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Fusicoccin Counteracts the Toxic Effect of Cadmium on the Growth of Maize Coleoptile Segments

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Abstract The effects of cadmium (Cd; 0.1–1000 μM) and fusicoccin (FC) on growth, Cd^{2+} content, and membrane potential (E_m) in maize coleoptile segments were studied. In addition, the E_m changes and accumulation of Cd and calcium (Ca) in coleoptile segments treated with Cd^{2+} combined with 1 μM FC or 30 mM tetraethylammonium (TEA) chloride (K^+-channel blocker) were also determined. In this study, the effects of Ca^{2+}-channel blockers [lanthanum (La) and verapamil (Ver)] on growth and content of Cd^{2+} and Ca^{2+} in coleoptile segments were also investigated. It was found that Cd at high concentrations (100 and 1000 μM) significantly inhibited endogenous growth of coleoptile segments and simultaneously measured proton extrusion. FC combined with Cd^{2+} counteracted the toxic effect of Cd^{2+} on endogenous growth and significantly decreased Cd^{2+} content (not the case for Cd^{2+} at the highest concentration) in coleoptile segments. Addition of Cd to the control medium caused depolarization of E_m, the extent of which was dependent on Cd concentration and time of treatment with Cd^{2+}. Hyperpolarization of E_m induced by FC was suppressed in the presence of Cd^{2+} at 1000 μM but not Cd^{2+} at 100 μM. It was also found that treatment of maize coleoptile segments with 30 mM TEA chloride caused hyperpolarization of E_m and decreased Cd^{2+} content in coleoptile segments, suggesting that, in the same way as for FC, accumulation of Cd^{2+} was dependent on plasma membrane (PM) hyperpolarization. Similar to FC, TEA chloride also decreased Ca^{2+} content in coleoptile segments. La and Ver combined with Cd^{2+} (100 μM) significantly decreased Cd content in maize coleoptile segments, but only La completely abolished the toxic effect of Cd^{2+} on endogenous growth and growth in the presence of FC. Taken together, these results suggest that the mechanism by which FC counteracts the toxic effect of Cd^{2+} (except at 1000 μM Cd^{2+}) on the growth of maize coleoptile segments involves both stimulation of PM H^+-ATPase activity by FC as well as Cd^{2+}-permeable, voltage-dependent Ca channels, which are blocked by FC and TEA chloride-induced PM hyperpolarization.

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Cadmium (Cd) is widely acknowledged as being one of the most phytotoxic agents (reviewed in Das et al. 1997; Sanità di Toppi and Gabbrielli 1999; Seregin and Ivanov 2001; Pál et al. 2006) inhibiting plant growth when present in excess (Sandalio et al. 2001; Wójcik and Tukiendorf 2005; Nouairi et al. 2006; Karcz and Kurtyka 2007; Kurtyka et al. 2008). Because Cd is one of the most readily absorbed and most rapidly translocated heavy metals in plants, it exerts strong toxicity (Di Cagno et al. 1999; Pál et al. 2006). Among other detrimental effects of Cd^{2+}, membrane damage and changes in enzyme activities affecting uptake and transport of mineral nutrients have been reported (Lindberg and Wingstrand 1985; Ros et al. 1990; Fodor et al. 1995; Llamas et al. 2000; Gonçalves et al. 2009). Several reports have shown that Cd^{2+} caused a decrease of K^+ content in various plant materials (Rubio et al. 1994; Llamas et al. 2000; Kurtyka et al. 2008; Gonçalves et al. 2009).

Furthermore, it has also been shown that Cd induces depolarization of membrane potential (E_m) Kennedy and Gonsalvez 1987; Aidid and Okamoto 1992; Llamas et al. 2000; Pavlovkin et al. 2006; Karcz and Kurtyka 2007), which probably results from decreased plasma membrane (PM) H^+-ATPase activity (Ros et al. 1992; Fodor et al. 1995; Astolfi et al. 2003; Kabała et al. 2008), leading to decreased H^+ extrusion (Astolfi et al. 2003; Karcz and Kurtyka 2007). In contrast to Cd^{2+}, fusicoccin (FC), the fungal toxin produced by *Fusicoccum amygdale* Del., enhances elongation growth by stimulating H^+ efflux driven by PM H^+-ATPase (Marré 1979; Kutschera and Schopfer 1985; Lüthen et al. 1990; Hager et al. 1991; Karcz et al. 1995; Palmgren 1998; Karcz and Burdach 2002; Hager 2003). Simultaneously with FC-induced proton extrusion, hyperpolarization of the PM is observed (Cleland et al. 1977; Karcz and Burdach 2007), which in turn activates an inwardly rectifying K^+ channel (K^+_{in}) (Philippar et al. 1999; Tode and Lüthen 2001). It is also establishes that an FC-binding site arises from interaction of the 14-3-3 protein dimer with the C-terminal autoinhibitory domain of the H^+-ATPase and that FC stabilizes this complex (Jahn et al. 1997; Baunsgaard et al. 1998; Fuglsang et al. 1999; Oecking and Hagemann 1999; Würtele et al. 2003). Taking into account that FC causes effects opposite to those produced by Cd, it was interesting to study whether this phytotoxin is able to counteract the toxic effect of Cd on the growth of maize coleoptile segments. This experimental design can provide new data on the toxic effects of Cd on plant growth.

