entire course of recording (Fig. 10a). Moreover, this inducible effect of Cd^{2+} on the

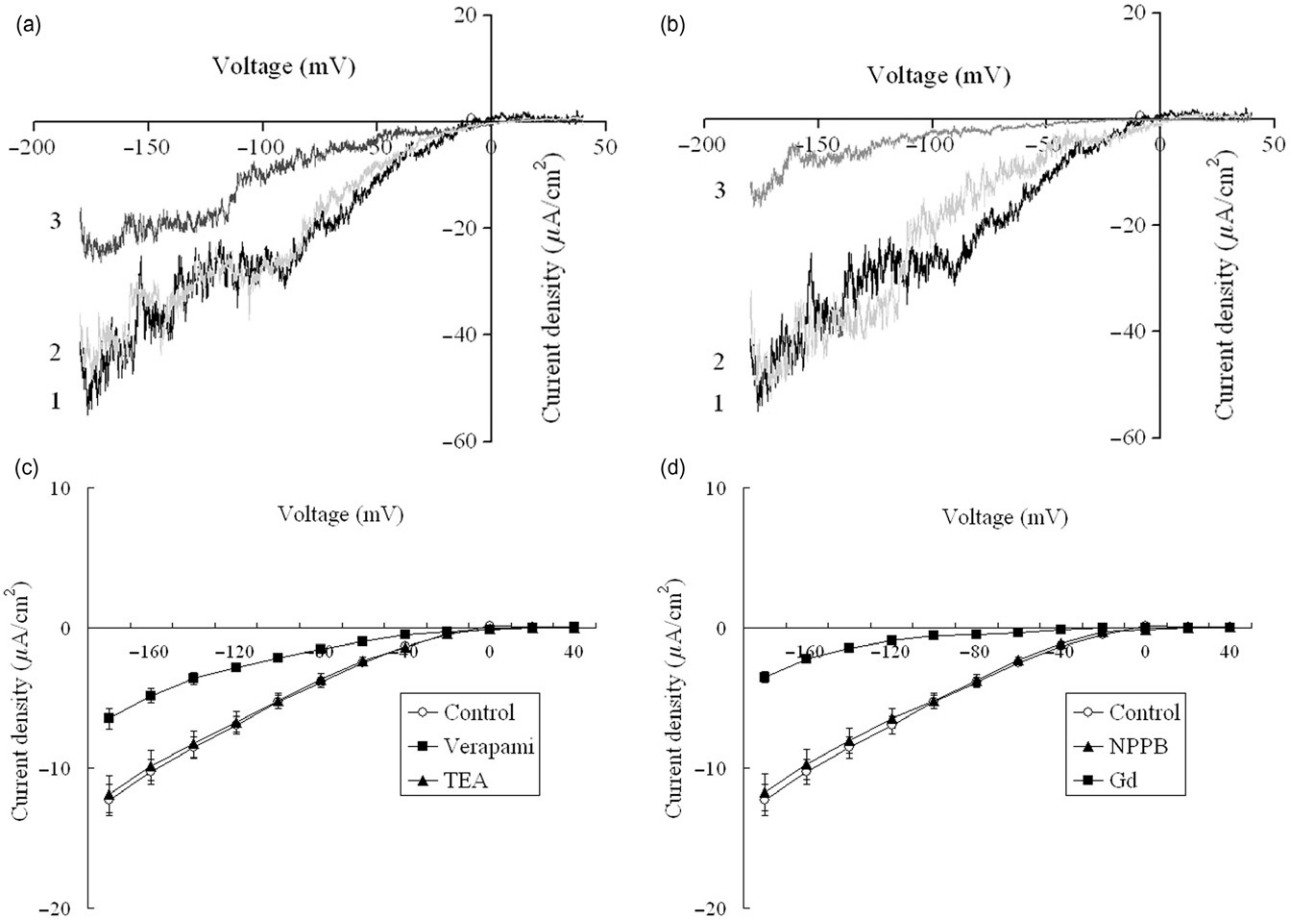

Figure 8. Effects of several known channel blockers on the inward Ca^{2+} currents in rice root hair cells. (a) Whole-cell Ca^{2+} currents were recorded under application of ramp-mode voltage protocol from −180 to 40 mV with increments at 0.01 mV ms^{-1} after a 1 s holding pulse at −180 mV (trace 1, control; trace 2, TEA^+, 10 mM; trace 3, verapamil, 0.02 mM). (b) Whole-cell Ca^{2+} currents were recorded as Figure 8a (trace 1, control; trace 2, NPPB, 10 μM; trace 3, $GdCl_3$, 0.1 mM). (c,d) Current–voltage relationship from cells as in (a) (trace 1, $n = 20$ cells; trace 2, $n = 25$ cells; trace 3, $n = 29$ cells) and (b) (trace 1, $n = 20$ cells; trace 2, $n = 35$ cells; trace 3, $n = 25$ cells). The data present mean ± SD.

