Planta (2004) 219: 526–532
DOI 10.1007/s00425-004-1256-z

ORIGINAL ARTICLE

Sylvia Lindberg · Tommy Landberg · Maria Greger

A new method to detect cadmium uptake in protoplasts

Received: 15 December 2003 / Accepted: 27 February 2004 / Published online: 7 April 2004

Abstract The mechanism for cadmium (Cd^{2+}) uptake into the cytosol of protoplasts from 5- to 7-day-old wheat seedlings (*Triticum aestivum* L. cv. Kadett) was investigated by a new method, using fluorescence microscopy and the heavy metal-specific fluorescent dye, 5-nitrobenzothiazole coumarin, BTC-5N. Cadmium fluorescence gradually increased in the cytosol of shoot and root protoplasts upon repeated additions of $CdCl_2$ to the external medium, reflecting an uptake of Cd^{2+}. The uptake was inhibited by calcium and potassium chloride, as well as by Verapamil and tetraethylammonium (TEA), inhibitors of calcium and potassium channels, respectively. Calcium competitively inhibited the cadmium uptake. The metabolic inhibitors vanadate and dinitrophenol partly inhibited the uptake, suggesting it was dependent on membrane potential. The results indicate that cadmium is taken up by channels permeable to both calcium and potassium. The total uptake of cadmium into the protoplasts was also detected by unidirectional flux analyses using $^{109}Cd^{2+}$, and showed approximately the same maximal concentration of Cd^{2+} as the fluorescence measurements. By combining the two methods it is possible to detect both uptake into the cytosol and into the vacuole.

Keywords Cadmium uptake · Fluorescence microscopy · Heavy metal · Protoplast · *Triticum*

Abbreviations BTC-5N, AM: Acetoxymethyl ester of 5-nitrobenzothiazole coumarin · DNP: 2,4-Dinitrophenol · TEA: Tetraethylammonium

S. Lindberg (✉)
Department of Plant Biology and Forest Genetics,
SLU, Box 7080, 750 07 Uppsala, Sweden
E-mail: sylvia.lindberg@vbsg.slu.se
Fax: +46-18-673279

T. Landberg · M. Greger
Department of Botany, Stockholm University,
106 91 Stockholm, Sweden

Introduction

Cadmium is a heavy metal that is toxic not only to man but also to animals and plants. It is easily taken up by plant roots and negatively affects physiological processes such as photosynthesis, respiration, growth, water transport and uptake of magnesium, iron and potassium (Lindberg and Wingstrand 1985; Reese and Roberts 1985; Greger and Lindberg 1987; Barcelo and Poschenrieder 1990; Greger and Ögren 1991; Marchiol et al. 1996; Larsson et al. 2001). Heavy metals such as copper and zinc, and especially cadmium, are toxic when present at high amounts in the cytosol. Plants, therefore, have developed several detoxification and tolerance mechanisms to exclude heavy metals from this part of the cell (Clemens 2001; Hall 2002).

As a result of human activities, cadmium levels in agricultural soils are increasing and, thereby, the cadmium content in crops used for food and fodder has also risen. To prevent the presence of cadmium in the food chain it is necessary to understand the mechanisms by which cadmium is taken up into plant roots and cells. From physiological experiments several mechanisms have been suggested for uptake of cadmium into plant cells. Since cadmium interferes with the uptake of other ions, such as iron, zinc and calcium in plants, it was thought that Cd^{2+} could be taken up by the same pathways as used by those elements (Costa and Morel 1993; Askwith et al. 1996; Assman et al. 1996; Lasat et al. 2000). There is a competition between Ca^{2+} and Cd^{2+} within the root tissue of sugar beets, suggesting that these two ions can compete for the same binding sites (Greger and Lindberg 1987; Greger and Bertell 1992). Cadmium also inhibits the active uptake of potassium ($^{86}Rb^{+}$/K^{+}) in sugar beet roots and in birch (Lindberg and Wingstrand 1985; Gussarsson and Jensén 1992). In experiments with plasma membranes from sugar beet roots, cadmium competitively inhibited the K^{+} stimulation of ATPase activity, suggesting that Cd^{2+} and

K^+ could bind to the same uptake site on the plasma membrane (Lindberg and Wingstrand 1985). It is possible that Cd^{2+} can be transported via K and Ca channels within the plasma membrane, since these channels are not very specific.

