0026-895X/02/6902-629–639
MOLECULAR PHARMACOLOGY
U.S. Government work not protected by U.S. copyright
Mol Pharmacol 69:629–639, 2002

Vol. 69, No. 2
14241/3080836
Printed in U.S.A.

Acquired Cadmium Resistance in Metallothionein-I/II(−/−) Knockout Cells: Role of the T-Type Calcium Channel Cacnα_{1G} in Cadmium Uptake

Elaine M. Leslie,[1] Jie Liu, Curtis D. Klaassen, and Michael P. Waalkes

Inorganic Carcinogenesis Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute and National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina (E.M.L., J.L., M.P.W.); and Department of Pharmacology, Toxicology, and Therapeutics, University of Kansas Medical Center, Kansas City, Kansas (C.D.K.)

Received May 22, 2005; accepted November 10, 2005

ABSTRACT

Metallothioneins (MTs) are cytoplasmic proteins that sequester certain divalent cations and are considered a primary cellular defense against the toxic transition metal cadmium (Cd^{2+}). MT-I/II(−/−) knockout [MT(−/−)] cells are available and serve as an excellent tool to study non–MT-related mechanisms in metal tolerance. In the current study, Cd^{2+}-resistant MT(−/−) (CdR) and CdR revertant (CdR-rev) cell lines were developed and characterized to investigate non–MT-mediated cellular protection mechanisms. Resistance to Cd^{2+} was approximately 70-fold higher in CdR than the parental MT(−/−) cell line (IC_{50} = 20 versus 0.3 μM, respectively) and was stable in the absence of Cd^{2+} for 35 days. Accumulation of Cd^{2+} by the CdR cell line was reduced by approximately 95% compared with parental cells, primarily because of a decreased Cd^{2+} uptake. Cd^{2+} uptake by the MT(−/−) parental cell line was independent of sodium, energy, and electrogenic potential. Uptake was saturable (K_m = 65 nM; V_{max} = 4.9 pmol/mg/min) and pH-dependent (maximal at pH 6.5–7). Potent inhibitors of Cd^{2+} uptake included Zn^{2+} (IC_{50} = 7 μM), Mn^{2+} (IC_{50} = 0.4 μM), and the T-type Ca^{2+} channel antagonist mibefradil (IC_{50} = 5 μM), whereas other metals (including Fe^{2+}) and L-type Ca^{2+} channel antagonists had little effect. Immunoblot and real-time reverse transcription-polymerase chain reaction analysis indicated that the Cacnα_{1G} T-type Ca^{2+} channel was expressed at a reduced level in CdR compared with the parental MT(−/−) cell line, suggesting it is important for Cd^{2+} uptake. The CdR1-rev cell line was found to have a Cd^{2+} uptake and sensitivity level in between that of the CdR1 and MT(−/−) cell lines. Consistent with this was an intermediate expression of Cacnα_{1G} in the CdR-rev cell line. These data suggest that decreased expression of Cacnα_{1G} protects cells from Cd^{2+} exposure by limiting Cd^{2+} uptake.

The heavy metal cadmium (Cd^{2+}) is naturally present in soil, sediment, air, and water and has been concentrated in the human environment through its industrial use. Cd^{2+} exposure leads to a variety of adverse health effects, including osteoporosis, nonhypertrophic emphysema, irreversible renal tubular injury, and anemia (Waisberg et al., 2003). In addition, Cd^{2+} is a human carcinogen, and occupational exposure has been associated with cancers of the lung and possibly prostate, pancreas, and kidney (Waalkes, 2003). The main nonoccupational human exposure to Cd^{2+} is from cigarette smoke, whereas for nonsmokers the diet is the predominant source (Satarug and Moore, 2004).

The metallothioneins (MTs) are a family of low-molecular-weight (6–7 kDa), thiol-rich, metal binding proteins (Klaassen et al., 1999). All vertebrates express two or more distinct MT isoforms designated MT I through MT IV (Palmiter, 1998). In mammals, MT I and MT II are ubiquitously expressed in all tissues and are considered the major forms (Palmiter, 1998). Cell lines and transgenic mice deficient in MT are sensitive to Cd^{2+}, whereas mice and cells overexpressing MT I and MT II are resistant (Klaassen et al., 1999). In addition, selection of cultured mammalian cells in Cd^{2+} typically results in resistance based on multiple amplifications of the entire MT locus (Palmiter, 1998). MT can bind large amounts of Cd^{2+} and is considered the predominant

This research was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research. E.M.L. is the recipient of a Canadian Institutes of Health Research Postdoctoral Fellowship and a Davies Charitable Foundation Research Fellowship Award.