The main goal of our experiments was to study the mechanisms of Cd-induced inhibition of elongation growth and the mechanism of Cd uptake. This goal was realized by: (1) studying the effects of Cd^{2+} on growth in the presence or absence of FC while simultaneously measuring growth-medium pH changes; (2) establishing E_m changes in parenchymal cells treated with Cd^{2+} or Cd^{2+} applied together with FC and tetraethylammonium (TEA) chloride; (3) determining Cd^{2+} and Ca^{2+} content in maize coleoptile segments incubated in medium containing Cd^{2+} or Cd^{2+} together with FC and TEA chloride; (4) studying the effects of Cd^{2+} on both growth and content of Cd^{2+} and Ca^{2+} in maize coleoptile segments incubated in the presence of FC combined with Ca^{2+}-channel blockers [lanthanum chloride (La) and verapamil (Ver)].

Materials and Methods

Plant Material

Seeds of maize (*Zea mays* L. cv. K33 × F2) were soaked in tap water for 2 h, sown on wet wood in plastic boxes, and placed in a growth chamber at 27 ± 1°C. The experiments were performed with 10 mm-long coleoptile segments cut from 96 h old etiolated maize seedlings. Intact coleoptile segments, with the first leaves removed, were excised 3 mm below the tip and incubated in a control medium made of 1000 μM KCl, 100 μM NaCl, and 100 μM $CaCl_2$.

Chemicals

FC (Sigma, USA) was dissolved in ethanol and added to the incubation medium at a final concentration of 1 μM. The maximal ethanol concentration of 0.2% did not affect growth of coleoptile segments (data not shown). $CdCl_2 \cdot 2.5$ H_2O (Fluka, Switzerland) was dissolved in control medium. TEA chloride (Sigma, USA), La (Sigma, USA), and Ver (Sigma, USA) were dissolved in deionized water and used at a final concentration of 30 mM, 5 mM, and 50 μM, respectively. Stock solutions of TEA chloride, La, and Ver were prepared in concentrations that were 100-fold greater compared with those used in the experiments.

Growth and pH Measurements

Growth experiments were performed using an apparatus that allowed simultaneous measurement of elongation growth and pH of the incubation medium from the same tissue sample (Karcz et al. 1990; Karcz and Burdach 2002, 2007). The optical system used for growth measurement (shadow graph methods) permitted recording of the longitudinal extension of a stack of 21 segments. In this setup, the segments, after their excision, were incubated in 6.3 cm^3 (0.3 cm^3 $segment^{-1}$) of an intensively aerated control medium (1000 μM KCl, 100 μM NaCl, and 100 μM $CaCl_2$). As described previously by Karcz et al. (1995) and by Karcz and Burdach (2002), in this system the incubation medium also flowed through the lumen of the coleoptile cylinders. This feature permitted treatment solutions to be in direct contact with the interior of the segments, which significantly enhanced both the elongation growth of coleoptile segments as well as proton secretion (Karcz et al. 1995).

All manipulations and growth experiments were conducted under dim green light. The temperature of all solutions in the elongation and pH-measuring system was thermostatically controlled at 25 ± 0.5°C. pH measurements were performed with a pH meter (type CI-316; Elmetron, Poland) and pH electrode (OSH 10-10; Metron, Poland). Cd, FC, and calcium (Ca)-channel blockers (La or Ver) were introduced into the incubation medium at 120 min into the experiment. The initial pH of the incubation solution was adjusted to 5.8–6.0 with either 0.1 M NaOH or 0.1 M HCl.

Electrophysiology

The electrophysiological experiments were carried out with intact, 10 mm-long coleoptile segments that were prepared the same as for the growth experiments. E_m was measured by recording the voltage between a 3 M KCl-filled glass micropipette inserted into the parenchymal cells and a reference electrode in the bathing medium containing the same composition as used in the growth experiments. For electrophysiological experiments, the segments were preincubated for 2 h in an intensively aerated bathing medium, whereupon the segments were transferred into a perfusion Plexiglass chamber mounted on a vertically placed microscope stage. The microelectrodes were inserted into cells under microscope by means of micromanipulator (Hugo Sachs Electronik; March-Hugstteten, Germany). Medium changes were performed by means of a peristaltic pump (Type Peri-Star PRO; World Precision Instruments, USA), which allowed changing of the bathing medium in the chamber (usually fourfold within <2 min). Micropipettes were pulled on a vertical pipette puller (model L/M-3P-A; List-Medical, Germany) from borosilicate glass capillaries (type 1B150F-3; World Precision Instruments, USA). Tip diameters measured <1 μm. Cd, FC, and TEA chloride were introduced into the control medium after stabilization (<10 min) of E_m.

