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RESEARCH PAPER

Response of plasma membrane H^+-ATPase to heavy metal stress in *Cucumis sativus* roots

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Abstract

The effect of heavy metals on the modification of plasma membrane H^+-ATPase (EC 3.6.3.14) activity in cucumber roots was studied. In plants stressed for 2 h with 10 μM or 100 μM Cd, Cu or Ni the hydrolytic as well as the transporting activity of H^+-ATPase in the plasma membranes of root cells was decreased. Transcript levels of *Cucumis sativus* plasma membrane H^+-ATPase in roots treated with 10 μM Cd, Cu, or Ni as well as with 100 μM Cu or Ni were similar to the control, indicating that the action of metals did not involve the gene expression level. Only in roots exposed to 100 μM Cd was the level of plasma membrane H^+-ATPase mRNA markedly decreased. The inhibition of the plasma membrane proton pump caused by 100 μM Cd, Cu and Ni was partially diminished in the presence of cantharidin, a specific inhibitor of protein phosphatases. Western blot analysis with the antibody against phosphothreonine confirmed that decreased activity of plasma membrane H^+-ATPase under heavy metals resulted from dephosphorylation of the enzyme protein. Taken together, these data strongly indicated that alteration of the enzyme under heavy metal stresses was mainly due to the post-translational modification of its proteins in short-term experiments.

Key words: Cd, Cu, H^+-ATPase activity, heavy metals, Ni, plasma membrane.

Introduction

Damage of the cell membrane system, especially the plasma membrane, is one of the primary events in heavy metal toxic action in plants. Disruption of membrane integrity is thought to be an effect of a complex interaction between heavy metals and functional groups of membranes. It is well known that metal ions are easily bound to both the sulphhydryl groups of proteins and the hydroxyl part of phospholipids (Devi and Prasad, 1999). They can also replace the calcium ions at essential sites on the cell membranes (Breckle and Kahle, 1991). All those events result in an increase of a non-specific membrane permeability and the parallel decrease of specific transporting activities which disrupt the ionic homeostasis and, subsequently, the activities of many enzymes crucial for basic cell metabolism.

The ATP-dependent proton pump of the plasma membrane has a central function in the regulation of ion homeostasis in the cytosol. This enzyme is encoded by a multigene family (Portillo, 2000; Arango *et al*., 2003) and the expression of at least some isogenes is differentially regulated according to tissue type and developmental stage (Oufattole *et al*., 2000). Many studies have also shown changes in the gene expression of the plasma membrane H^+-ATPase in response to a variety of environmental factors, including salt stress (Binzel, 1995), dehydration (Surowy and Boyer, 1991), light conditions (Harms *et al*., 1994), mechanical stress (Oufattole *et al*., 2000), and externally applied hormones (Frías *et al*., 1996). Increasing evidence indicates that, besides the genetic regulation of the proton pump, its activity might be fast modulated post-translationally at the protein

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Abbreviations: BTP, bis-tris propane; DAB, 3,3′-diaminobenzidine; FW, fresh weight; MES, 2-(*N*-morpholino) ethanesulphonic acid; PM, plasma membrane; PP2A, protein phosphatases type 2A; PP1, protein phosphatases type 1; TRIS, hydroxymethylaminomethane; BSA, bovine serum albumin; DTT, dithiothreitol; SDS, dodecylsulphate sodium salt; SDS-PAGE, dodecylsulphate sodium salt- polyacrylamide gel electrophoresis; PMSF, phenylmethylsulphonyl fluoride; EDTA, ethylene diamine tetraacetic acid.

level, mainly through reversible phosphorylation (Schaller and Sussman, 1988; Portillo, 2000). This important role in the regulation of the plasma membrane H^+-ATPase has its autoinhibitory domain in the C-terminal region of the enzyme (Jahn *et al*., 1997; Oecking *et al*., 1997; Baunsgaard *et al*., 1998; Camoni *et al*., 1998). In the low-activity state, the C-terminal tail interacts with the catalytic region of the enzyme, limiting its activity (Portillo, 2000). Many data have suggested that the activation of the plant plasma membrane H^+-ATPase is mediated through phosphorylation of the Thr-948 in the enzyme, which allows the binding of 14-3-3 protein (Svennelid *et al*., 1999). This causes displacement of the C-terminal tail and activates the PM H^+-ATPase (Palmgren, 2001; Arango *et al*., 2003). Dephosphorylation of the enzyme protein, mediated by the specific phosphatases belonging to the PP2 and followed by the release of 14-3-3 protein from the complex, inactivates H^+-ATPase.