[1] Current affiliation: School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.014241.

ABBREVIATIONS: MT, metallothionein; Dmt, divalent metal transporter; CdR, Cd^{2+}-resistant; Cdr-rev, Cd^{2+}-resistant revertant; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; Ct, cycle time; Zirtl, zinc-iron regulated transporter-like.

cellular defense mechanism against Cd^{2+} toxicity (Klaassen et al., 1999).

The level of MT gene expression is impacted by a variety of circumstances and differs widely with factors such as tissue type, cell type, gender, and pathological state (Waalkes and Pérez-Ollé, 2000). In addition, remarkable interindividual variation in MT expression level in human tissues such as liver, kidney, and red blood cells has been reported previously (Onosaka et al., 1985; Bem et al., 1988; Silevis-Smitt et al., 1992; Yoshida et al., 1998; Allan et al., 2000; Wu et al., 2000). Thus, it is important to understand potential non–MT-mediated cellular protection pathways, because these could be important alternative or auxiliary mechanisms for persons who have relatively poor MT expression. Altered cellular transport characteristics could reduce accumulation of Cd^{2+}, through either increased efflux or decreased uptake, and could be important for tissue protection. Such mechanisms have not been extensively characterized at the molecular level. A large body of literature exists describing the importance of glutathione-dependent biliary excretion of metals, including Cd^{2+}, in vivo (for review, see Leslie et al., 2005). Studies in rats deficient in Mrp2 (Abcc2), an ATP-binding cassette transporter protein involved in the cellular efflux of many xenobiotics, implicate Mrp2 as the major canalicular membrane transporter involved in biliary excretion of Cd^{2+} (Dijkstra et al., 1996; Paulusma and Oude Elferink, 1997). A candidate transporter for cellular uptake of Cd^{2+} is the divalent metal transporter (Dmt1/Slc11a2), also known as the natural resistance-associated macrophage protein 2 or divalent cation transporter 1, a proton-coupled metal-ion transporter (Gunshin et al., 1997). Several in vitro studies have firmly established human DMT1/SLC11A2 as a transporter of Cd^{2+} (Bressler et al., 2004). In addition, a variety of studies have suggested that Cd^{2+} transport into cells occurs through transporters and ion channels involved in the passage of physiological metals such as Zn^{2+}, Mn^{2+}, and Ca^{2+} (Waalkes and Poirier, 1985; Friedman and Gesek, 1994; Souza et al., 1997; Yanagiya et al., 2000; Zalups and Ahmad, 2003).

Except for the recently identified solute carrier protein ZIP8/Slc39a8 (Dalton et al., 2005), transporters and channels that Cd^{2+} could gain cellular entry through "molecular mimicry" have not been specifically identified at the molecular level. For example, high concentrations of Ca^{2+} channel antagonists have been shown to inhibit cellular uptake of Cd^{2+} (Friedman and Gesek, 1994; Souza et al., 1997). However, concentrations of antagonists used in these studies could inhibit several classes of Ca^{2+} and/or other channel and transport proteins. By using an MT-I/II(−/−) knockout [MT(−/−)] cell line made resistant to Cd^{2+} by continuous exposure in a stepwise manner (Yanagiya et al., 1999), potential non–MT-mediated cellular protection mechanisms from Cd^{2+} have been studied (Yanagiya et al., 1999, 2000; Himeno, 2002; Himeno et al., 2002). The Cd^{2+}-resistant MT(−/−) cell line had a reduced ability to take up manganese (Mn^{2+}), and it was concluded that the MT(−/−) cell line expresses a novel Mn^{2+} transport system by which Cd^{2+} can enter mammalian cells (Yanagiya et al., 2000). However, such a transporter is yet to be identified.

In the present work, a Cd^{2+}-resistant MT(−/−) murine fibroblast cell line (CdR) and a revertant cell line (CdR-rev) have been established and Cd^{2+} transport extensively characterized. Resistance was predominantly because of a reduced Cd^{2+} uptake, probably through a specific decrease in expression of the T-type Ca^{2+} channel Cacnaα_{1G}, suggesting that Cacnaα_{1G} could be an important pathway for Cd^{2+} entry into cells.