outward K^+ currents in the root hair cells was reversible by washout with bath solution (Fig. 10a). To investigate effects of Cd^{2+} at different concentrations on outward K^+ conductance, we added a series of concentrations of Cd^{2+} in bath solution ranging from 1 μM to 100 μM. As shown in Fig. 9b, the inducible effect of exogenous Cd^{2+} on outward K^+ conductance reached a peak level at a Cd^{2+} concentration of 10 μM and did not change significantly with the increase of Cd^{2+} concentration. When the exogenous Cd^{2+} was below 10 μM, the inducible effect showed a dose-dependent manner (Fig. 10b). To further confirm the characteristics of the recorded outward K^+ conductance, traditional pharmacological approach was used in this test. As shown in Fig. 11, the outward K^+ currents induced by Cd^{2+} were dramatically blocked by 10 mM TEA^+, and partially blocked by 0.1 mM Gd^{3+}, while 0.01 mM NPPB and 0.02 mM verapamil showed no effect. When 30 mM TEA^+ replaced K^+ in the pipette solution, the Cd^{2+}-inducible outward currents could not been detected (data not shown). This demonstrates that outward currents were mainly mediated by K^+ efflux.

Together, these results clearly indicated that extracellular Cd^{2+} inhibited the uptake of Ca^{2+} and K^+ in rice root hair cells, whereas the efflux of K^+ was induced by Cd^{2+} exposure.

DISCUSSION

It has been widely documented that Cd^{2+} entering plants will cause a series of detrimental consequences (Sanitá di Toppi & Gabbrielli, 1999), but the mechanisms of Cd^{2+} toxicity are not clearly understood. In this study, we showed that Cd^{2+} exposure reduced Ca^{2+} and K^+ uptake into rice roots by inhibiting the inward Ca^{2+} -permeable and K^+ channel activities and by inducing the outward K^+ currents in root hair cells. In addition, Cd^{2+}-induced depolarization of the membrane potential attenuated the driving force for Ca^{2+} and K^+ uptake and directly induced K^+ efflux mediated by outward-rectifying K^+ channels. Thus, by repressing Ca^{2+} and K^+ influxes into roots, inducing the efflux of K^+ and Cd^{2+} may interfere with K^+ and Ca^{2+} homeostasis in rice plants.