To date, data concerning heavy metal action on the plasma membrane H^+-ATPase are very limited. Only a few observations have indicated that enzyme activity was decreased under heavy metal stresses (Kennedy and Gonsalves, 1989; Fodor *et al*., 1995). However, no attempts were undertaken to elucidate the mechanisms involved in this inhibition. In this work, the results of such investigations are presented. To explain the mechanism of metal action on the plasma membrane proton pump, the hydrolytic and transporting activities of H^+-ATPase were measured simultaneously with the expression of genes encoding the enzyme. Moreover, the effect of cantharidin, a specific inhibitor of phosphatases, on the inhibitory action of metals on the H^+-ATPase was determined. Since the activity of the plasma membrane proton pump depends on ATP, the changes in its level were measured in plants treated with metals. Finally, the accumulation of cadmium, copper, and nickel in cucumber roots was asseyed to estimate to what degree the efficiency of metal absorption may affect the H^+-ATPase activity.

Materials and methods

Cucumber seeds (*Cucumis sativus* L. var. Krak), germinated in darkness at 25 °C for 48 h, were transferred to a nutrient medium for 6 d. The nutrient solution contained 1 mM K_2SO_4, 0.2 mM $Ca(H_2PO_4)_2.H_2O$; 1.5 mM $CaSO_4.0.5H_2O$, 0.3 mM $MgSO_4.7H_2O$, and microelements 75 μM ferric citrate, 10 μM $MnSO_4.5H_2O$, 5 μM H_3BO_4, 1 μM $CuSO_4.5\ H_2O$, 0.01 μM $ZnSO_4.7H_2O$, 0.05 μM $Na_2MoO_4.2H_2O$. After this the plants were exposed for 2 h to the 0.33 mM MES-NaOH (pH 5.5) solution without (control) or with 10 μM Cd, Cu or Ni and 100 μM Cd, Cu or Ni. The plants were grown hydroponically with a 16 h photoperiod (180 μmol m^{-2} s^{-1}) at 25 °C during the day and 22 °C during the night. The relative humidity in the light and dark was 70%.

Plasma membrane (PM) vesicles were isolated from cucumber root microsomes by phase partitioning according to the procedure by Larsson (1985) and modified by Kłobus (1995). A 16 g phase system containing 6.2% (w/w) Dextran T500, 6.2% (w/w) polyethylene glycol 3350, 330 mM sorbitol, 5 mM KCl, 5 mM BTP-MES (pH 7.5) was used. The plasma membranes obtained by this procedure were mainly composed of right-side-out vesicles and were used to determine the hydrolytic ATPase activity. Part of the vesicles was turned to the inside-out oriented form by the method of Johansson *et al*. (1995) and used for the measurements of ATP-dependent H^+ transport in plasma membranes.

The hydrolytic activity of the vanadate-sensitive ATPase (PM-H^+-ATPase) was determined according to the procedure of Gallagher and Leonard (1982), as modified by Sze (1985). The reaction mixture contained 50 μg of protein (plasma membrane), 33 mM TRIS-MES (pH 7.5), 3 mM ATP, 2.5 mM $MgSO_4$, 50 mM KCl, 1 mM NaN_3, 0.1 mM Na_2MoO_4, 50 mM $NaNO_3$, ±200 μM Na_3VO_4, and 0.02% Triton X-100. PM H^+-ATPase activity was expressed as the difference between the activity measured in the absence and in the presence of Na_3VO_4. The P_i released during the reaction was determined according to Ames (1966) with 0.2% (w/v) sodium dodecyl sulphate included to prevent precipitation (Dulley, 1975).

H^+ transport activity was measured spectrophotometrically as the change in acridine orange absorbance at 495 nm (A_{495}) according to Kłobus and Buczek (1995). The assay medium contained: PM vesicles (about 50 μg of protein), 25 mM BTP-MES (pH 7.5), 330 mM sorbitol, 50 mM KCl, 0.1% BSA, 10 μM acridine orange, and 0.05% Brij 58. Proton transport was initiated by the addition of 3 mM Mg-ATP. For every combination, passive proton movement through the membrane was determined without ATP in the reaction medium.

Protein was measured according to Bradford (1976) in the presence of 0.02% Triton X-100 with bovine serum albumin as the standard.

Determination of adenosine triphosphate (ATP) was made according to Glaab and Kaiser (1999). The fresh root material (1 g) was ground in liquid nitrogen and 5 cm^3 of 4.5% perchloric acid was added and mixed until it thawed. The mixture was then supplemented with 0.125 cm^3 2 mM TRIS and centrifuged for 5 min at 5000 *g*. The pH of the supernatant was adjusted to 7.4 with 5 mol dm^{-3} K_2CO_3, and recentrifuged. ATP was determined with the 'firefly' luciferin-luciferase assay using a TD-20/20 Luminometer (Turner Designs, USA).