More recent experiments using yeast mutants and transformed yeast cells, especially *Saccharomyces cerevisiae* and to some degree *Schizosaccharomyces pombe*, have introduced new possibilities of finding transporters for cadmium. Based mainly on uptake studies and growth effects, transporter proteins of the ZIP family, e.g. the iron transporter IRT1, the zinc transporter ZNT1 and the Nramp-transporters from *Arabidopsis* and rice, were proposed as possible transporters also for cadmium (Korshunova et al. 1999; Pence et al. 2000; Thomine et al. 2000). It is also interesting that the wheat LCT1, which originally was cloned by complementation of a K high-affinity uptake-deficient yeast mutant, mediates Na^+ influx, as well as an increased Cd^{2+} and Ca^{2+} uptake activity in *S. cerevisiae* (Schachtman et al. 1997; Clemens et al. 1998).

Studies of cadmium uptake in root cells by use of the cadmium isotope $^{109}Cd^{2+}$ suggested that the total uptake of Cd^{2+} in cytosol and vacuoles might depend on both passive and active pathways (Costa and Morel 1993, 1994; Hart et al. 1998). The uptake of Cd^{2+} in the nanomolar concentration range was thought to be mediated by carriers (Hart et al. 1998). However, little is known about the mechanism for the primary uptake of Cd^{2+} into the cytosol. We have used the new fluorescent 5-nitrobenzothiazole coumarin probe, BTC-5N (Molecular Probes, The Netherlands) to measure the uptake of Cd^{2+} in protoplasts of wheat. By the use of BTC-5N, it should be possible to estimate the concentration of free Cd^{2+} in the cytosol. Previously, BTC-5N was used only for studies of cadmium concentrations in animal cells, as well as for analysis of water quality (Kanthamsy et al. 1995; Prestel et al. 2000). The acetoxymethyl ester of 5-nitrobenzothiazole coumarin (BTC-5N, AM) is split into the carboxylate form, which by binding to Cd^{2+} increases its fluorescence.

Since the main purpose was to investigate if BTC-5N, AM could be used to detect cadmium uptake into plant cells, we compared the uptake of Cd^{2+} into the cytosol as determined by use of BTC-5N, AM with the uptake of cadmium into the protoplast determined by using the isotope $^{109}Cd^{2+}$. Another purpose was to study if Cd^{2+} can be transported through Ca and K channels in the plasma membrane, or whether other transport mechanisms occur. We, therefore, detected the uptake of Cd^{2+} in the absence and presence of calcium and potassium in the medium, as well as in the absence and presence of Verapamil and tetraethylammonium (TEA), blockers of calcium and potassium channels, respectively. By use of the metabolic inhibitor dinitrophenol (DNP) and the H^+-ATPase inhibitor vanadate, we investigated if the uptake was dependent on membrane potential.

Materials and methods

Cultivation

Caryopses of wheat (*Triticum aestivum* L. cv. Kadett; Svalöf-Weibull, Landskrona, Sweden) were surface-sterilized, washed and soaked in 5 mM $CaSO_4$ as described by Pettersson and Strid (1989). The seedlings were grown in beakers on double layers of Miracloth (LIC, Stockholm, Sweden) using a complete nutrient solution containing 2 mM KNO_3, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 1.5 μM H_3BO_3, 0.5 μM $MnSO_4$ and 0.5 mM Fe-EDTA. They were cultivated in a climate-controlled chamber for 5–7 days and were illuminated for 16 h per day at an irradiance of 118 W m^{-2} at the top of the shoots. The temperature in the growth chamber was 20 ± 1°C. The relative humidity was 50–60% throughout the cultivation.

Protoplast isolation and loading

The root protoplasts were prepared as described by Lindberg and Strid (1997). Root tips were treated with 2% cellulysin cellulase (EC 3.2.1.4) from *Trichoderma viride* (Calbiochem; LabKemi) and 0.1% pectolyase Y23 (EC 3.2.1.15) from *Aspergillus japonicus* (Kemila, Sollentuna, Sweden) in a medium containing 500 mM sorbitol, 0.05% polyvinylpyrrolidone (PVP; Sigma, St. Louis, MO, USA), 0.05% bovine serum albumin (BSA; Sigma), 0.5 mM $CaCl_2$ and 20 mM Tris–Mes (Sigma), at pH 5.5 for about 3 h. The root tips were rinsed twice, for 30 s each time, with a solution of the same composition but without enzymes. The three suspensions were finally pooled and filtered through nylon net with 100-μm pores. The filtrate was centrifuged at 100 *g* for 6 min. The protoplasts were purified by a sorbitol gradient as described by Lindberg (1995). They were then suspended in a medium containing 500 mM sorbitol, 0.1 or 1.0 mM $CaCl_2$, 0.2% BSA, 0.05% PVP and 5 mM Tris–Mes buffer (pH 5.5; medium A). The protoplast suspension was washed once with the same medium before dye loading.