Determination of Cd and Ca Content

Cd and Ca concentrations in maize coleoptile segments were determined by emission spectrometry with excitation by way of an argon inductively coupled plasma technique by means of a Spektroflame-M spectrophotometer (Spectro Analytical Instruments, Germany). Before chemical analysis, 110 intact coleoptile segments were split along the long axis and preincubated for 2 h in an intensively aerated growth medium (control). The composition and volume (0.3 cm^3 $segment^{-1}$) of the incubation medium were the same as that used in the growth experiments. After preincubation of the coleoptile segments, Cd (0.1–1000 μM), Cd together with FC (1 μM), or TEA chloride (30 mM) were introduced into the incubation medium for 5 h. The variant in which these three components (Cd^{2+}, FC, and TEA chloride) were combined was also studied. Moreover, Cd and Ca content in maize coleoptile segments exposed (for 5 h) to 100 μM Cd or Cd combined with (1 μM) FC or/and Ca-channel blockers (La and Ver) was also determined. In this case, the segments were first preincubated for 2 h in control medium, whereupon Cd or Cd with FC or/and Ca-channel blockers were added. After each treatment, the halves of the segments were removed from the solution and washed three times with deionized water, whereupon they were dried at 80°C to determine dry weight. For Cd and Ca analyses, dry plant tissue was mineralized. Each sample (approximately 0.2 g dry matter) was treated with 5 ml ultrapure concentrated nitric acid (Merck, Germany) and left for 24 h. Next, the samples were digested until complete mineralization was achieved. After mineralization, the samples were diluted with redistilled water to a volume of 10 ml. Concentrations of Cd and Ca were measured by inductively coupled plasma–atomic emission spectroscopy (frequency 27.12 MHz; power 1.0 kW; plasma gas 14.0 l/min; auxiliary gas 0.5 l/min; carrier gas 1 l/min; and analytical line Cd^{2+} 228.802 nm and Ca^{2+} 422.673 nm). As standards for control of the elemental analysis, Virginia tobacco leaves (CTA-VTL-2) were used; the results fit the range of certified recommended values. All experiments concerning the accumulation of Cd^{2+} and Ca^{2+} were replicated at least four times, and results are expressed as means ± SEs.

Statistical Analysis

Data were analyzed using computer software Statistical for Windows (StatSoft 2008; STATISTICA data analysis software system, version 8.0. http://www.statsoft.com, USA). Differences between individual treatments and control were analyzed using one-way analysis of variance and Fisher's least significant difference (LSD) test. Statistical significance was defined at $P < 0.05$.

Results

Effect of Cd^{2+} on Endogenous and FC-Induced Growth of Coleoptile Segments Incubated With or Without Ca-Channel Blockers

The effect of Cd^{2+} (0.1–1000 μM) on the growth of maize coleoptile segments incubated in control medium (endogenous growth) is shown in Fig. 1. As can be seen in this figure, Cd^{2+} applied to the medium (after 2 h; preincubation of segments in control medium) at concentrations of 10, 100, and 1000 μM decreased endogenous growth of maize coleoptile segments by 14, 40, and 60%, respectively. However, Cd at 1 μM did not cause any significant changes in endogenous growth (856.8 ± 32.6 μm cm^{-1}) compared with the control (935.0 ± 43.9 μm cm^{-1}). Even the lowest Cd concentration (0.1 μM) used stimulated (by 16%) the growth of maize coleoptile segments. The inset in Fig. 1 shows the time courses of the endogenous growth of maize coleoptile segments as a function of Cd^{2+} concentration.

FC added to the control medium at 120 min rapidly enhanced endogenous growth of maize coleoptile segments (Fig. 2). It was found that FC-induced growth of maize

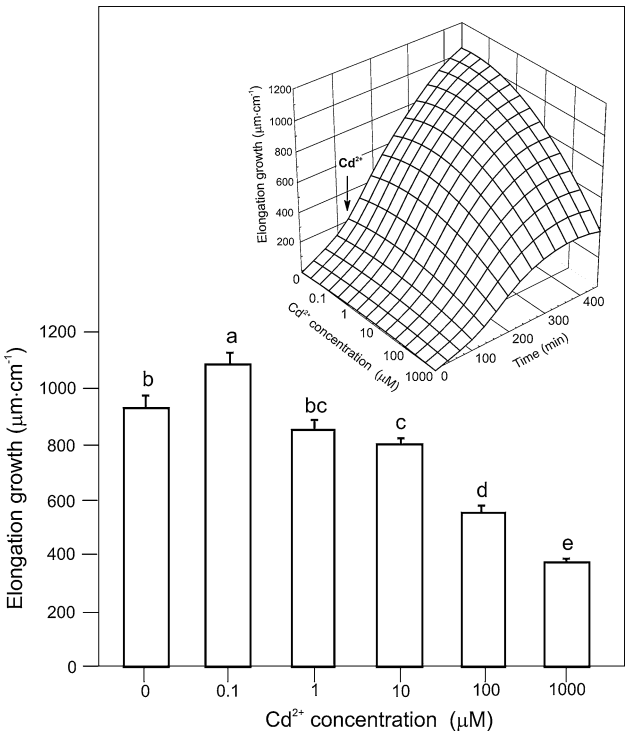

Fig. 1 Effect of Cd (0.1–1000 μM) on the endogenous growth (μm cm^{-1}) of maize coleoptile segments. The elongation of a stack of 21 segments was measured as described in Materials and Methods. The growth of maize coleoptile segments after 7 h is shown (2 h of preincubation in control medium plus 5 h of incubation with Cd). The *inset* shows Cd dependence of endogenous growth as a function of time. After preincubation (2 h) of coleoptile segments in control medium, Cd was added (*arrow*). Values are means of ten independent experiments. *Bars* indicate means ± SEs. Statistical analysis (using software Statistica) showed that differences between values of elongation growth for control and 1.0 μM Cd^{2+} were statistically not significant at 420 min (LSD test $P < 0.05$)