Total and symplastic metal levels were determined spectrophotometrically (Perkin-Elmer 3300) in fresh root tissues digested with concentrated HNO_3 in a microwave. Prior to the determination of the symplastic metal pool after 2 h exposure to Cu, Cd or Ni, the roots were washed with 5 mM $PbCl_2$ at 0 °C for 30 min, in order to remove the extracellular bound ions (Harrison *et al*., 1979). The apoplastic pool was calculated from the differences between the total and the symplastic metal level.

Total RNA was isolated from 50 mg of roots using Tri Reagent (Sigma). To evaluate the expression of the PM-H^+-ATPase gene (*CsHA3*), semi-quantitative RT-PCR analysis with specific primers for each gene was performed (Titan one tube RT-PCR system, Roche, Germany). As an internal standard the actin gene was used. All primers were constructed on the basis of the sequences published in GenBank. The following specific primers were designed for the PM-H^+-ATPase gene (cucumber root H^+-ATPase *CsHA3*, accession number EF375892) 5′-AAGTTTGTGGGGTTC-ATGTGGAAT-3′ (forward primer); 5′-GTAAGAGGAAGTGAC-TCTCCAGTC-3′ (reverse primer), and for actin the primers were 5′-CCGTTCTGTCCCTCTACGCTAGTG-3′ (forward primer); 5′-GGAACTGCTCTTTGCAGTCTCGAG-3′ (reverse primer).

cDNA was synthesized using 150 ng of total RNA with TthDNA polymerase. For the cDNA reaction 18 cycles were run (UNO II Thermoblock, Biometra, Germany), which corresponded to the exponential phase for the PM-H^+-ATPase and actin genes. Each

cycle was composed of a 30 s denaturation at 94 °C, 30 s of annealing at 59 °C, a 2 min 40 s extension at 68 °C followed by 7 min of prolonged extension at 68 °C. The PCR products were electrophorised in 1.5% agarose stained with ethidium bromide. The gel images were digitally captured with a Sony XC-ST50CE camera and analysed using the Biocapt version 99 program.

For western blot analysis, 20 μg of plasma membrane proteins were incubated in SDS buffer containing 2% (w/v) SDS, 80 mM DTT, 40% (w/v) glycerol, 5 mM PMSF, 10 mM TRIS, 1 mM EDTA, and 0.05% (w/v) bromophenol blue for 30 min at room temperature and separated on 7.5% SDS-polyacrylamide gels (Laemmli, 1970). After 1 h of electrophoresis at room temperature (25 mA) proteins were electrotransferred at room temperature (60 V, 200 mA) for 1.5 h to nitrocellulose using a Sigma-Aldrich SV10-EB10 blotting apparatus. Transfer buffer contained 25 mM TRIS, 150 mM Gly, and 10% (v/v) methanol.

To identify the plasma membrane H^+-ATPase, the blots were incubated overnight at 8 °C with monoclonal antibody against plasma membrane H^+-ATPase, (46E5B11D) kindly provided by W Michalke (Universität Freiburg, Germany). The antiserum was diluted 2000-fold. After repeated washing the nitrocellulose membrane was incubated at room temperature for 1 h with 1:4000 diluted secondary antibody (anti-mouse, conjugated to horseradish peroxidase, Sigma-Aldrich) and visualized by staining with DAB.

Phosphorylation of plasma membrane H^+-ATPase was detected with the antibody against phosphothreonine (rabbit polyclonal to phosphothreonine, Abcam) used at A concentration of 2 μg ml^{-1} after overnight incubation at 8 °C. The membranes were rinsed and incubated for 1 h at room temperature with 5000-fold secondary antibody conjugated to horseradish peroxidase (goat polyclonal to rabbit IgG, Abcam). The results were visualized by staining with DAB.

Statistics

Data reported in the figures are the results from at least three experiments performed on independent preparation. The results are means ±SD. All assays were run with at least three replicates.

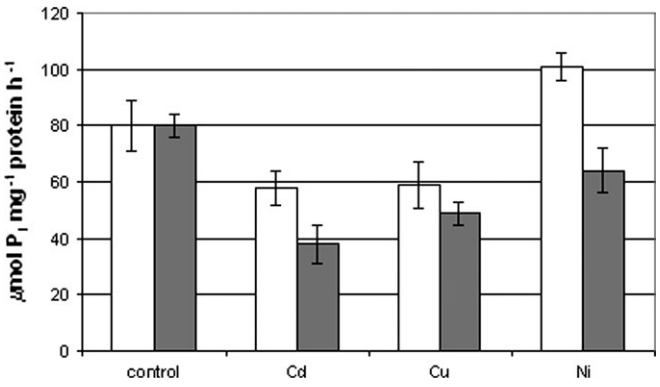

Fig. 1. Effect of Cd, Cu and Ni on the hydrolytic activity of H^+-ATPase in plasma membrane vesicles. The plasma membrane was isolated from the control roots (control), and roots treated for 2 h with 10 μM metals (white bars) or with 100 μM metals (grey bars). Hydrolytic activity of H^+-ATPase was measured as described in the Materials and methods. Results are means ±SD of three independent experiments with each experiment performed in triplicate.