Materials and Methods

Materials. [^{109}Cd]$CdCl_2$ (3 mCi/mg) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Verapamil, mibefradil, diltiazem, nicardipine, rotenone, sodium azide, choline, $MnCl_2$ (Mn^{2+}), $ZnCl_2$ (Zn^{2+}), $NiCl_2$ (Ni^{2+}), $Pb(CH_3COO)_2$ (Pb^{2+}), $FeSO_4$ (Fe^{2+}), $CuCl_2$ (Cu^{2+}), $AlCl_3$ (Al^{3+}), $HgCl_2$ (Hg^{2+}), $NaAsO_2$ (As^{3+}), and $CdCl_2$ (Cd^{2+}) were purchased from Sigma-Aldrich (St. Louis, MO).

Establishment of Cd^{2+}-Resistant and -Revertant Cell Lines. Previously established simian virus 40-transformed MT(−/−) and MT(+/+) fibroblast cells that originated from whole embryonic cells (Kondo et al., 1999) were cultured in high-glucose DMEM with 10% fetal bovine serum under 5% CO_2 at 37°C. CdR cells were developed by continuous exposure to increasing concentrations of Cd^{2+} up to a final concentration of 10 μM, according to the method of Yanagiya et al. (1999). Cells were cultured for several weeks in the presence of 10 μM Cd^{2+} and then cloned by limiting dilution. Cloned cells (CdR1 and CdR2) were grown in the absence of Cd^{2+} (4–10 days) before use in experiments. Revertant cell lines (CdR1-rev and CdR2-rev) were established by growing CdR1 and CdR2 in the absence of Cd^{2+} for up to 238 days.

Cytotoxicity Testing. MT(−/−), MT(+/+), CdR1, CdR2, CdR1-rev, and CdR2-rev cell lines were plated at 1×10^4 cells/well in a 96-well plate, and 24 h later they were treated with Cd^{2+} (0.1–300 μM) for 72 h. The tetrazolium-based microtiter plate assay CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI), was then used to measure acute cytotoxicity, according to the manufacturer's instructions. Results are expressed as a percentage of untreated control.

Cd^{2+} Total Accumulation, Efflux, and Uptake by MT(−/−), CdR, and CdR1-rev Cells. Cells were plated at 2×10^5 cells/well in six-well plates and cultured 24 h before treatment with carrier-free ^{109}Cd. For total accumulation studies, cells were incubated with ^{109}Cd (0.1, 0.3, and 1 μM; 40–400 nCi) for 24 h. Cells were then harvested by washing three times with 2 ml of ice-cold PBS containing 0.05% (w/w) EDTA and lysed with PBS containing 2% SDS. Protein content was measured using the DC Protein Assay kit (Bio-Rad, Hercules, CA), and radioactivity was normalized per milligram of protein. For measurement of cellular Cd^{2+} efflux, cells were incubated for 6 h with ^{109}Cd (0.1 μM; 40 nCi) in serum-free media, cells were then washed three times with PBS containing 0.05% EDTA (37°C), incubated at 37°C in serum-free media for the indicated time points, and harvested as described above for total accumulation. For uptake studies, cells were incubated with ^{109}Cd (0.1 μM) in serum-free media for 1 h or at the indicated time points and harvested as described above for total accumulation.

Sodium dependence of uptake was measured using transport buffer (137 mM NaCl replaced with equimolar choline chloride or LiCl, 5.33 mM KCl, 1.8 mM $CaCl_2$, 0.814 mM $MgCl_2$, 10 mM HEPES, and 25 mM glucose). The effect of the metabolic inhibitors sodium azide (10 mM) and rotenone (10, 30, and 100 μM) on ^{109}Cd uptake was assessed in glucose-free DMEM with the addition of mannitol (25 mM) to maintain osmolarity. Cells were preincubated for 1 h with these inhibitors before the addition of ^{109}Cd and measurement of uptake. The effects of other potential modulators of ^{109}Cd uptake [Zn^{2+} (0–30 μM), Mn^{2+} (0–30 μM), Ni^{2+} (0–300 μM), Fe^{2+} (0–100 μM), Pb^{2+} (0–100 μM), pH change (pH 5, 6, 6.5, 7, 7.5, or 8), and the Ca^{2+} channel antagonists mibefradil (0–30 μM), verapamil (0–100 μM), diltiazem (0–300 μM), and nicardipine (0–100 μM)] were evaluated, without preincubation of modulators, in serum-free DMEM.

Kinetic parameters of uptake were determined by measuring the initial rate of ^{109}Cd uptake at eight different substrate concentrations (3–300 nM) at a single time point of 1 h. Kinetic parameters were determined using nonlinear regression analysis (Prism; GraphPad Software Inc., San Diego, CA).