The leaf protoplasts were isolated from 5- to 7-day-old wheat seedlings by an enzymatic method similar to that described by Lindberg (1995) with some modifications [using 1% cellulase from *Trichoderma resei* (Sigma; EC 3.2.1.4.) and 0.3% macerase "Maceroenzyme R-10" (Serva; EC 3.2.1.4)].

The protoplasts were loaded with BTC-5N in the acetoxymethyl ester form (BTC-5N, AM; Molecular Probes, Leiden, the Netherlands). A stock solution of BTC-5N was prepared by solving 50 μg of the dye in 39.5 μl dry ($< 0.1\%$ v/v water) dimethyl sulfoxide. The solution was mixed with 10 μl Pluronic F-127 (Molecular Probes) solution (20% w/v) in dimethyl sulfoxide. Five μl of the solution was used per 1 ml buffer. Loading continued for 50 min at 4 ± 1°C. At the end of the

incubation time the samples were centrifuged and pellets were resuspended into 1 ml of a solution containing 0.5 M sorbitol, 0.05% PVP, 0.2% BSA, 5 mM Hepes and 0.1 or 1 mM $CaCl_2$ at pH 7.0 (solution B). Before measurements, samples were kept in darkness at room temperature for 25 min.

Fluorescence measurements

An epi-fluorescence microscope (Axiovert 10; Zeiss, Oberkochen, Germany) supplied with an electromagnetic filter exchanger (Zeiss), xenon lamp (XBO 75; Zeiss), photometer (Zeiss), microprocessor (MSP 201; Zeiss) and a personal computer was used to determine the fluorescence intensity ratio after excitation at 415 nm. Emission wavelengths were 500–530 nm. The fluorescence measurements were made on single protoplasts with a 40×/0.75 planneofluar objective for phase contrast. The measurements taken every 250 ms were performed only with protoplasts of similar size and properly loaded in the cytosol.

For standard measurements, the BTC-5N, AM was hydrolysed according to Molecular Probes product information sheet [Acetoxy methyl (AM) esters, G002 09/29/96], to the cadmium-binding form, BTC-5N. This form was used for detection of Cd in samples of solution B (see above) supplied with $CdCl_2$ at different concentrations. In the presence of 0.05–1 μM Cd^{2+}, there was a linear relationship between fluorescence and Cd^{2+} concentration. Since $CaCl_2$ at 1 mM slightly interfered with the 415-nm Cd^{2+}-fluorescence, different calibrations were performed at 0.1 and 1.0 mM $CaCl_2$.

Measurement of unidirectional flux

The uptake experiments using the isotope ^{109}Cd were performed in 2-ml beakers, connected to a suction system with glass microfibre filters (GF/C; Whatman). Protoplasts were prepared as previously described and the protoplast density was counted. Protoplast samples (333 ml, 28,000–85,000 protoplasts mm^{-3}) were added to each of 10 beakers, and 2 other beakers containing only the protoplast medium were added as a control. Thereafter, 1 ml protoplast medium A (see above) containing the isotope ^{109}Cd (46,000 Bq) was added to each beaker. The treatment was ended by washing the samples three times with the protoplast medium. Samples (and glass microfibre filter) were then mixed with 5 ml H_2O. Five ml Emulsifier Safe (Packard) was added and the isotope was analysed by liquid scintillation (Wallac 1409). Also, protoplasts with no ^{109}Cd treatment were analysed as a control.

Statistics

Figures 2 and 4 show representative traces of specific experiments repeated more than five times with protoplasts from independent cultivations. Each value in Fig. 4 corresponds to ≥20 measurements. Figures 3, 4, 5 and 6 show the mean value ± SE for ≥4 independent experiments.

Results

The acetoxymethyl ester of the dye, BTC-5N, AM, could be loaded into the protoplasts after 50 min at 4 ± 1°C. The fluorescence was low and increased upon cadmium addition to the medium. In most of the protoplasts the dye was loaded into the cytosol, and not into vacuoles, as shown by the micrographs (Fig. 1a,b).

Cadmium uptake into the cytosol

The uptake experiments were performed with single root and shoot protoplasts, but since there was little difference in the uptake between root and shoot protoplasts, most of the experiments shown are on shoot protoplasts. In the presence of 0.1 mM $CaCl_2$ in the external solution, the fluorescence immediately increased upon addition of a low concentration of $CdCl_2$ (0.5 μM) to the protoplasts, indicating an increase of Cd^{2+} in the cytosol of the protoplast (shown for leaf protoplasts in Fig. 2a). Even in the presence of relatively high concentrations of Cd^{2+} in the external medium (220.5 μM), a very low amount of Cd^{2+} was taken up into the cytosol. The optimal increase in fluorescence within 6 min corresponded to approximately 0.35 μM Cd when compared with a standard curve (See Materials and methods).