Results

Treatment of the cucumber seedlings with heavy metals (Cd, Cu and Ni) changed the hydrolytic and transporting activities of the plasma membrane H^+-ATPase (Figs 1, 2). The effect of 10 μM Cd and Cu was similar, and both metals decreased hydrolytic activity by about 30% (Fig. 1, white bars), whereas 10 μM Ni increased the hydrolysis of ATP in plasma membrane vesicles by about 30% (Fig. 1, white bars). Higher concentrations of every metal (100 μM) in nutrient solution caused the distinct inhibition of H^+-ATPase activity (Fig. 1, grey bars). The greatest inhibition of ATP hydrolysis was observed in plasma

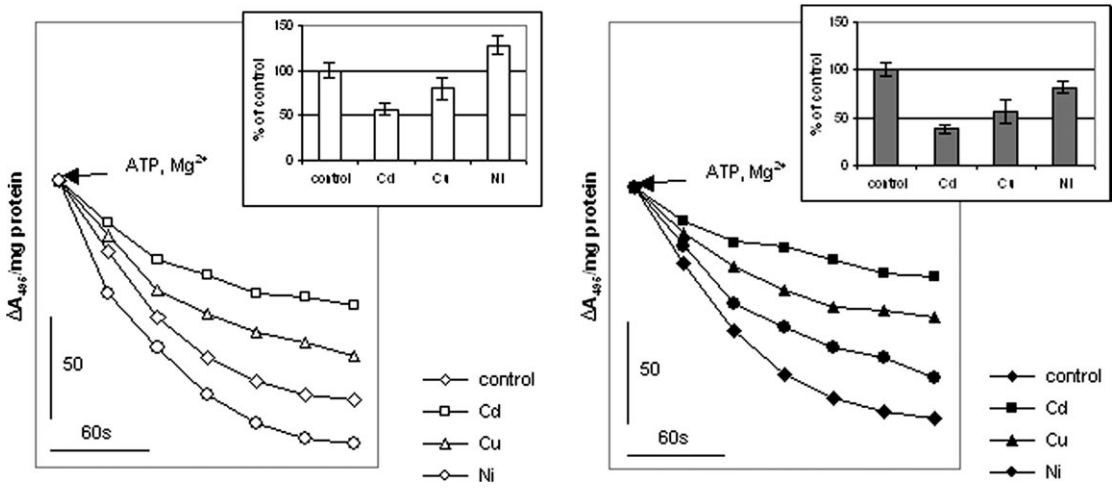

Fig. 2. Effect of Cd, Cu and Ni on the proton transport activities measured in the plasma membrane vesicles. The plasma membranes (50 μg of protein) were isolated from control roots (control), roots treated for 2 h with 10 μM (A, open symbols) or with 100 μM (B, closed symbols) of Cd, Cu or Ni. After equilibration of membranes with the reaction medium (at least for 5 min), vesicle acidification was initiated by the addition of Mg-ATP to give a final concentration of 3 mM. The formation of ΔpH gradient in the vesicles was monitored as the changes in acridine orange absorbance (A_{495}). The values presented are representative for the results obtained in three independent experiments with each experiment done in triplicate. The results in the inner diagrams (the steady-state of H^+-transport) are means ±SD from those three independent experiments.

membranes isolated from cucumber roots treated with Cd (about 60%), while in the case of Cu and Ni it was 45% and 20%, respectively. All metals at both concentrations affected the transporting activity of the plasma membrane proton pump in a similar manner (Fig. 2A, B).

The effect of metals on the plasma membrane proton pump could comprise the expression levels of a gene (mRNA) as well as the enzyme protein level. To verify whether an alteration of enzyme activity under heavy metals was caused by changes in gene expression, the level of specific transcript of *Cucumis sativus* plasma membrane ATPase (GenBank accession number EF375892) was determined using RT-PCR approaches. The transcript level of the constitutively expressed actin gene was used as the internal standard. The results of RT-PCR are presented as the gel image (Fig. 3A, C) and as a ratio of the PM-H^+-ATPase gene signal in the selected line compared with the actin gene signal (Fig. 3B, D). Transcript levels of the *CsHA3* gene in roots treated with 10 μM Cd, Cu and Ni were similar to the control, indicating that the metal effect on plasma membrane H^+-ATPase in our short-term experiments did not involve the gene level (Fig. 3A, B). Also, higher concentrations (100 μM) of Cu and Ni did not affect the expression of the *CsHA3* gene, whereas in roots treated with 100 μM Cd the level of the transcript markedly declined (Fig. 3C, D).