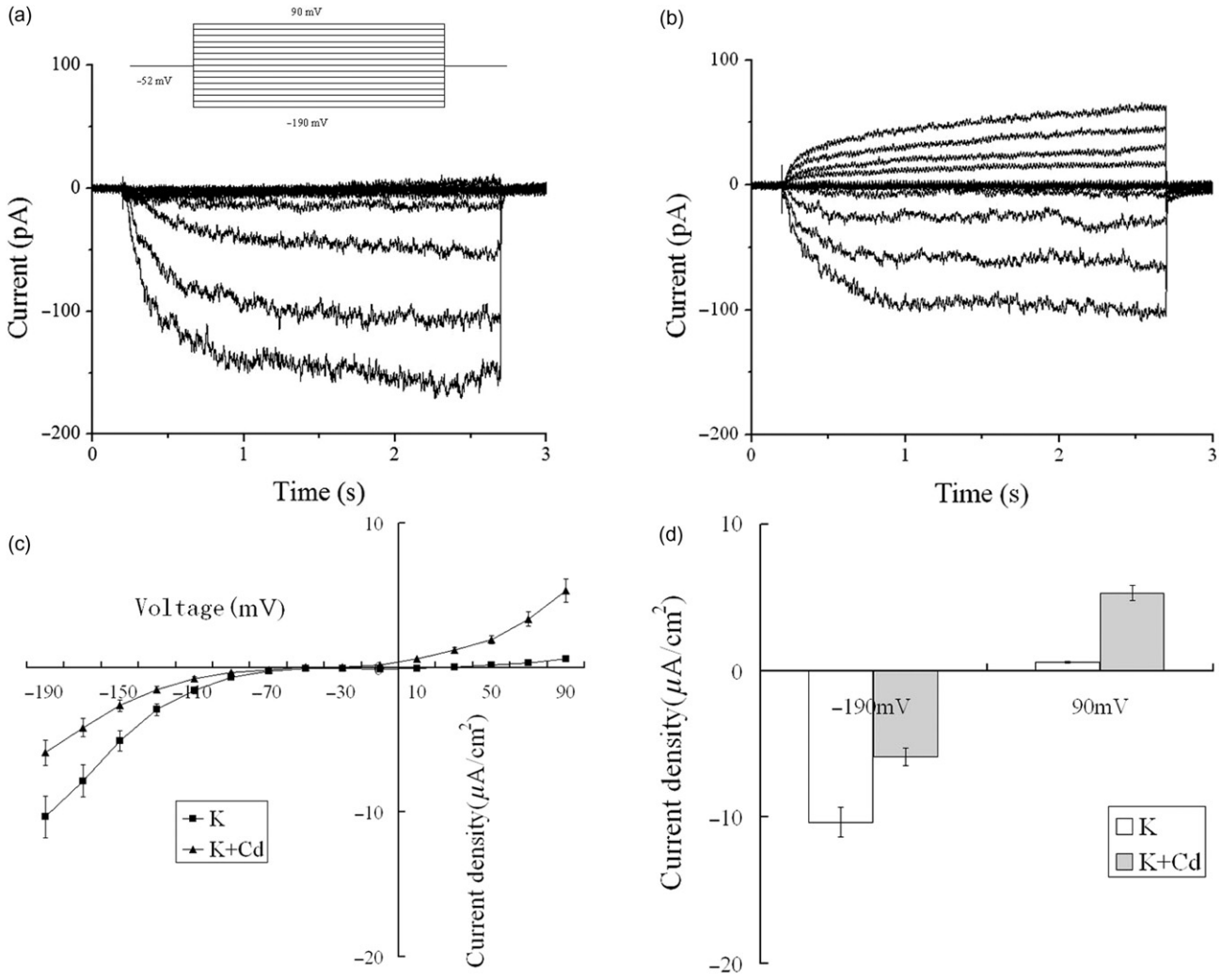

Figure 9. Effects of extracellular cadmium on the K^+ currents in rice root hair cells. (a) A typical example of whole-cell inward K^+ currents (capacitance = 5.5 pF). (b) The same cell as in (a) was treated with 0.05 mM $CdCl_2$ in the bath solution for 30 min. (c) The current amplitudes (mean ± SD) from the control cells (■) and the cells treated with 0.05 mM $CdCl_2$ in the bath solution (▲) are presented as current–voltage curves ($n = 18$). (d) Whole-cell K^+ current values at –190 and 90 mV before and 30 min after treatment with 0.05 mM $CdCl_2$. Mean ± SD ($n = 21$). Open bars, control cells; closed bars, cells treated with $CdCl_2$. During the recordings, the holding potential was –52 mV, the currents were recorded at the membrane potentials from –190 to 90 mV with increment of 20 mV, the p/n ($n = 4$) leak subtraction was used before obtaining time-dependent K^+ currents.

We propose that one of the mechanisms of Cd^{2+} toxicity is to impair the uptake and transport of Ca^{2+} and K^+, thereby disturbing ion homeostasis in higher plants.

Potassium and calcium are the very important essential macronutrients required in all living cells. In general, potassium and calcium deficiency will cause leaf rolling and chlorosis, and reduce growth, which are very similar to the symptoms of Cd^{2+} toxicity (Fig. 1c). In this study, we detected the potassium and calcium contents in Cd^{2+}-treated rice seedlings under hydroponic conditions and found that Cd^{2+} exposure, at the whole-plant level, significantly decreased potassium and calcium levels in rice plants (Fig. 2). It is reasonable to propose that Cd^{2+}-induced macronutrient deficiency may significantly contribute to Cd^{2+} toxicity.