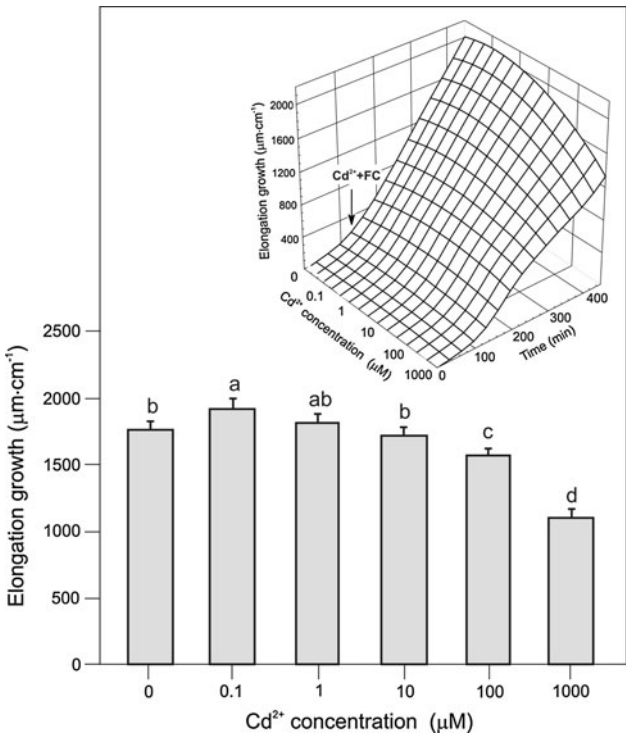

Fig. 2 Effect of Cd (0.1–1000 μM) on growth (μm cm^{-1}) of maize coleoptile segments incubated in the presence of 1 μM FC. The elongation of a stack of 21 segments was measured as described in Materials and Methods. The growth of maize coleoptile segments after 7 h is shown (2 h of preincubation in control medium plus 5 h of incubation with Cd combined with FC). The *inset* shows Cd dependence of growth in the presence of FC as a function of time. After preincubation (2 h) of coleoptile segments in the control medium, Cd combined with FC (1 μM) was added (*arrow*). Values are means of ten independent experiments. *Bars* indicate means ± SEs. Means followed by the *same letter* are not significantly different from each other (LSD test $P < 0.05$)

coleoptile segments was enhanced approximately twofold compared with growth seen in control medium. Application of FC together with Cd^{2+} counteracted the toxic effect of Cd on endogenous growth, which means that growth of coleoptile segments in the presence of both substances was always greater than endogenous growth (935.0 ± 43.9 μm cm^{-1}). In contrast, Cd^{2+} at 100 and 1000 μM inhibited FC-induced growth by 10 and 30%, respectively (Fig. 2). Moreover, the data presented here (Fig. 2) show that the growth of coleoptile segments treated with FC combined with 0.1 μM Cd^{2+} was greater than FC-induced growth (1764.5 ± 67 μm cm^{-1}).

When Ver was added to the control medium at 50 μM, it did not change either endogenous or FC-induced growth of maize coleoptile segments (Fig. 3). However, La at 5 mM significantly stimulated (by 30%) both endogenous and FC-induced growth. As indicated in Fig. 3, La combined with Cd^{2+} abolished the toxic effect of Cd on both endogenous growth and growth in the presence of FC. Compared with

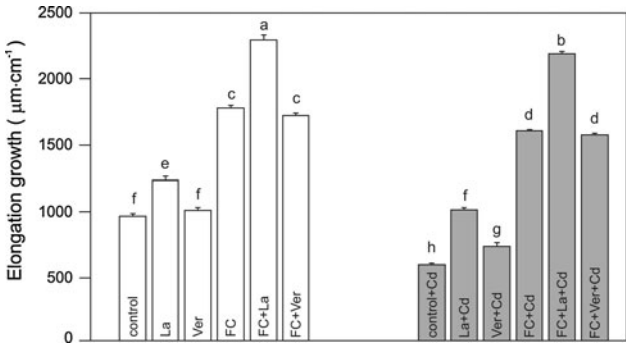

Fig. 3 Effect of 100 μM Cd on endogenous and FC-induced growth (μm cm^{-1}) of maize coleoptile segments incubated in the presence of Ca-channel blockers (La and Ver). Values are means of five independent experiments. *Bars* indicate means ± SEs. Means followed by the *same letter* are not significantly different from each other (LSD test $P < 0.05$)