Since the activity of H^+-ATPase can also be rapidly changed through reversible protein phosphorylation, it was tested if cantharidin, the specific inhibitor of protein phospatases (PP2A, PP1), could affect the inhibitory effect of metals on the enzyme activity. When cucumber plants were exposed to 100 μM Cd, Cu and Ni in the presence of cantharidin the metal-dependent inhibition was partially diminished (Fig. 4) suggesting that the effect of metals could involve dephosphorylation of H^+-ATPase protein.

The differences in phosphorylation of plasma membrane H^+-ATPase after metal treatment of plants (2 h with 100 μM Cd, Cu and Ni) were also confirmed with the antibody against phosphothreonine (Fig. 5B). To verify the specificity of the antibody against phosphoamino acids, a control with antibody against plasma membrane H^+-ATPase, 46E5B11D, was used (Fig. 5A). Figure 5B shows that the phosphorylation of plasma membrane ATPase decreased in plants treated with heavy metals compared with the control. This result strongly indicates that, under heavy metal stress, dephosphorylation of plasma membrane H^+-ATPase occurs.

ATP is absolutely required by the plasma membrane H^+-ATPase for proton transport. Therefore the amount of ATP in tissues could be one of the factors limiting an active phospho-enzyme level. For that reason, ATP levels in cucumber roots treated for 2 h with heavy metals were also measured. In general, ATP contents in roots of plants stressed with every metal were lower than in the control (Fig. 6). The lowest ATP was found after treatment of roots with 100 μM Cu.

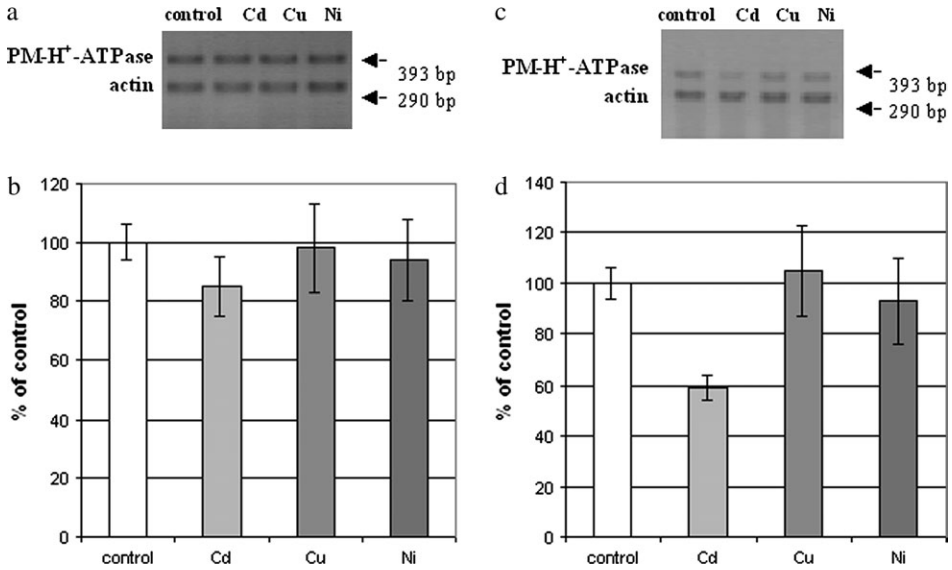

Fig. 3. Expression of the PM-H^+-ATPase gene in cucumber roots treated with 10 μM Cd, Cu or Ni (A, B) and 100 μM Cd, Cu or Ni (C, D). Total RNA was isolated from cucumber roots untreated (control, white bar) or treated for 2 h with 10 or 100 μM heavy metals (grey bars). To evaluate the expression of PM-H^+-ATPase and actin (internal standard) genes, semi-quantitative RT-PCR (Titan one tube RT-PCR system, Roche) with specific primers for each gene was performed. Ethidium bromide-stained bands for the ATPase transcripts were quantified with respect to the band of actin. RT-PCR results are presented as the gel image (A, C) and as a ratio of PM-H^+-ATPase gene signal in the selected line to the actin gene signal (B, D). All experiments were repeated three times independently with comparable results (±SD).