Real-Time RT-PCR Analysis. Gene expression was quantified using real-time RT-PCR analysis as described previously (Walker, 2001). In brief, total RNA was isolated from cell lines using an RNeasy midi kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions, reverse transcribed with murine leukemia virus reverse transcriptase and oligo(dT) primers. The forward and reverse primers for selected genes were designed using Primer Express software (Applied Biosystems, Foster City, CA) and are shown in Table 1. The SYBR Green DNA polymerase chain reaction kit (Applied Biosystems) and the MyIQ single-color real-time polymerase chain reaction detection instrument (Bio-Rad) were used for real-time RT-PCR analysis. The relative differences in expression between groups were expressed using cycle time (Ct) values, and the Ct values for genes of interest were first normalized with that of β-actin in the same sample, and then differences between groups were expressed relative to controls set as 100%. Assuming that the Ct value is reflective of the starting copy number and there is 100% efficiency, a difference of one cycle is equivalent to a 2-fold difference in starting copy number using the $2^{(-dCt)}$ formula. Real-time RT-PCR was performed in triplicate, and similar results were obtained from standard curves produced for each gene analyzed.

Immunoblot Analysis. Cell homogenates were prepared, and relative levels of Cacnα_{1G} were determined by immunoblot analysis essentially as described previously (Yunker et al., 2003). In brief, cells were washed twice with ice-cold PBS and then collected with a plastic cell scraper in 1 ml of HEPES (50 mM; pH 7.4) buffer containing EGTA (1 mM) and protease inhibitors (Complete cocktail tablets; Roche Diagnostics, Indianapolis, IN). Cell homogenates (50 μg) and a positive control homogenate prepared from murine cerebellum (75 μg) were subjected to SDS-polyacrylamide gel electrophoresis and electrotransferred to a nylon membrane. Blots were blocked in 5% (w/v) skim milk powder for 1 h followed by incubation with the CW53 Cav3.1/Cacnα_{1G} antibody in 3% (w/v) bovine serum albumin overnight at 4°C. After washing, blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit antibody (GE Healthcare, Little Chalfont, Buckinghamshire, UK) followed by application of chemiluminescence blotting substrate (SuperSignal Dura Extended Duration; Pierce Chemical, Rockford, IL).

Results

Establishment of a Cd^{2+}-Resistant Cell Line. A CdR variant of the murine fibroblast MT(−/−) cell line was established by culturing these cells in gradually increasing concentrations of Cd^{2+} up to 10 μM. After establishment and continuous culture in 10 μM Cd^{2+} for 10 weeks, two clones (CdR1 and CdR2) were produced that had similar resistance levels to Cd^{2+} (IC_{50} of ~20 μM), approximately 70- and 7-fold more resistant than the sensitive MT(−/−) parental cell line (IC_{50} = 0.3 μM) and the MT(+/+) cell line (IC_{50} = 3 μM), respectively (Fig. 1). To evaluate the stability of the CdR phenotype, the CdR1 cell line was also grown in the absence of Cd^{2+}, and the resistance to Cd^{2+} was evaluated over time (Table 2). Resistance was stable for a minimum of 35 days without Cd^{2+} exposure. At 63 days of culture in the absence of Cd^{2+}, resistance had decreased 40% with an IC_{50} of 9 μM versus 15 μM for cells continuously cultured in the presence of Cd^{2+}. After 238 days of culture in the absence of Cd^{2+}, resistance had decreased by 96% (IC_{50} = 0.8 versus 18 μM for absence and presence of Cd^{2+}, respectively), and this was considered the revertant cell line.

Resistance Is Mediated Primarily through Reduced Cd^{2+} Uptake. To discern the mechanism of resistance to Cd^{2+}, the total accumulation of ^{109}Cd over 24 h was measured in MT(−/−) parental, CdR1, and CdR2 cell lines (Fig. 2A). CdR1 and CdR2 cell lines accumulated approximately 95% less Cd^{2+} than the MT(−/−) parental cell line over Cd^{2+} concentrations of 0.1, 0.3, and 1 μM. Further experiments revealed that uptake of Cd^{2+} (0.1 μM; 40 nCi) by CdR1 and CdR2 cell lines was reduced by approximately 58, 70, 85, and 92% compared with the MT(−/−) parental cell line at 15, 30, 60, and 120 min, respectively (Fig. 2B). Over 30 min, efflux of Cd^{2+} from the MT(−/−) parental cell line was not detected; however, approximately 40% of Cd^{2+} was effluxed from CdR1 and CdR2 cell lines at 1 min at which point a plateau was reached (Fig. 2C). Although increased efflux does occur in the CdR clones compared with the sensitive parental MT(−/−) cell line, initial Cd^{2+} accumulation was only approximately 5% in the CdR1 and CdR2 cells compared with the parental cell line because of decreased uptake (Fig. 2C). Despite the lack of MT, once Cd^{2+} had entered the MT(−/−) parental cell