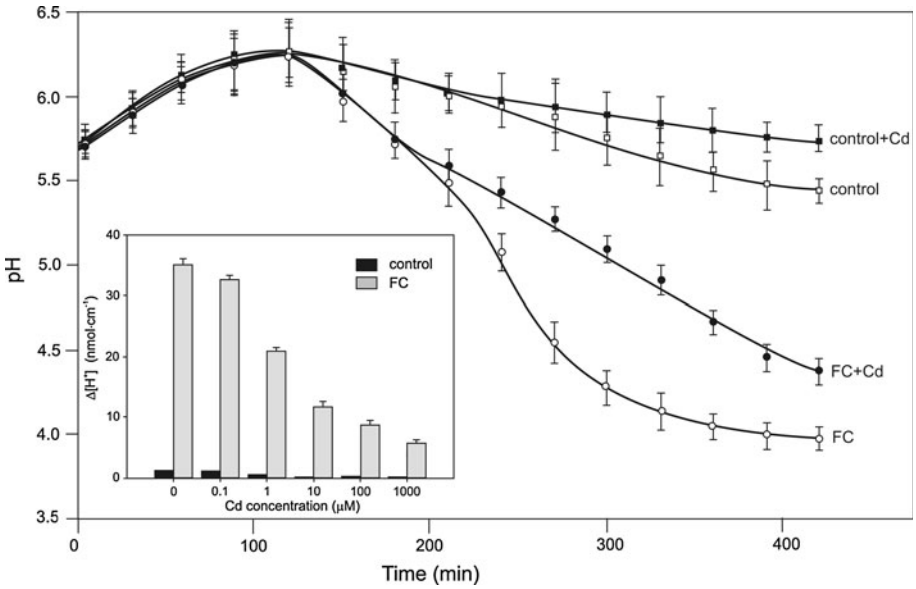

Fig. 4 Effect of 100 μM Cd on pH changes of the incubation medium measured simultaneously (using the same tissue sample) with endogenous growth and growth in the presence of FC (1 μM). The segments were first preincubated (2 h) in control medium, whereupon FC, Cd, or Cd together with FC were added (*arrow*). Values for pH are means of ten independent experiments. *Bars* indicate means ± SEs. To avoid illegibility of the figure, only some curves have been shown. The *inset* shows Cd dependence of medium pH expressed as Δ $[H^+]$, where Δ $[H^+]$ indicates the difference between H^+ concentration $[H^+]$ at 420 and 120 min. The differences between pH values for control medium, FC, Cd, or Cd combined with FC were statistically significant at 420 min (LSD test $P < 0.05$)

La, Ver did not abolish the toxic effect of Cd^{2+} on FC-induced growth, and it only partly diminished the inhibitory effect of Cd^{2+} on endogenous growth (Fig. 3).

Effect of Cd^{2+} on pH Changes of the Incubation Medium

pH changes of the incubation medium were measured simultaneously with the elongation growth of coleoptile segments. The data in Fig. 4 indicate that coleoptile segments incubated in control medium characteristically changed the medium's pH; usually within the first 2 h an increase in pH to 6.0–6.5 was observed, followed by a slow decrease in pH to approximately 5.5 after 7 h. When FC was added (after 2 h of preincubation) to the control medium, an additional decrease in pH to 4.0 was observed. However, when Cd^{2+} alone (at concentrations >0.1 μM) or Cd^{2+} together with FC was added at 120 min, significant suppression of medium acidification was found (Fig. 4 inset). For example, the H^+ concentration in the medium found in the presence of Cd^{2+} (100 μM) combined with FC was approximately fourfold lower than with FC alone (Fig. 4 inset).

Effect of Cd^{2+}, FC, and TEA Chloride on E_m

Cd^{2+} ions administered into the control medium caused depolarization of E_m, the extent of which was dependent on Cd^{2+} concentration and time after its addition (Table 1). For example, treatment of parenchymal cells of maize coleoptile segments with 100 or 1000 μM Cd^{2+} caused depolarization of E_m by 55 and 67 mV, respectively. In turn, addition of FC to the control medium caused immediate hyperpolarization of E_m by 23 mV (from -121 ± 4.1 to -144 ± 6.2 mV). This hyperpolarization of E_m was suppressed in the presence of Cd^{2+} at 1000 μM but not with Cd^{2+} at 100 μM (Table 1). Cd^{2+} at 1000 μM not only suppressed FC-induced hyperpolarization of the E_m but also caused additional membrane depolarization.

TEA chloride applied to the control medium caused hyperpolarization of E_m, during which E_m became 33.2 ± 4.0 mV more negative than the original potential (-116.1 ± 4.6 mV) (Table 1). TEA chloride combined with Cd^{2+} counteracted this Cd^{2+}-induced depolarization of E_m (Table 1). Interestingly, Cd^{2+} at the highest concentration (1000 μM), but not Cd^{2+} at 100 μM, suppressed hyperpolarization of E_m induced by TEA chloride. When FC and TEA chloride were combined, they did not cause any additional effect on E_m compared with that induced by each substance separately. In turn, combined FC, TEA chloride, and Cd^{2+} counteracted depolarization of E_m induced by Cd^{2+} (Table 1). This was similar to TEA chloride applied only with Cd^{2+}, but it was not the case for FC applied with Cd^{2+} at 1000 μM.

Table 1 E_m changes of parenchymal coleoptile cells with addition of Cd (100 and 1000 μM) and Cd together with FC (1 μM) and/or 30 mM TEA chloride

Treatments	E_m (mV)			
	E_m after stabilization in control medium (time 0)	E_m after 20 min	E_m after 40 min	E_m after 60 min
FC	−121.0 ± 4.1	−137.0 ± 5.5	−144.2 ± 5.1	−144.0 ± 6.2
TEA chloride	−116.1 ± 4.6	−123.8 ± 5.7	−152.3 ± 4.8	−149.3 ± 4.0
TEA chloride + FC	−117.0 ± 6.2	−129.1 ± 5.5	−146.2 ± 11.7	−146.2 ± 11.9
Cd (100 μM)	−119.5 ± 8.4	−112.8 ± 5.3	−68.0 ± 3.2	−64.4 ± 3.3
FC + Cd	−118.8 ± 4.3	−134.2 ± 7.5	−145.3 ± 7.2	−142.0 ± 8.5
TEA chloride + Cd	−114.5 ± 6.3	−127.7 ± 6.1	−149.7 ± 6.8	−143.4 ± 7.3
TEA chloride + FC + Cd	−115.4 ± 5.0	−121.6 ± 4.7	−144.1 ± 6.3	−146.0 ± 7.9
Cd (1000 μM)	−122.0 ± 7.7	−102.6 ± 4.2	−55.9 ± 1.8	−55.1 ± 2.6
FC + Cd	−120.6 ± 8.1	−117.8 ± 4.5	−72.5 ± 3.8	−60.5 ± 3.5
TEA chloride + Cd	−119.2 ± 6.6	−128.6 ± 8.5	−136.3 ± 7.5	−125.9 ± 6.3
TEA chloride + FC + Cd	−121.4 ± 8.4	−130.7 ± 8.2	−134.9 ± 8.2	−128.7 ± 6.9