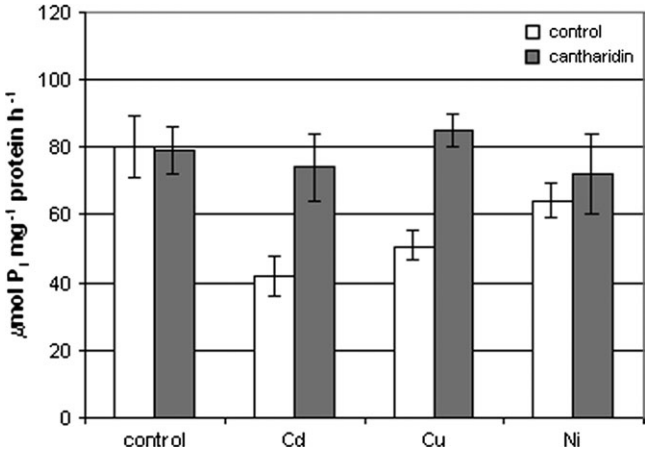

Fig. 4. Effect of metals and cantharidin on the hydrolytic activity of H^+-ATPase in plasma membranes of cucumber roots. The plasma membranes were isolated from the control roots (control), from roots treated for 2 h with 100 µM Cd, Cu and Ni, (white bars) or from roots treated for 2 h with metals and 50 µM cantharidin (grey bars). Hydrolytic activity of H^+-ATPase was measured as described in the Materials and methods. Data are expressed as a percentage of the enzyme activity determined in plasma membranes of control roots. Results are means ±SD of three independent experiments with each experiment done in triplicate.

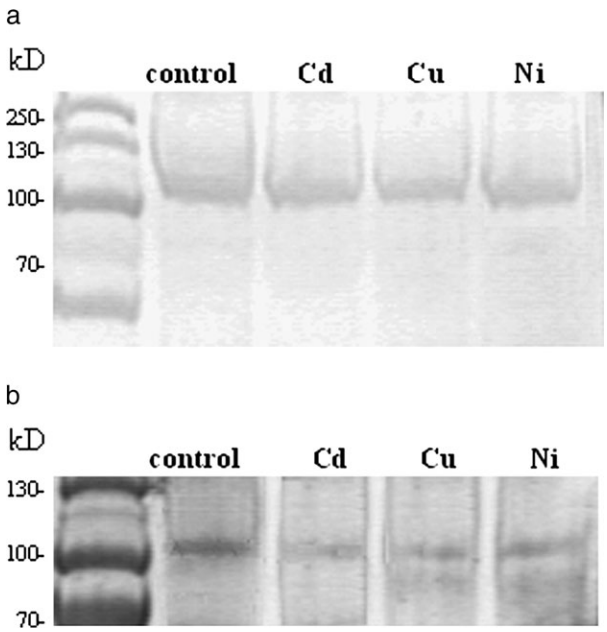

Fig. 5. Western blot of plasma membrane protein (obtained from control plants or plants treated with 100 µM Cd, Cu, Ni) with antibodies raised against plasma membrane H^+-ATPase -46E5B11D (A), and phosphothreonine (B). The location of the molecular mass standard is indicated on the left. The figures are representative for the results obtained in three independent experiments.

Discussion

Plasma membrane functions are rapidly altered by heavy metals present in the environment at high concentrations. The first diagnostic symptom of membrane damage by

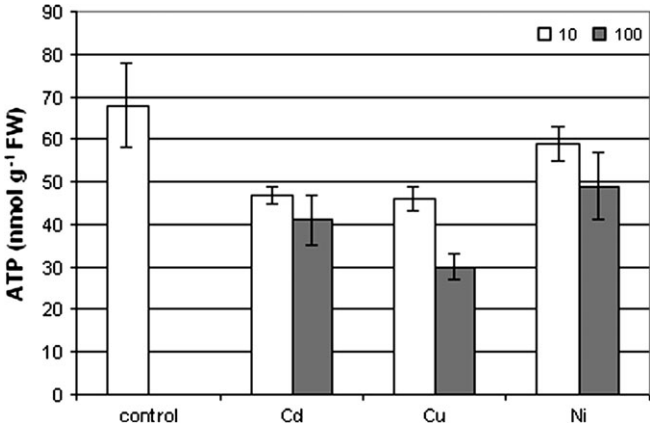

Fig. 6. Levels of ATP in the cucumber roots treated with heavy metals. Plants were exposed for 2 h to the MES-NaOH solution without (control, white bar) or with 10 µM Cd, Cu or Ni (grey bars) and 100 µM Cd, Cu or Ni (black bars). ATP content in roots was measured as described in the Materials and methods. The data represent an average (±SD) of three experiments; each assay was performed three times.