Under most environmental conditions, Cd^{2+} enters the roots first, and then transports to shoots via xylem (Uraguchi *et al.* 2009). As a result, the root cells are likely to experience Cd^{2+} toxicity first. One of the major functions of roots is nutrient uptake. In this study, we tested the possibility that Cd^{2+} may affect nutrient uptake of roots. We first examined the net uptake of Ca^{2+} and K^+ across the root hair cells by ~~SIET~~, experiments, and found that Cd^{2+} exposure resulted in a rapid Ca^{2+} and K^+ efflux from root hair cells (Fig. 3), contributing to the decrease of net uptake of these cations, which is similar with the previous report (Fan *et al.* 2011). The Cd^{2+} induced a decrease in net H^+ efflux, and membrane depolarization may further contribute to the reduced uptake of K^+ and Ca^{2+} across the plasma membrane after Cd^{2+} exposure (Figs 3d & 4a). Our result is completely

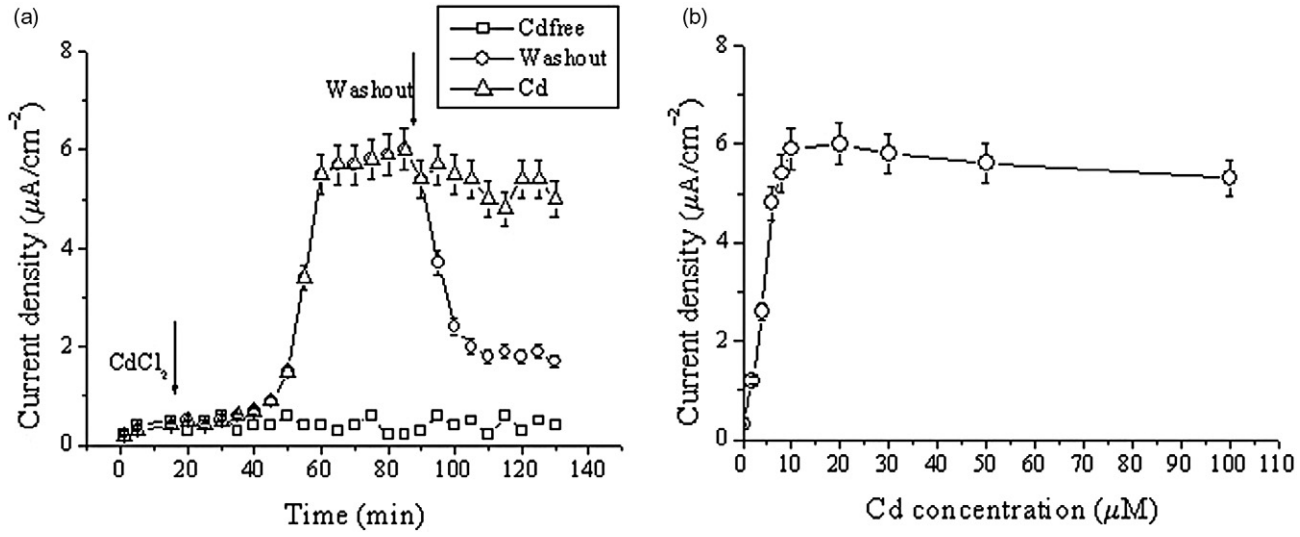

Figure 10. Time and Cd^{2+} concentration dependence of Cd^{2+}-induced outward K^+ currents. (a) The time dependence of induced whole-cell outward K^+ currents at 90 mV after treatment with 0.05 mM $CdCl_2$ and after washout with bath solution ($n = 13$). (b) The current density of induced outward K^+ currents at 90 mV in response to different concentrations of Cd^{2+} ($n = 13$). The data present mean ± SD.

consistent with the previous study (Llamas, Ullrich & Sanz 2000), implying that one of the reasons for Cd^{2+} inhibition of K^+ and Ca^{2+} uptake is depolarization of the root hair cell plasma membrane, thereby attenuating the driving force for Ca^{2+} and K^+ uptake. For Cd^{2+}-induced membrane depolarization, the most likely explanation is that ATPase-dependent H^+ efflux is the major force driving the cation uptake into cells by its role in establishing the plasma membrane potential. In this study, Cd^{2+} application resulted in a significant decrease of H^+ efflux, implying that the activity of H^+-ATPase was inhibited by Cd^{2+}. In fact, it has been widely accepted that plasma membrane H^+-ATPase was inhibited by heavy metal stress, which in turn resulted in significant membrane depolarization (Janicka-Russak *et al.* 2008).