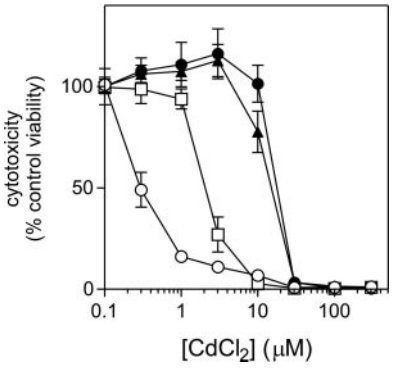

Fig. 1. Cytotoxic effect of Cd^{2+} on MT(+/+), MT(−/−) parental, CdR1, and CdR2 cell lines. The cell lines MT+/+ (□), MT−/− parental (○), CdR1 (●), and CdR2 (▲) were plated at 1 × 10^4 cells/well in a 96-well plate, and 24 h later they were treated with Cd^{2+} for 72 h. A tetrazolium-based microtiter plate assay was used to measure acute cytotoxicity as described under *Materials and Methods*. Symbols represent means of quadruplicate determinations (±S.D.) in a single experiment, and similar results were obtained in three additional experiments.

TABLE 1
Sequence of primers used in real-time RT-PCR analysis

Accession No.	Gene	Forward (5′-3′)	Reverse (5′-3′)
BC005474	*Zirtl/Slc39a1*	GGGACATTAGAAGGAGGCAGACT	GCTGGATGCCTAACTCTCAGTTC
NM_006979	*Ae4/Slc39a7*	CGTGTGTCTGGGTACCTGAATCT	ACGAAAGGAAGCACCAATGG
NM_008732	*Dmt1/Slc11a2*	CTTGGGTTGGCAGTGTTTGA	AGTATTGCCACCGCTGGTATCT
NM_026228	*Zip8/Slc39a8*	CTAACGGACACATCCACTTCGA	CCCTTCAGACAGGTACATGAGCTT
NM_007472	*Aquaporin1*	GTGGCAAGGAAGGGATAGCTT	TGCTGTCAAGACTGTCCCTTCTAG
NM_007581	*Cacnβ_3*	CTCCATGGCTCCTGCAAGAT	GTTCACTGTGTCCTGTTCCTCTCA
NM_009783	*Cacnα_{1G}*	CTGCCTCTGAACACCAAGACTGTA	GCTCAGGTTGCCCTCATCA
M12481	*β-Actin*	GTATGACTCCACTCACGGCAAA	GGTCTCGCTCCTGGAAGATG

line it was accumulated (Fig. 2, A–C). This could be because of the lack of an efflux mechanism for Cd^{2+} in this cell line and/or a non-MT mechanism for intracellular sequestration. Overall, these data suggest that a decreased uptake was the primary mechanism by which CdR1 and CdR2 acquired resistance to Cd^{2+}.

Cd^{2+} Uptake Is Independent of Sodium, Energy, and Electrogenic Potential. Because a large part of the CdR phenotype seemed to be conferred by a decreased uptake of Cd^{2+} into CdR1 and CdR2 cell lines, Cd^{2+} influx was characterized using the MT(−/−) parental cell line. When NaCl was substituted with choline or LiCl in transport buffer, cellular Cd^{2+} uptake was increased or unchanged, respectively, indicating that this is not a Na^+-dependent process (Fig. 3A). When cells were depolarized with 10-fold higher external K^+(54 mM) than control (5.4 mM) concentrations, little difference in Cd^{2+} uptake was observed, indicating that this process is not dependent on the electrogenic potential of the cell (Fig. 3B). An energy requirement for uptake was evaluated using the metabolic inhibitors sodium azide (10 mM) and rotenone (10, 30, and 100 μM) (Fig. 3C). These inhibitors had no significant effect on Cd^{2+} uptake.

Kinetic Analysis of Cd^{2+} Uptake. Cd^{2+} uptake was further characterized by determining initial rates of uptake with several concentrations of Cd^{2+} (Fig. 4A). Uptake was saturable and according to nonlinear regression analysis, the apparent K_m for Cd^{2+} was 65 ± 0.32 nM, and the V_{max} was 4.9 ± 0.1 pmol mg^{-1} min^{-1}. These kinetic parameters are consistent with values reported previously (Yanagiya et al., 2000).