At time 0, the control medium was changed for a new one, at the same salt composition, containing Cd, FC, TEA chloride, or Cd together with FC or/and TEA chloride. E_m was measured continuously after 2 h of segment preincubation in control medium. Data are means of at least five independent experiments. *Error* indicates means ± SEs

Cd and Ca Content in Maize Coleoptile Segments

Cd and Ca content in maize coleoptile segments was also determined (Figs. 5, 6, 7). It was found that Cd content in maize coleoptile segments depended on Cd concentration in the control medium. An increase in Cd concentration from 10 to 100 μM caused an approximately 11-fold increase of its accumulation in maize coleoptile segments, whereas an increase in Cd^{2+} concentration from 100 to 1000 μM caused only a 2.3-fold increase of Cd^{2+} tissue content (Fig. 5). When FC was combined with Cd^{2+} at 100 μM, a 50% decrease of the Cd accumulation in coleoptile segments [from 1901 ± 103 to 895 ± 27 mg kg^{-1} dry mass (dm)] was observed compared with the segments treated with Cd^{2+} alone. FC did not change the content of Cd^{2+} in coleoptile segments treated with Cd^{2+} at 1000 μM. Furthermore, the effect of TEA chloride on Cd^{2+} and Ca^{2+} accumulation in segments of maize coleoptiles was also studied. These results indicate that TEA chloride decreased accumulation of both elements (Figs. 5, 6). In segments treated with TEA chloride combined with 100 or 1000 μM Cd^{2+}, Cd^{2+} content was relatively lower compared with segments treated with Cd alone. As illustrated in Fig. 6, a total Ca^{2+} content of 573.32 ± 49.9 mg kg^{-1} (dm) was decreased approximately twofold after TEA chloride application. The application of TEA chloride together with FC or/and Cd^{2+} to the control medium led to significant decrease ($P < 0.05$) in Cd (Fig. 5) and Ca concentrations (Fig. 6).

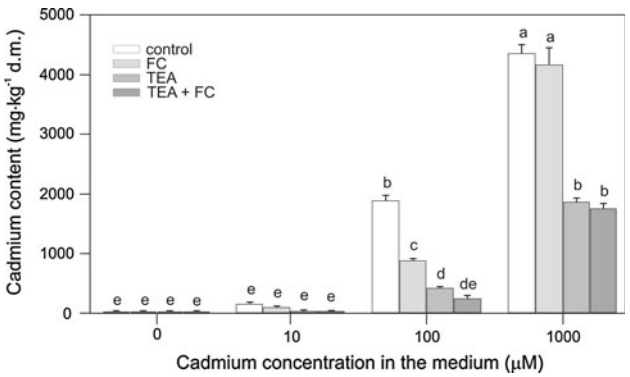

Fig. 5 Cd content in maize coleoptile segments exposed (5 h) to either 10, 100, or 1000 μM Cd, Cd combined with FC (1 μM), and/or 30 mM TEA. The segments were first preincubated (2 h) in control medium, whereupon Cd or Cd with FC or/and TEA were added. Results are the means of four independent experiments. *Bars* indicate means ± SEs. Means followed by the *same letter* are not significantly different from each other (LSD test $P < 0.05$)

Interestingly, FC alone or FC combined with Cd^{2+} decreased Ca content in maize coleoptile segments (Fig. 6). In addition, Cd^{2+} at the highest concentration (1000 μM) decreased Ca content by 50%, probably as a result of Cd competitively inhibiting Ca uptake. Both FC and Ca-channel blockers (La and Ver) significantly decreased (LSD test $P < 0.05$) Cd content in maize coleoptile segments (Fig. 7). La and Ver practically did not change Ca content in maize coleoptile segments (data not shown).

Discussion

Despite there being much research on Cd toxicity, our knowledge concerning the effect of this heavy metal on

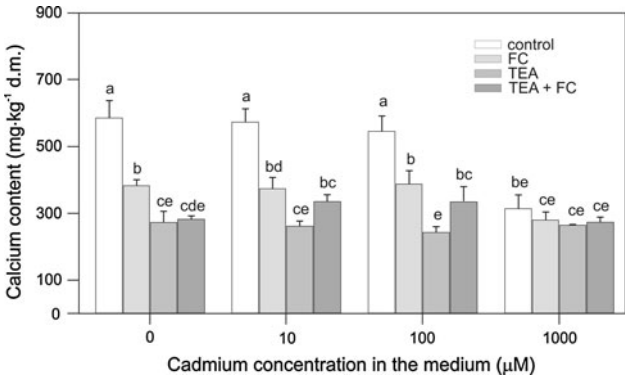

Fig. 6 Ca content in maize coleoptile segments exposed (5 h) to either 10, 100, or 1000 μM Cd, Cd combined with FC (1 μM), and/or 30 mM TEA. The segments were first preincubated (2 h) in control medium, whereupon Cd or Cd with FC or/and TEA were added. Results are the means of four independent experiments. *Bars* indicate means ± SEs. Means followed by the *same letter* are not significantly different from each other (LSD test $P < 0.05$)