In patch-clamp experiments, we detected the whole-cell Ca^{2+}-permeable conductance in root hair cells, which showed a large inward conductance at hyperpolarized potentials (below –80 mV). Calcium is a major component of all plant cells; therefore, in roots of growing plants, there

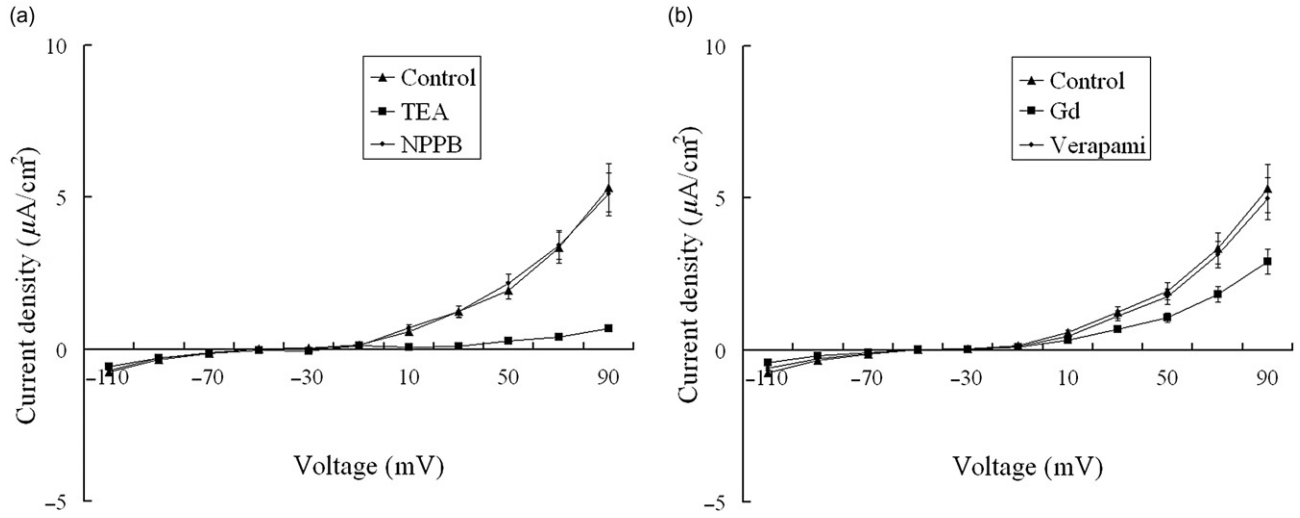

Figure 11. Effects of several known channel blockers on the Cd^{2+}-induced outward K^+ currents in rice root hair cells. (a) 10 mM TEA dramatically blocked Cd^{2+}-induced outward K^+ currents, while 10 μM NPPB showed no effect (control, $n = 17$ cells; TEA treatment, $n = 22$ cells; NPPB treatment, $n = 20$ cells). (b) The outward currents were partially inhibited by 0.1 mM Gd^{3+}, but made no response to 0.02 mM verapamil (control, $n = 17$ cells; Gd^{3+} treatment, $n = 17$ cells; verapamil treatment, $n = 28$ cells). During the recordings, the holding potential was –52 mV, the currents were recorded at the membrane potentials from –110 to 90 mV with increment of 20 mV. The data present mean ± SD.

is a need for large Ca^{2+} influxes to provide for the Ca^{2+} requirements of both expanding root cells and for delivery to the shoot. The large inward conductances activated by hyperpolarization in this experiment are most likely responsible for this need. In pharmacological experiments, the Ca^{2+} conductances were completely blocked by non-selective cation channel blocker Gd^{3+} and partially blocked by Ca^{2+} channel blocker verapamil (Fig. 8). These characteristics of Ca^{2+}-permeable conductance were similar with those of nonselective cation channels (NSCCs) widely reported previously (Roberts & Tester 1997; Davenport & Tester 2000; Demidchik *et al*. 2002; Demidchik & Maathuis 2007); Zhao *et al*. 2007) and with HACCs (Very & Davies 2000), implying that the NSCCs may mediate Ca^{2+} conductance. In addition, a time- and voltage-dependent inward K^+ channel activity was also detected in the K^+-based bath solution. Both inward Ca^{2+}-permeable and K^+ channel activities were inhibited by exogenous Cd^{2+} application (Figs 6 & 9), consistent with the results showing reduced uptake of Ca^{2+} and K^+ in the whole plants and in ~~SIET~~ experiments.