Upon each repeated addition of cadmium the fluorescence usually increased and then formed a plateau at the higher level. Only upon addition of a low concentration of Cd^{2+} (5 μM), was the increase in fluorescence somewhat higher in root protoplasts than in shoot protoplasts; otherwise, there was little difference between data for root and shoot protoplasts (Fig. 3).

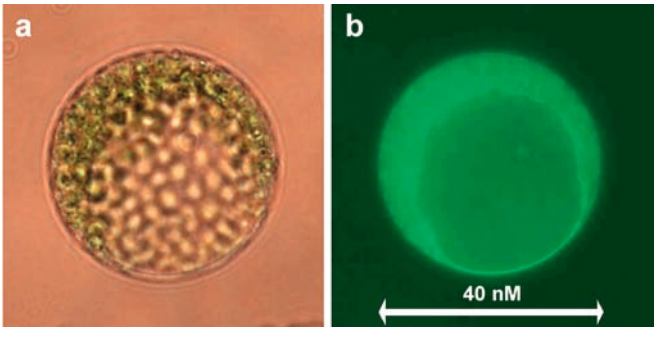

Fig. 1a, b Micrographs of a wheat (*Triticum aestivum*) leaf protoplast loaded with BTC-5N, AM for 50 min, as shown in transmitted light (**a**) and fluorescent light using a fluorescein isothiocyanate (FITC) filter (**b**)

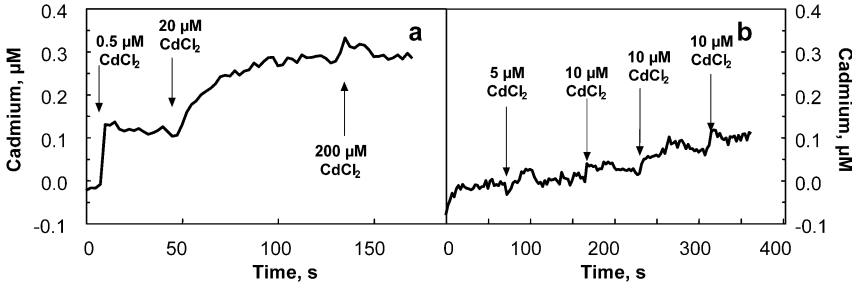

Fig. 2a, b Increase in free concentration of Cd^{2+} in the cytosol of a wheat leaf protoplast upon repeated additions of $CdCl_2$ to the protoplast medium in the presence of 0.1 mM (**a**) or 1.0 mM (**b**) $CaCl_2$. Excitation wavelength was 415 nm. Estimation of cadmium concentrations was made from standard determination using hydrolysed dye (BTC-5N)

In the presence of a higher concentration of $CaCl_2$ (1 mM), a smaller increase in fluorescence was obtained upon addition of cadmium than in the presence of 0.1 mM calcium, and after repeated additions there were further, but small, increases in fluorescence (Figs. 2b, 3). The optimal increase in fluorescence in the presence of 1 mM calcium corresponded to approximately 0.1 µM Cd^{2+} (Fig. 2b). Moreover, in the presence of 5–10 mM KCl instead of calcium (not shown), there was a similar decrease in fluorescence as in the presence of $CaCl_2$ (Fig. 3).

Since the presence of high calcium (1 mM), or potassium, in the external solution prevented the uptake of Cd^{2+}, the measurements were also made after pre-treatment of protoplasts for 15 min in a buffer containing either the calcium channel blocker, Verapamil, or the potassium channel blocker, TEA. Each channel blocker inhibited the Cd^{2+}-dependent increase in fluorescence upon cadmium addition (Fig. 3). Simultaneous addition of Verapamil and TEA completely inhibited the uptake of cadmium (not shown).

Moreover, calcium inhibited the uptake of Cd^{2+} in a competitive way as shown by a Dixon plot, where 1/percent fluorescence increase is plotted against inhibitor concentration (Fig. 4). From this plot the half-maximal inhibition constant, K_i for inhibition by calcium could be estimated to approximately 1 mM.

After pre-treatment of leaf protoplasts with vanadate or DNP, the increase in fluorescence upon cadmium addition was only half of that obtained in the control protoplasts, indicating that the uptake of cadmium into the cytosol was partly dependent on membrane potential (Fig. 5).