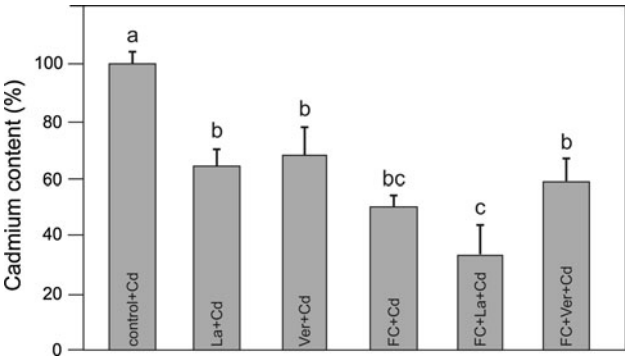

Fig. 7 Cd content in maize coleoptile segments exposed (5 h) to either 100 μM Cd, Cd combined with FC (1 μM), and/or Ca-channel blockers (La and Ver). The segments were first preincubated (2 h) in control medium, whereupon Cd or Cd with FC or/and Ca-channel blockers (La and Ver) were added. Results are the means of four independent experiments. *Bars* indicate means ± SEs. Means followed by the *same letter* are not significantly different from each other (LSD test $P < 0.05$)

plant growth is still limited. The main goal of this work was to determine the mechanisms of Cd-induced inhibition of elongation growth as well as the mechanism of Cd uptake.

It is currently well established that an FC-binding site arises from the interaction of 14-3-3 protein dimer with the C-terminal autoinhibitory domain of PM H^+-ATPase, and FC stabilizes this complex, thus causing an increase in proton pump activity (Jahn et al. 1997; Baunsgaard et al. 1998; Fuglsang et al. 1999; Oecking and Hagemann 1999; Würtele et al. 2003). The data presented in this article, i.e., that FC causes (1) acceleration of elongation growth compared with endogenous growth (Figs. 1, 2), (2) enhancement of proton extrusion compared with FC-free medium (Fig. 4), and (3) immediate hyperpolarization of the PM (Table 1), are in good agreement with the results obtained by other investigators (Cleland et al. 1977; Kutschera and Schopfer 1985; Felle 1989; Lüthen et al. 1990; Rück et al. 1993; Karcz and Burdach 2002; Hager 2003; Karcz and Burdach 2007).

The simultaneous measurements of elongation growth and pH level of the incubation medium showed that in maize coleoptile segments, Cd^{2+} at >1 μM produced a significant inhibition of endogenous growth (Fig. 1) as well as a decrease in proton extrusion (Fig. 4). Cd^{2+} also caused depolarization of E_m (Table 1). These findings match strikingly with Cd^{2+} content in maize coleoptile segments (Figs. 5, 7) and support the results of our previous experiments (Karcz and Kurtyka 2007). It should be pointed out that the large range of Cd concentrations used in our experiments showed that concentrations effectively producing growth inhibition are high compared with concentrations usually found in soil solutions. This discrepancy, among others, results from the fact that in the case of environmental studies, durations of growth experiments are usually significantly longer compared with studies using a model system, such as coleoptile segments, which, as our experiments showed, need greater Cd concentration. The inhibition of proton extrusion and depolarization of E_m observed here in the presence of Cd^{2+} are in line with the findings of other investigators, who reported an inhibition of PM H^+-ATPase (Ros et al. 1992; Fodor et al. 1995; Astolfi et al. 2003) and depolarization of E_m by Cd^{2+} (Kennedy and Gonsalvez 1987; Aidid and Okamoto 1992; Llamas et al. 2000; Pavlovkin et al. 2006).

Addition of FC together with Cd^{2+} counteracted the toxic effect of Cd on endogenous growth (growth in control) of maize coleoptile segments, although Cd^{2+} at concentrations of 100 and 1000 μM inhibited FC-induced growth by 10 and 30%, respectively (Fig. 2). The first phenomenon is probably due to stimulation of the segment elongation by FC, which, according to the "acid growth theory," enhances growth by stimulating proton extrusion through PM H^+-ATPase. It is suggested that this proton extrusion is large enough to overcome the toxic effect of Cd^{2+} on endogenous growth (Fig. 4). In contrast, Cd^{2+} at concentrations of 100 and 1000 μM diminished FC-induced growth and proton extrusion, probably as a result of PM H^+-ATPase inhibition. This last suggestion is also supported by the experiments in which FC-induced E_m hyperpolarization was suppressed by Cd^{2+} at a concentration of 1000 μM. Until now, there has been no doubt that FC-induced PM hyperpolarization is a consequence of a stimulated proton extrusion through PM H^+-ATPase (Cleland et al. 1977; Marré 1979; Kutschera and Schopfer 1985; Karcz et al. 1995; Karcz and Burdach 2007).