heavy metals is an increase in its permeability (Fodor *et al*., 1995) with a subsequent disturbance in the ionic balance of the cell. It is mainly caused by metal-induced changes in the composition of the membrane lipids and the saturation of fatty acids (de Vos *et al*., 1993). However, the action of metals is much more complex and comprise various events including the oxidation and cross-linking of protein thiols resulting in an inhibition of the key membrane proteins (Harms *et al*., 1994). One of the membrane enzymes which is altered in plants stressed with metals seems to be the H^+-ATPase, the only proton pump operating in plasma membranes and playing a central role in the regulation of ion homeostasis. It was shown that the hydrolytic activity of H^+-ATPase in roots of different plants was inhibited by Cd (Kennedy and Gonsalves, 1989; Fodor *et al*., 1995) as well as Cu (Burzyński and Kolano, 2003). These results generally confirmed those findings. The short-term treatment of cucumber with Cd or Cu reduced the hydrolysis of ATP in the plasma membranes of root cells (Fig. 1). Moreover, the ATP-dependent proton transport in membranes isolated from roots stressed with metals was also inhibited in a similar manner (Fig. 2). The effect of metals on both hydrolytic and transporting activities was dependent on their concentrations in the nutrient solution and correlated well with an accumulation in the symplast of the cells (Table 1). A similar effect was observed in roots treated with higher concentration of Ni (100 µM), whereas lower concentrations of the metal slightly elevated both activities of the plasma membrane proton pump. Comparable observations were made earlier by Burzyński and Buczek (1994) and by Ros *et al*. (1992) for the plasma membrane ATPase in cucumber and rice grown with nickel. Interestingly, the action of nickel on the proton pumps was parallel to its effect on plant growth: low concentration of

Table 1. *Total, symplastic and apoplastic metal levels in root tissues after 2 h of cucumber exposition to Cd, Cu or Ni*

10 seedlings were incubated in 50 cm^3 of 10 or 100 μmol dm^{-3} of metal chloride solutions (pH 5.5). Cd, Cu or Ni levels were determined as described in the Materials and methods. Values are means with ±SD of eight replicates of four independent experiments.

Distribution	Accumulation of metals in roots (nmol 100 mg^{-1} FW)								
	Cd (μM)			Cu (μM)			Ni (μM)		
	0	10	100	0	10	100	0	10	100
Total	0.02±0.01	33.5±2.1	160±11	2.5±0.21	23.9±2.2	181±2	1.2±0.08	34.2±2.8	162.2±8.6
Symplastic fraction	–	9.1±1.8	70.9±9.2	–	10.0±1.4	62.4±4.1	–	13.1±2.9	72.0±5.2
Apoplastic fraction	–	24.4±1.9	89.1±9.1	–	13.9±1.7	118.6±5.2	–	21.1±2.8	90.2±5.4

Ni stimulated the growth of various crop species, while higher concentrations of the metal inhibited the growth of plants (Brown *et al*., 1987; Saito *et al*., 2005). Since one of the main function of plasma membrane H^+-ATPase is the regulation of growth (Rayle and Cleland, 1992), it could be suggested that the enhanced growth of plants subjected to low concentrations of Ni and the lowered growth in the presence of higher concentrations of Ni is, at least partially, a result of the modification of plasma membrane H^+-ATPase activity by nickel. Shen *et al*. (2005) also observed the increase of plasma membrane H^+-ATPase activity when plants were subjected to another metal – aluminium. Results from real-time RT-PCR and immunodetection analysis indicated that the stimulation of the plasma membrane proton pump activity by Al, in soybean roots, is caused by transcriptional and translational regulation.

As mentioned above, the effect of metals on the plasma membrane proton pump was correlated with their concentration in the nutrient solution. However, possible reasons for the differential action of the metals on enzyme activity, in spite of similarities in their uptake, is not clear. The strongest inhibition of H^+-ATPase was obtained after treatment of plants with cadmium. There is no correlation between enzyme protein inhibition and Lewis acidity of metal ions or when the inhibition by metals is compared with the various ligands (hydroxyl, carboxyl, phosphoryl, amino, sulphydryl) present in cells (Filippis, 1979).

Heavy metal toxicity can be due to membrane distortion as shown earlier by the altered lipid content (Burzyński and Kolano, 2003). Similarly Ros *et al*. (1992) and Fodor *et al*. (1995) have indicated that saturation of fatty acid in the membrane of plants growing with metals increased, whereas the level of sterols significantly decreased. Since lipid composition and membrane fluidity are considered as significant factors regulating the plasma membrane H^+-ATPase (Hernandez *et al*., 2002), an inactivation of the proton pump could be the result of the metal-induced changes in the plasma membrane.