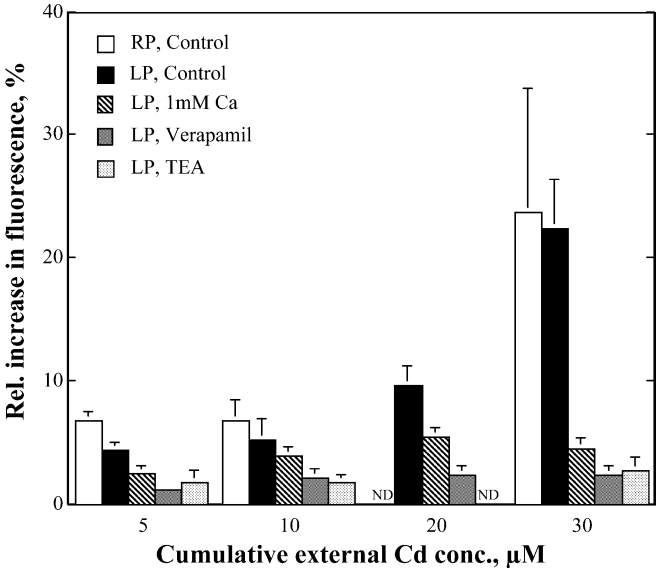

Fig. 3 Increase in free concentration of Cd^{2+} in the cytosol, given as relative increase in fluorescence at 415 nm excitation, of wheat leaf (*LP*) and root (*RP*) protoplasts upon repeated additions of $CdCl_2$ to the protoplast medium in the presence of 1 µM Verapamil, 10 mM TEA or 1 mM calcium. The medium contained 0.1 mM calcium, if no other information is given. *ND* No data. Mean ± SE, $n \geq 4$

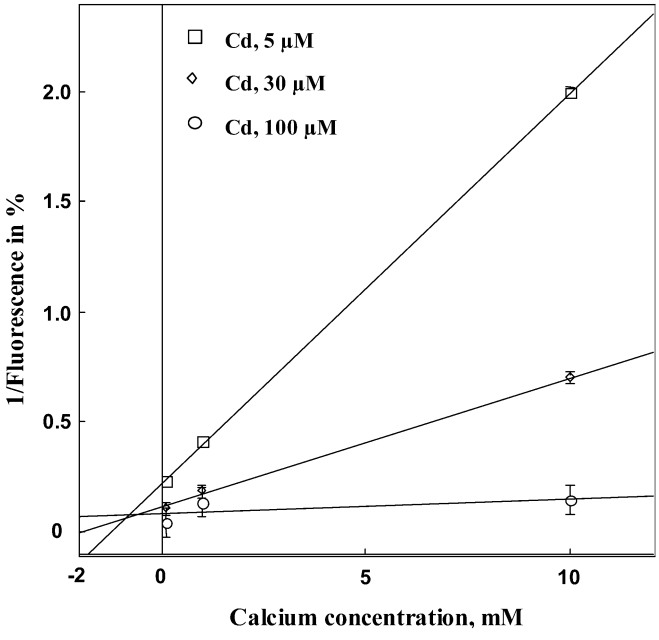

Fig. 4 Dixon plot showing the uptake of Cd^{2+} into the cytosol, given as relative increase in fluorescence at 415 nm excitation of wheat leaf protoplasts, at different concentrations of Ca^{2+} and Cd^{2+} in the protoplast medium. 1/percent fluorescence increase plotted against inhibitor concentration. Each value represents 20 measurements from independent cultivations. Mean ± maximal SE

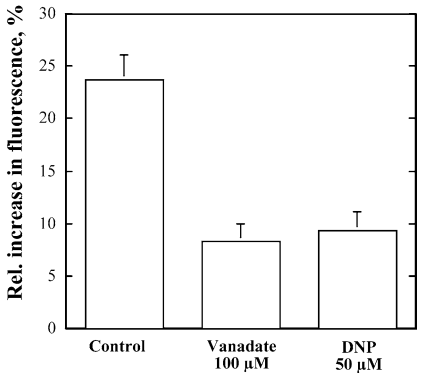

Fig. 5 Increase in free concentration of Cd^{2+} in the cytosol, given as relative increase in fluorescence at 415 nm, of wheat leaf protoplasts upon addition of 50 μM $CdCl_2$ to the protoplast medium in the absence, and in the presence of 100 μM vanadate or 50 μM DNP. Pre-treatment time with vanadate and DNP was 10 min. The medium contained 0.1 mM calcium. Mean ± SE, $n \geq 4$

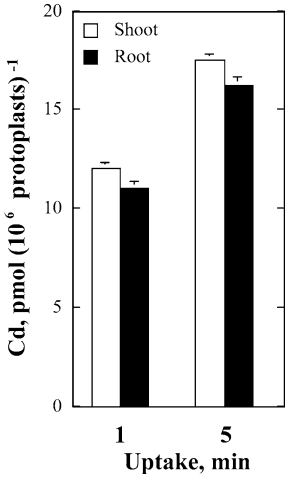

Fig. 6 Total uptake of cadmium in root and shoot protoplasts, in picomoles Cd^{2+} per million protoplasts, obtained 1 or 5 min after addition of cadmium to protoplasts. Cadmium was detected as $^{109}Cd^{2+}$. Mean ± SE, $n \geq 10$