Cd^{2+} Uptake Is pH-Dependent. To determine whether uptake of Cd^{2+} by the MT(−/−) parental cell line was influenced by pH, transport was measured over a pH range of 5 to 8. Uptake of Cd^{2+} was minimal at pH 5 to 6 (0.3 pmol mg^{-1} min^{-1}), maximal between pH 6.5 and 7 (6.5 pmol mg^{-1} min^{-1}), and decreased to 1.6 pmol mg^{-1} min^{-1} between pH 7.5 and 8 (Fig. 4B). Uptake of Cd^{2+} by the CdR1 cell line was minimal over the pH range examined (Fig. 4B).

Cd^{2+} Uptake Is Modified by Several Metals. The ability of several transition metals and metalloids to alter the uptake of Cd^{2+} was evaluated. As a preliminary experiment, the effect of a single concentration (10 μM) of Pb^{2+}, Cu^{2+}, Fe^{2+}, Al^{3+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Hg^{2+}, and As^{3+} on Cd^{2+} (0.1 μM; 40 nCi) uptake was quantified (Fig. 5A). At this concentration, Fe^{2+}, Al^{3+}, Ni^{2+}, Hg^{2+}, and As^{3+} had no effect on Cd^{2+} uptake. Several compounds were selected for further characterization through generation of concentration-response curves and IC_{50} value determination (Fig. 5, B–F).

TABLE 2
Stability of CdR1 resistance to $CdCl_2$

Cytotoxicity of $CdCl_2$ was measured as described under *Materials and Methods*. The CdR1 cell line was grown continuously in the presence of $CdCl_2$ (10 μM) until 4 to 10 days before use in experiments, and the CdR1-rev was grown in the absence of $CdCl_2$ for the times indicated. Similar results were obtained with the CdR2 and CdR2-rev cell lines. IC_{50} values (micromolar) are followed in parentheses by -fold resistance.

Time	MT(−/−) Parental	CdR1	CdR1-rev
11 days	0.7	20 (29)	20 (29)
28 days	0.7	20 (29)	20 (29)
35 days	0.2	18 (90)	18 (90)
63 days	0.3	15 (50)	9 (30)
84 days	0.3	20 (67)	7 (23)
238 days	0.2	18 (90)	0.8 (4)

Consistent with the results of Yanagiya et al. (2000), Zn^{2+} and Mn^{2+} were found to be potent inhibitors of Cd^{2+} uptake with IC_{50} values of 7 and 0.4 μM, respectively (Fig. 5, B and C). Although Fe^{2+} had no effect on Cd^{2+} uptake at 10 μM, it was tested at increasing concentrations because the Fe^{2+} uptake transporter Dmt1/Slc11a2 is known to be important