The results presented here also show that FC combined with Cd^{2+} at 100 μM decreased (by 50%) Cd content in

maize coleoptile segments (Figs. 5, 7). This lower Cd^{2+} concentration could also decrease the toxic effect of Cd^{2+} (at least for Cd^{2+} at 100 μM) on elongation growth of coleoptile segments. In the case of greater Cd^{2+} concentration (1000 μM), FC (but not TEA chloride) was not effective in either recovering Cd^{2+}-induced membrane depolarization or decreasing Cd content in maize coleoptile cells. This finding is probably due to irreversible inhibition of PM H^+-ATPase by such high Cd concentrations. In trying to explain the effect of FC on Cd^{2+} accumulation in maize coleoptile segments, we assumed that Cd enters plant cells by way of Ca channels. Such a possibility has previously been described in plants and animals (Perfus-Barbeoch et al. 2002 and literature therein). It is also known that activation of PM Ca channels and resulting Ca^{2+} influx depends on the E_m (for review see White 2000).

Taking into account that FC combined with Cd^{2+} at 100 μM hyperpolarized E_m potential of the parenchymal cells (Table 1), it could be suggested that the Ca channels (probably depolarization-activated Ca^{2+} channels) are closed, thus decreasing Cd^{2+} influx into the cell. These findings are also supported by the fact that FC alone, or FC combined with Cd^{2+}, decreased Ca content cells of coleoptile segments (Fig. 6). To prove our hypothesis that Cd^{2+} enters plant cells by way of Ca channels, we performed experiments with Ca-channel blockers (La and Ver) and also with TEA chloride, which blocks potassium channels (Thiel et al. 1996; Claussen et al. 1997; White 2000; Tode and Lüthen 2001; Osawa and Matsumoto 2002; Lindberg et al. 2004; Shishova and Lindberg 2004) as well as Ca channels (White 2000; White et al. 2002). As indicated in Fig. 7, both La and Ver significantly decreased Cd^{2+} content in maize coleoptile segments. This finding is probably due to the blocking of Ca channels, by which Cd^{2+} ions enter cells. TEA chloride was not used in growth experiments because it inhibits FC-induced growth in maize coleoptile segments, probably as a result of K^+ inward rectifying (ZMK1) channel inhibition (Philippar et al. 1999; Tode and Lüthen 2001; Siemieniuk 2006). However, it was found that TEA chloride was much more effective than FC in (1) PM hyperpolarization (Table 1), (2) decreasing Cd and Ca content in coleoptile segments treated with Cd^{2+} (Figs. 5, 6), and (3) recovery of E_m after its depolarization induced by the highest Cd^{2+} concentration (Table 1). Our data showing the effects of TEA chloride on E_m of plant cells are consistent with results obtained by Olivetti et al. (1995) and Siemieniuk (2006), who showed that TEA chloride hyperpolarized the E_m of root cap cells of *Phaseolus vulgaris* and parenchymal cells of *Zea mays* coleoptile segments, respectively. It also has been shown previously (Pavlovkin et al. 2006) that FC added to Cd^{2+}-treated (1 mM) cortical cells in maize roots accelerated recovery of E_m.

The results reported by Lindberg et al. (2004) with protoplasts isolated from wheat seedlings should also be mentioned. These investigators showed that Cd^{2+} uptake into the cytosol of wheat leaves and root protoplasts was inhibited by Ca and potassium chloride as well as by Ver and TEA chloride (inhibitors of Ca and potassium channels, respectively). They suggested that Cd uptake into the cytosol of wheat protoplasts is mediated in part by channels permeable to Ca and potassium and is dependent on E_m. Lindberg et al. (2004) suggested that Cd^{2+} can be partly taken up by K^+-channels because TEA chloride inhibits Cd^{2+} uptake. However TEA chloride could also inhibit PM Ca^{2+}-channels (White 2000; White et al. 2002) excluding a role of K^+-channels in decreasing Cd^{2+} uptake. Our results with TEA chloride also support this hypothesis because TEA chloride significantly diminished uptake of Cd^{2+} and Ca^2. Because TEA chloride inhibits FC-induced growth (Tode and Lüthen 2001; Siemieniuk 2006), probably as a result of ZMK1 channel inhibition, it is unlikely that Cd^{2+} enters the cells by way of K^+-channels. We showed that hyperpolarization of PM, rather than its depolarization observed in the presence of metabolic inhibitors and TEA chloride (Lindberg et al. 2004), is responsible for decreasing Cd^{2+} uptake by coleoptile segments.

The results presented here are generally in agreement with data obtained by Perfus-Barbeoch et al. (2002), who showed in patch-clamp experiments with *Vicia faba* guard cell protoplasts that PM K^+-channels were insensitive to external Cd^{2+} application, whereas Ca^{2+}-channels were found to be permeable to Cd^{2+}. They also showed that Cd^{2+} enters cells by way of hyperpolarization-activated Ca^{2+}-channels. In contrast to Perfus-Barbeoch et al. (2002), we suggest that Cd^{2+} enters the cells of maize coleoptile segments through depolarization-activated Ca^{2+}-channels. Such channels have been characterized from suspension cultures of mesophyll and root cells (Thuleau et al. 1994a, b; Pineros and Tester 1997; White 2000). All of them are activated at voltages less negative than −140 mV.

In conclusion, the results presented in this article demonstrate that the mechanism by which FC counteracts the toxic effects of Cd^{2+} (except for 1000 μM Cd^{2+}) on the growth of maize coleoptile segments involves both stimulation of PM H^+-ATPase activity by FC and Cd^{2+}-permeable, voltage-dependent Ca channels, which are blocked by FC- and TEA chloride-induced PM hyperpolarization.

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