Uptake of cadmium into whole protoplasts

The uptake of Cd^{2+} into root and shoot protoplasts was measured by use of the cadmium isotope $^{109}Cd^{2+}$ (Fig. 6). The pattern of cadmium uptake in both root and shoot protoplasts after 1 min corroborates the pattern of cytosolic uptake (Figs. 2, 6). The total uptake of cadmium after 1 and 5 min, respectively, was approximately the same in root and shoot protoplasts from control plants. After 1 min in the presence of $^{109}Cd^{2+}$ the control protoplasts contained approximately the same maximal concentration of Cd (0.36 μM) as the cytosol (0.35 μM), as determined by fluorescence (Fig. 2 and see Appendix). However, the uptake measurement using $^{109}Cd^{2+}$ showed a higher concentration in the whole protoplast after 5 min, compared with the cytosol after 6 min (Figs. 6, 2).

Discussion

The present results show that the fluorescent dye BTC-5N can be used to detect free cadmium concentrations in plant protoplasts. Approximately the same maximal concentration of Cd^{2+} was found in the cytosol and whole protoplast, using the fluorescence and the isotope techniques, respectively (Figs. 2, 6). There was little difference in short-term cadmium uptake into root and shoot protoplasts (Fig. 6).

It has been suggested that no free cadmium occurs inside a cell because this ion has high affinity for S-, N- and O-containing ligands and could bind strongly to, for example, glutathione and other SH-containing molecules in the cell. It is possible, however, that BTC-5N has a higher affinity for cadmium than for those molecules, or that the binding of Cd^{2+} to BTC-5N is faster. The fluorescence increases severalfold upon binding of Cd^{2+} to BTC-5N.

In the presence of a *low* external concentration of calcium (0.1 mM), addition of Cd^{2+} to protoplasts resulted in an increased level of free Cd^{2+} in the cytosol, due to less competition with calcium (Figs. 2a, 4). Further additions of Cd^{2+} increased the concentration of Cd^{2+} in the cytosol and in the whole protoplast to approximately the same maximal concentration in the control protoplasts after 1 min (Figs. 2, 6, and Appendix). However, 5 min after addition of cadmium to protoplasts, the concentration in the whole protoplast became higher, probably due to transport to the vacuoles (Fig. 6 and Appendix).

Since calcium inhibited the uptake of cadmium in a competitive way, it is likely that cadmium and calcium use the same channels (Fig. 4). Clemens et al. (1998) also found a strong inhibition of cadmium uptake by calcium exceeding 100 μM in transformed yeast cells. The estimated half-maximal inhibition of the *LTC1*-mediated Cd-uptake was only 25 μM. However, half-maximal inhibition in native yeast cells occurred at a Ca^{2+} concentration of 700 μM, a value similar to that for wheat protoplasts, where the inhibition constant was ≈1 mM (Fig. 4).

The results suggest that the uptake of cadmium into the cytosol of wheat leaves and root protoplasts, in the nano- to micromolar range, proceeds through channels permeable to both calcium and potassium. This was confirmed, because the inhibitors of these channels, Verapamil and TEA, as well as high concentrations of Ca^{2+} and K^+, blocked the increase (uptake) of Cd^{2+} in control protoplasts (Figs. 2, 3). It was earlier shown that Verapamil inhibits the uptake of calcium into protoplasts by Ca-channels and that TEA inhibits potassium uptake by K^+-channels (Graziana et al. 1988; Rengel and Elliott 1992; Thiel et al. 1996). In the presence of both Verapamil and TEA, there was no Cd^{2+}-influx at all, showing that the effect of the inhibitors was additive (not shown). Therefore, it is unlikely that there is any uptake through iron or zinc transporters in this case.

Cadmium also enters the plasma membrane mainly via Ca channels in animal, human and fungal cells. Cadmium uptake into the symplasm of a mycorrhizal fungus was partially linked to potential-dependent calcium uptake, since the calcium ionophore A23187 inhibited cadmium accumulation (Blaudez et al. 2000). In animal and human cells, calcium channels, at least partly, take up Cd^{2+}, and the uptake of cadmium is inhibited by Verapamil (Souza et al. 1996, 1997; Weidner and Sillman 1997; Braeckman et al. 1999; Craig et al. 1999). Clemens et al. (1998) showed that in transformed yeast cells the plant cDNA *LCTI* mediates the uptake of both calcium and cadmium. Moreover, Perfus-Barbeoch et al. (2002) recently proposed that cadmium could enter guard cell protoplasts via hyperpolarized calcium channels, but not by potassium channels.