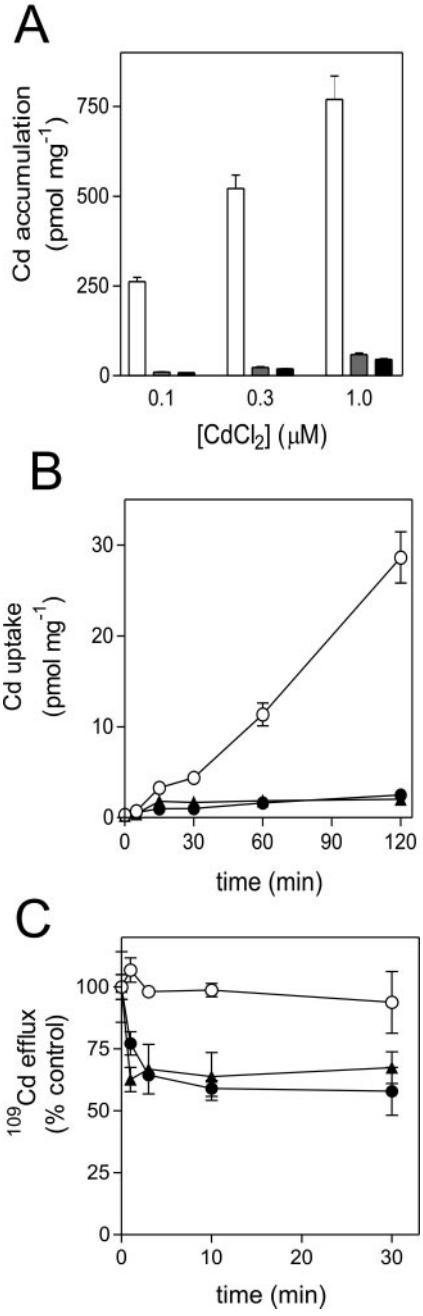

Fig. 2. Cd^{2+} total accumulation, uptake, and efflux by MT(−/−) parental and CdR cell lines. MT(−/−) parental (white columns or ○), CdR1 (gray columns or ●), and CdR2 (black columns or ▲) cell lines were plated at 2×10^5 cells/well in six-well plates and cultured for 24 h before experiments. A, total accumulation of Cd^{2+} in cells was measured after incubating cells with ^{109}Cd (1 μM, 400 nCi; 0.3 μM, 120 nCi; and 0.1 μM 40 nCi) for 24 h. (B) Cellular uptake of Cd^{2+} was measured at the indicated time points by incubating cells with ^{109}Cd (0.1 μM; 40 nCi). C, cellular Cd^{2+} efflux was measured after preincubation of cells for 6 h with ^{109}Cd (0.1 μM; 40 nCi). Columns and symbols represent means of triplicate determinations (±S.D.) in a single experiment, and similar results were obtained in two additional experiments.

for Cd^{2+} uptake in other cell systems (Bressler et al., 2004). However, Fe^{2+} did not inhibit the uptake of Cd^{2+} at the concentrations tested (up to 100 μM) (Fig. 5D). Pb^{2+} had an unexpected stimulatory effect on Cd^{2+} uptake with an activity 155% of control at 100 μM (Fig. 5E). Although Ni^{2+} had little effect at 10 μM (Fig. 5A), it was further characterized at increasing concentrations because of previous reports that Ni^{2+} can inhibit certain Ca^{2+} channels, which are possibly involved in cellular translocation of Cd^{2+} (Lacinová et al., 2000). Ni^{2+} was not a potent inhibitor of Cd^{2+} uptake with an IC_{50} value of 300 μM (Fig. 5F).

Cd^{2+} Uptake Is Altered by Several Ca^{2+} Channel Antagonists. A series of Ca^{2+} channel antagonists were tested for their ability to inhibit Cd^{2+} uptake by the parental MT(−/−) cell line. Mibefradil, which has been shown to be a relatively specific inhibitor of T-type Ca^{2+} channels, proved to be a potent inhibitor of Cd^{2+} uptake by the parental MT(−/−) cell line with an IC_{50} value of 5 μM (Fig. 6A). The phenylalkylamine verapamil, an L-type Ca^{2+} channel antagonist, inhibited Cd^{2+} uptake with an IC_{50} value of 60 μM (Fig. 6B). Diltiazem and nicardipine are drugs from different chemical classes of L-type Ca^{2+} channel antagonists, the benzothiazepines and dihydropyridines, respectively. Neither diltiazem ($IC_{50} > 300$ μM) nor nicardipine ($IC_{50} > 100$ μM) was a potent inhibitor of Cd^{2+} uptake (Fig. 6, C and D). The overall pattern of inhibition by these Ca^{2+} channel antagonists indicates that a T-type Ca^{2+} channel is involved in Cd^{2+} uptake.

CdR1-rev Shows Intermediary Cd^{2+} Uptake and Sensitivity. As a tool to identify changes of functional relevance for the Cd^{2+} resistance in CdR cells, a CdR1-rev cell line was developed by growing the CdR1 cell line in the absence of Cd^{2+} for 238 days. The CdR1-rev was compared with the MT(−/−) parental and CdR1 cell lines for the following: Cd^{2+} cytotoxicity (Fig. 7A; Table 2); Cd^{2+} efflux (Fig. 7B); Cd^{2+} uptake (Fig. 7C); and the impact of Mn^{2+}, Zn^{2+}, and the Ca^{2+} channel antagonist mibefradil on Cd^{2+} uptake (Fig. 7D). The CdR1-rev cell line was of intermediate sensitivity to Cd^{2+} cytotoxicity (IC_{50} = 0.8 μM) compared with the CdR1 (IC_{50} = 18 μM) and MT(−/−) parental (IC_{50} = 0.2 μM) cell lines (Fig. 7A). Cd^{2+} efflux from the CdR1-rev cell line was not significantly different from that of the MT(−/−) parental cell line, suggesting that efflux is not the predominant protective mechanism (Fig. 7B). Consistent with the sensitivity to Cd^{2+}, uptake by the CdR1-rev cell line was intermediate between the CdR1 and MT(−/−) parental cell lines. Uptake in CdR1-rev cells was approximately 50% higher than that of CdR1 and 50% lower than that of MT(−/−) parental cell lines (Fig. 7C). Mn^{2+}, Zn^{2+}, and mibefradil inhibited Cd^{2+} uptake by the CdR1-rev cell line by 44, 65, and 38%, respectively, relative to vehicle control (Fig. 7D). Cd^{2+} uptake by the MT(−/−)-sensitive parental cell line was inhibited to a similar extent (relative to vehicle control) by Mn^{2+}, Zn^{2+}, and mibefradil (66, 74, and 47%, respectively). As expected, the uptake of Cd^{2+} by the CdR1 cell line was not