In agreement with our results, Cd^{2+} has been well characterized as a blocker of Ca^{2+} channel in mammalian nerve cells (Chow 1991; Murphy 1997). In yeast expressing wheat *LCT1* cDNA clone, the Ca^{2+} uptake was inhibited competitively by Cd^{2+} exposure (Clemens *et al*. 1998). In higher plants, however, there is no direct evidence supporting the notion that extracellular Cd^{2+} could inhibit Ca^{2+}-permeable channel activity, although several previous reports propose that Cd^{2+} may compete for the same transmembrane carrier with other cations, such as K^+, Ca^{2+}, Zn^{2+} (Rivetta, Negrini & Cocucci 1997; Welch *et al*. 1999; Cie'cko *et al*. 2004). Therefore, our electrophysiological results here provide a direct evidence that Cd^{2+} exposure can block the Ca^{2+}-permeable conductance in rice root hair cells (Fig. 6), and this blockage was in a dose-dependent manner (Fig. 7), and was reversible on washout of bath solution. These results implied that Cd^{2+} might be a competitive blocker of Ca^{2+}-permeable channel, which is similar to the mode of blockage in animal cells and in yeast (Chow 1991; Clemens *et al*. 1998). We propose that Cd^{2+} may permeate the channels and bind transiently to a site in the pore, reversibly obstructing the passage of Ca^{2+}. Our experiments on detecting of Ca^{2+} conductances, apparently, were preliminary results. Further experiments are needed to test this idea.

Previous reports have shown that Cd^{2+} blocks outward K^+ currents in shrinking cells of *Samanea saman* leaflets (Moran, Fox & Satter 1990) and in mammalian cells (Wang *et al*. 2008). We show in this study that exogenous Cd^{2+} significantly decreased inward K^+ currents in rice root hair cells (Fig. 9b), leading to significant drop in net K-uptake (Fig. 2b). At the same time, the outward K^+ currents were activated significantly (Fig. 9b), suggesting that the efflux of K^+ from root hairs was enhanced. It has been widely reported that heavy metals, including Hg^{2+}, Cu^{2+} and Cd^{2+}, can provoke K^+ efflux from yeast, *Scenedesmus quadricauda*, and proximal tubule (White & Gadd 1987; Kone, Brenner & Gullans 1990; Reddy & Prasad 1992). However, to the best of our knowledge, no reports on Cd^{2+}-induced outward K^+ conductance from higher plant cells have been reported. Therefore, our electrophysiological results here provide a direct evidence that Cd^{2+} exposure can induce outwardly directed K^+ conductance in rice root hair cells, which mostly likely is responsible for Cd^{2+}-induced K^+ efflux. Previous studies have reported that outward-rectifying K^+ channels (KOR) are responsible for NaCl-induced K^+ loss from *Arabidopsis* roots and leaves, and that this triggers an oxidative burst, which in turn provokes programmed cell death (PCD) (Demidchik *et al*. 2003, 2010; Shabala *et al*. 2006). PCD helps plants to survive under stress (Mur *et al*. 2008). In this study, Cd^{2+} induced outwardly directed K^+ channel activity, leading to K^+ loss, and this may stimulate protease and endonuclease activity, like it functions in animal tissues (Remillard & Yuan 2004), which in turn provokes the PCD. We propose that KOR channel may play the same crucial role in plant stress tolerance, including heavy metal toxicity tolerance, and K^+ loss-stimulated PCD may be an efficient detoxifying mechanism for Cd^{2+}. Further biochemical and physiological analysis will illustrate whether Cd^{2+} exposure stimulates the process of PCD in rice root hairs.

We should not exclude other possible modes of action of Cd^{2+} on K^+ losses. Peroxidation of membrane lipids triggered by heavy metal could also responsible for net K^+ losses. It has been reported that the permeability of membranes depends on the degree of lipid peroxidation (Dhindsa, Dhindsa & Thorpe 1982). Reddy & Prasad (1992) reported that Cd^{2+} induced potassium efflux from *S. quadricauda*, and suggested that the increased permeability of *S. quadricauda* may possibly be due to lipid peroxidation.