Our results show that cadmium uptake into the cytosol of wheat protoplasts partly takes place by channels permeable to calcium and potassium, and is dependent on membrane potential. It cannot be excluded that Cd^{2+} can be partly taken up by K^+-channels, since TEA inhibited the uptake. Verapamil could also have blocked K^+-channels (Terry et al. 1992), since the inhibition by Verapamil and TEA is between 56 and 89%. It is also likely that potassium, DNP, vanadate and TEA inhibit the uptake of cadmium by depolarising the plasma membrane. This would be expected if, for instance, Cd enters through hyperpolarized calcium channels, such as those described by Perfus-Barbeoch et al. (2002).

Conclusion

Both methods of cadmium detection show that the maximal accumulation of free cadmium into wheat protoplasts, as well as into the cytosol, is very low (0.35–0.36 μM). The uptake into the cytosol occurs via channels permeable to Ca^{2+} and K^+, and partly depends on membrane potential. Calcium competitively inhibits the uptake of cadmium into the cytosol, suggesting that uptake takes place through the same channels.

Acknowledgements We are grateful to Mr. Lars Bengtsson and Miss Clara Neuschütz, who took part in the experimental work.

Appendix

The diameter of protoplasts used for the experiments was approximately 40 μm. To estimate their volumes and Cd^{2+} concentrations it was assumed that the protoplasts contained 5% cytosol. By use of the formula $4\pi r^3/3$, the volume of 10^6 protoplasts could be estimated to 33.51 μl. The maximal concentration of cadmium (determined by $^{109}Cd^{2+}$) that was found in the protoplasts was then about 0.36 (0.3581) μM. Since the maximal cytosolic concentration, determined by fluorescence was 0.35 μM, the vacuolar concentration was approximately 0.36 (0.3585) μM.

References

Askwith CC, de Silva D, Kaplan J (1996) Molecular biology of iron acquisition in *Saccharomyces cerevisiae*. Mol Microbiol 20:27–34

Assman S, Sigler K, Hofer M (1996) Cd^{2+}-induced damage to yeast plasma membrane and its alleviation by Zn^{2+}: studies on *Schizosaccharomyces pombe* cells and reconstituted plasma membrane vesicles. Arch Microbiol 165:279–284

Barcelo J, Poschenrieder C (1990) Plant water relations as affected by heavy metal stress: a review. J Plant Nutr 13:1–37

Blaudez D, Botton B, Charlot M (2000) Effects of heavy metals on nitrogen uptake by *Paxillus involutus* and mycorrhizal birch seedlings. PEMS Microb Ecol 33:61–67

Braeckman B, Smagghe G, Brutsaert N, Cornelis R, Raes H (1999) Cadmium uptake and defence mechanism in insect cells. Environ Res 80:231–243

Clemens S (2001) Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212:475–486

Clemens S, Antosiewicz DM, Ward JM, Schachtman DP, Schroeder JI (1998) The plant cDNA LCT1 mediates the uptake of calcium and cadmium in yeast. Proc Natl Acad Sci USA 95:12043–12048

Costa G, Morel JL (1993) Cadmium uptake by *Lupinus albus* (L.): cadmium excretion, a possible mechanism of cadmium tolerance. J Plant Nutr 16:1921–1929

Costa G, Morel JL (1994) Efficiency of H^+ATPase activity on cadmium uptake by four cultivars of lettuce. J Plant Physiol 17:627–637

Craig A, Hare L, Tessier A (1999) Experimental evidence for cadmium uptake via calcium channels in the aquatic insect *Chironomus staegeri*. Aquat Tox 44:255–262

Graziana A, Fosset M, Ranjeva R, Hetherington AM, Lazdunski M (1988) Ca^{2+} channel inhibitors that bind to plant cell membranes block Ca^{2+} entry into protoplasts. Biochemistry 27:764–768

Greger M. Bertell G (1992) Effects of Ca^{2+} and Cd^{2+} on the carbohydrate metabolism in sugar beet (*Beta vulgaris*). J Exp Bot 43:167–173

Greger M, Lindberg S (1987) Effects of Cd^{2+} and EDTA on young sugar beets (*Beta vulgaris*). II. Net uptake and distribution of Mg^{2+}, Ca^{2+} and Fe^{2+}/Fe^{3+}. Physiol Plant 69:81–86

Greger M, Ögren E (1991) Direct and indirect effects of Cd^{2+} on the photosynthesis and CO_2-assimilation in sugar beet (*Beta vulgaris*). Physiol Plant 83:129–135