Inhibition of the plasma membrane proton pump in root cells under heavy metal stress could also result from alteration at the transcriptional as well as the post-transcriptional level. The plasma membrane H^+-ATPase is encoded by a multigene family and it is generally accepted that the expression of at least some of them is differentially regulated in response to a variety of environmental stresses. Thus it could be assumed that heavy metals also affect the activity of the proton pump through an alteration of specific gene expression. However, using genetic approaches, it has been proved that, among the metals used in these experiments, only cadmium markedly decreased the level of *CsHA3* mRNA. Those observations are in agreement with other findings that show that Cd is a very effective modulator (up- and down-regulation) of plant gene expression (Fusco *et al*., 2005; Kovalchuk *et al*., 2005). Kovalchuk *et al*. (2005) analysed the global genome expression in plants exposed to Cd and have shown that there are at least 65 up-regulated and 338 down-regulated genes. Also in *Brassica juncea* L., known as a hyperaccumulator plant, 73 transcript-derived fragments were identified as Cd-responsive (Fusco *et al*., 2005). Among them 52 genes have been identified as encoding proteins with varied physiological functions (transcriptional factors, expression regulators, stress-responsive proteins, and proteins involved in general metabolism). Such results indicate that the response of plants to Cd stress at the genetic level is very broad. The other metals used in our experiments (Cu and Ni) did not change the level of plasma membrane H^+-ATPase transcripts in cucumber roots (Fig. 3). Thus the alteration of the plasma membrane proton pump activity in cucumber roots stressed with those metals seem not to involve the gene expression level. However, H^+-ATPase is encoded by a gene family and only one isoform was analysed at the transcript level. Therefore it cannot be excluded that other isoforms could be differentially regulated.

Evidence has been presented that the plasma membrane H^+-ATPase activity is rapidly modulated through the phosphorylation/dephosphorylation mechanism (Schaller and Sussman, 1988; Kłobus and Janicka-Russak, 2004, Janicka-Russak and Kłobus, 2006). The activation of the enzyme is due to its phosphorylation followed by binding of 14-3-3 protein, whereas the dephosphorylation of H^+-

ATPase by specific phosphatases belonging to the PP2, and the release of the 14-3-3 protein from the complex, inactivates the enzyme. To determine if the metal-induced inhibition of the plasma membrane proton pump in cucumber roots was attributed to its post-translational modification, the effect on the membrane proton pump of cantharidin, a specific inhibitor of protein phosphatases, was studied. As shown, cantharidin applied together with 100 μM Cd, Cu and Ni distinctly diminished the inhibitory effect of the metals (Fig. 4), suggesting that the metals altered the proton pump activity mainly via dephosphorylation of its protein. Results of experiments with specific phosphothreonine antibodies confirmed this suggestion. The level of H^+-ATPase phosporylation was distinctly lower in plasma membranes from cucumber roots treated with Cd, Cu or Ni (Fig. 5) demonstrating that alteration of the plasma membrane proton pump under heavy metals is due to dephosphorylation of the enzyme protein. Considering that our plants (control plants and those treated with heavy metals) were exposed for 2 h to MES-NaOH without Ca^{2+} in solution, a set of supplementary experiments with plants exposed to metals in MES-NaOH with or without 200 μM Ca ($CaSO_4$) was performed. Nevertheless no differences were found in both the hydrolytic and the transporting activity of the H^+-ATPase. Also western blot analysis of plasma membranes with the antibody against phosphothreonine showed that the presence of calcium in the solution did not affect the metal action on H^+-ATPase phosphorylation in the short-term experiments presented in this paper (data not shown).

As protein phosphorylation depends on the energetic status of the cell, the level of endogenous ATP was measured. Each metal treatment decreased the amount of the adenosine triphosphate in root tissues, suggesting that, besides the dephosphorylation intensity of the proton pump, the ATP level is also an important factor in enzyme deactivation under heavy metals stresses. It is known that the main source of ATP in root tissues is mitochondrial respiration. Until now, the effect of heavy metals on respiration and ATP content in plant cells has not been sufficiently studied. However, it was revealed that Cd treatment of plants significantly reduced the oxygen consumption by roots (Reese and Roberts, 1985) as well as disordering the electron transport chain (Kessler and Brand, 1994). Thus it is suspected that altered respiration could enhance the inhibition of plasma membrane proton pump activity in plants treated with metals. There is the possible action of heavy metals on ATPase activity through decreasing the energy charge of the tissue. Such a correlation is indicated since the endogenous amount of ATP, the substrate for plasma membrane H^+-ATPase, decreased under heavy metal stress. In addition, it is well known that endogenous ATP influences ATPase activity, not only as the substrate but also through its function as an activator. Since protein phosphorylation depends on the endogenous ATP level, its reduction upon the addition of heavy metals is in line with our hypothesis. Data of experiments with cantharidin and western blot with antibodies against phosphothreonine support this supposition. We think that some changes in plant cells initiated under heavy metals lead to an increase in the activity of specific phosphatases, responsible for the dephosphorylation of H^+-ATPase protein.

Taken together, the data presented suggest that alterations of the plasma membrane proton pump under heavy metal stresses are mainly due to the post-translational modification of its protein. Only in the case of cadmium action, can the gene expression level also be involved in this short-term experiment.

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