The inducible effect of Cd^{2+} on the outward K^+ currents in the root hair cells was reversible by washout with bath solution (Fig. 10a), and acted in a dose-dependent manner when the Cd^{2+} concentration was lower than 10 μM (Fig. 10b), which is similar with the response of Ca^{2+} conductance (Fig. 7). We could not make sure if the inducible outward K^+ currents are mediated by the same population of K^+ channels mediating the K^+ inward current. The time dependence of induction implies that there might be a complicated process between the Cd^{2+} signal reception and the response of channel. Based on Fig. 4, we can see that Cd^{2+} causes depolarization that is not sufficient for outward K^+ channel activation. The most likely explanation is that Cd^{2+} may stimulate outward K^+ channel activity via directly binding or inhibiting some component, which in turn results in a conformation change and releases the active site of the channel. In some naturally occurring metal-binding sites in proteins, Cd^{2+} or Zn^{2+} ions are coordinated by imidazole groups from histidines and by sulfhydryl groups from cysteins (Krizek, Zawadzke & Berg 1993). We presume that Cd^{2+} may bind with some amino acid residues, like cysteine and histidine, located in the same or different subunits of K^+ channel, which in turn result in a change of conformation, and may trap K^+ channel in the open state. To understand more about the molecular mechanism by which KOR channel opens, the further work will focus on the Cd^{2+}

modification on cysteins and/or histidines located in positions of S6 transmembrane segment of GORK, which is known to conduct large outwardly rectifying K^+ currents, and is expressed in root hairs (Ivashikina *et al*. 2001), by substitution mutagenesis. The Cd^{2+}-induced activation of outward K channel in rice root hair cells is in contrast with the Cd^{2+} blockage of outward K channel reported previously (Moran *et al*. 1990; Wang *et al*. 2008). This difference may result from different subtypes of K channels in different organisms.

Our experimental results here have connected Cd^{2+} toxicity and inhibition of ion channel activity in plants. Future work on the molecular basis of Cd^{2+} interaction with ion channel proteins will provide further insight on the precise mechanisms of Cd^{2+} toxicity in higher plants.

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SUPPORTING INFORMATION

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

Figure S1. Effects of different heavy metal ions on K^+ efflux and outwardly rectifying K^+ channel activity in rice root hairs. (a) Effects of different heavy metal ion applications on net K^+ flux in rice root hair ($n = 11$). The sign convention to be used is 'efflux positive'. (b) Whole-cell K^+ current values at 90 mV before and 30 min after treatment with 0.05 mM Cd^{2+}, Cu^{2+} and Hg^{2+}. Mean ± SD (for Cd^{2+}, $n = 17$; for Cu^{2+}, $n = 15$; for Hg^{2+}, $n = 22$).

Figure S2. Kinetics of net Cd^{2+} flux on the surface of rice root hair. The sign convention to be used is 'inflflux positive'.

Figure S3. Effect of Gd^{3+} pretreatment on Cd^{2+}-induced Ca^{2+} efflux in rice root hairs. The sign convention to be used is 'efflux positive'. ** indicate the difference at $P < 0.01$ by Student's *t* test. Data represent mean ± SD.

Figure S4. Replacement of Ba^{2+} pipette solution with standard K^+ pipette solution brought a slight influence on Cd^{2+}-blocked Ca^{2+} conductance in rice root hair cells. (a) Steady current–voltage relationship (trace 1: standard K^+ pipette solution, $n = 17$; trace 2: Ba^{2+} pipette solution, $n = 20$). The arrow marks current reversal. (b) Whole-cell recording of Ca^{2+} current with 0.05 mM Cd^{2+} in bath solution using K^+ standard pipette solution. (c) Whole-cell Ca^{2+} current recorded during a rapid voltage ramp (–50 to +100 mV; 50 mV/s). A 1 s holding pulse at –180 before voltage ramp. E_{Cl^-}, Cl^- equilibrium potential.

Figure S5. Effect of extracellular cadmium on the K^+ currents in rice root hair cells. (a) An example of whole-cell K^+ currents without leak subtraction (capacitance = 6 pF). (b) The same cell as in (a) was treated with 0.05 mM $CdCl_2$ in the bath solution for 30 min. (c) Current–voltage relationship from cells as in (a,b) ($n = 18$). During the recordings, the holding potential was –52 mV, the currents were recorded at the membrane potentials from –190 to 90 mV with increment of 20 mV.

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