Gussarsson M, Jensén P (1992) Effects of copper and cadmium on uptake and leakage of K^+ in birch (*Betula pendula*) roots. Tree Physiol 11:305–313

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 366:1–11

Hart JJ, Welch RM, Norvell WA, Sullivan LA, Kochian LV (1998) Characterization of cadmium binding, uptake, and translocation in intact seedlings of bread and durum wheat cultivars. Plant Physiol 116:1413–1420

Kanthamsy AG, Isom GE, Borowitz JL (1995) Role of intracellular Cd^{2+} in catecholamine release and lethality in PC12 cells. Toxicology 81:151–157

Korshunova YO, Eide D, Clark WG, Guerinot ML, Pakrasi HB (1999) The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. Plant Mol Biol 40:37–44

Larsson EH, Bornman JF, Asp H (2001) Physiological effects of cadmium and UV-B radiation in phytochelatin-deficient *Arabidopsis thaliana*, cad 1-3. Austr J Plant Physiol 28:505–512

Lasat MM, Pence NS, Garvin DF, Ebbs SD, Kochian LV (2000) Molecular physiology in the Zn hyperaccumulator *Thlaspi caerulescens*. J Exp Bot 51:71–79

Lindberg S (1995) In situ determination of intracellular concentrations of K^+ in barley (*Hordeum vulgare* L. cv. Kara), using the fluorescent probe PBFI-AM. Planta 195:525–529

Lindberg S, Strid H (1997) Aluminum induces rapid changes in cytosolic pH and free calcium and potassium concentrations in root protoplasts of wheat (*Triticum aestivum*). Physiol Plant 99:405–414

Lindberg S, Wingstrand G (1985) Mechanism for Cd^{2+} inhibition of (K^+ + Mg^{2+}) ATPase activity and K^+($^{86}Rb^+$) uptake in roots of sugar beet (*Beta vulgaris*). Physiol Plant 63:181–185

Marchiol L, Leita L, Martin M, Peressotti A, Zerbi G (1996) Physiological responses of two soybean cultivars to cadmium. J Environ Qual 25:562–566

Pence NS, Larsen PB, Ebbs SD, Letham DL, Lasat MM, Garvin DF, Eide D, Kochian LV (2000) The molecular physiology of heavy metal transport in the Zn/Cd hyper accumulator *Thlaspi caerulescens*. Proc Natl Acad Sci USA 97:4956–4960

Perfus-Barbeoch L, Leonhardt N, Vavasseur A, Forestier C (2002) Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. Plant J 32:539–548

Pettersson S, Strid H (1989) Effects of aluminium on growth and kinetics of K^+($^{86}Rb^+$) uptake in two cultivars of wheat (*Triticum aestivum* L.) with different sensitivity to aluminium. Physiol Plant 76:255–261

Prestel H, Gahr A, Niessner R (2000) Detection of heavy metals in water by fluorescence spectroscopy: on the way to a suitable sensor system. J Anal Chem 368:182–191

Reese RN, Roberts LW (1985) Effects of cadmium on whole cell and mitochondrial respiration in tobacco cell suspension cultures (*Nicotiana tabacum* L. Var. Xanthi). J Plant Physiol 120:123–130

Rengel Z, Elliott DC (1992) Mechanism of aluminium inhibition of net $^{45}Ca^{2+}$ uptake by *Amaranthus* protoplasts. Plant Physiol 98:632–638

Schachtman DP, Kumar R, Schroeder JI, Marsh EL (1997) Molecular and functional characterization of a novel low-affinity cation transporter (LCT1) in higher plants. Proc Natl Acad Sci USA 94:11079–11084

Souza V, Bucio L, Jay D, Chavez E, Gutierrrez-Ruiz MC (1996) The effect of cadmium on calcium transport in a human fetal hepatic cell line (WRL-68 cells). Toxicology 11:97–104

Souza V, Bucio L, Gutierrrez-Ruiz MC (1997) Cadmium uptake by human hepatic cell lines (WRL-68 cells). Toxicology 120:215–220

Terry BR, Findlay GP, Tyerman SD (1992) Direct effects of Ca^{2+}-channel blockers on plasma membrane cation channels of *Amaranthus tricolor* protoplasts. J Exp Bot 43:1457–1473

Thiel G, Brudern A, Gradman D (1996) Small inward rectifying K^+ channels in coleoptiles: inhibition by external Ca^{2+} and function in cell elongation. J Membr Biol 149:9–20

Thomine S, Wang R, Ward JM, Crawford NM, Schroeder JI (2000) Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to *Nramp* genes. Proc Natl Acad Sci USA 97:4991–4996

Weidner WJ, Sillman AJ (1997) Low levels of cadmium chloride damage the corneal endothelium. Arch Toxicol 71:455–460