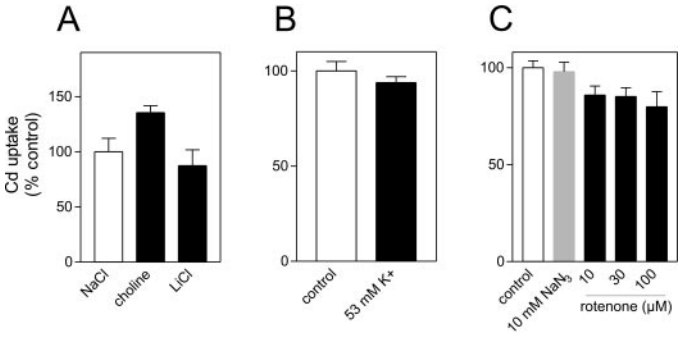

Fig. 3. Effect of sodium substitution, potassium excess, and ATP depletion on Cd^{2+} uptake. The MT(−/−) parental cell line was plated at 2 × 10^5 cells/well in a six-well plate and cultured for 24 h before experiments. Cells were incubated with ^{109}Cd (0.1 μM; 40 nCi) for 1 h, washed as described under *Materials and Methods*, and Cd^{2+} content was quantified. A, sodium dependence of uptake was measured using transport buffer with equimolar choline chloride or LiCl in place of NaCl. B, potassium concentration of the DMEM was adjusted from 5.4 mM (control) to 54 mM during Cd^{2+} uptake. C, effect of the metabolic inhibitors sodium azide (10 mM) and rotenone (10, 30, and 100 μM) on ^{109}Cd uptake was assessed in glucose-free DMEM with the addition of mannitol (25 mM) to maintain osmolarity. Columns represent means of triplicate determinations (±S.D.) in a single experiment, and similar results were obtained in at least one additional experiment.

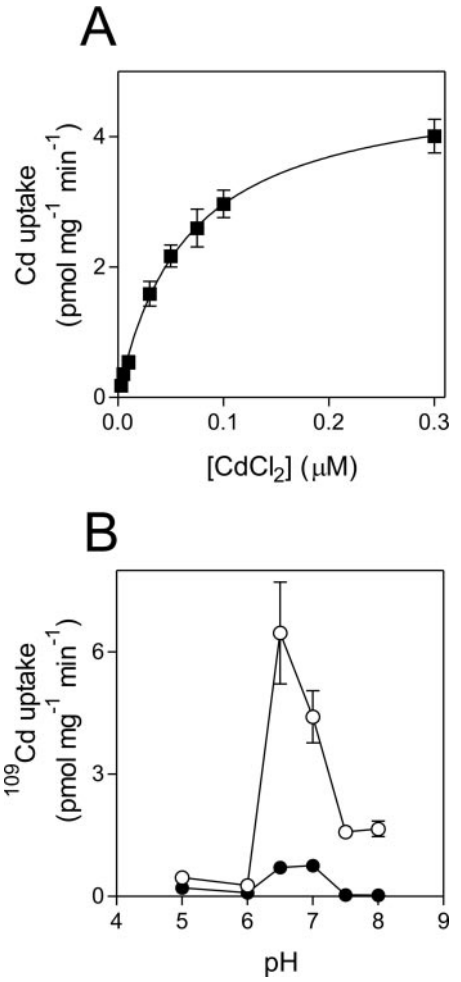

Fig. 4. Kinetic analysis and pH dependence of Cd^{2+} uptake. The MT(−/−) parental and CdR1 cell lines were plated at 2 × 10^5 cells/well in a six-well plate and cultured 24 h before experiments. A, Cd^{2+} uptake by the MT(−/−) parental cell line was measured at various Cd^{2+} concentrations (3–300 nM; 12–120 nCi) for 1 h at 37°C. Kinetic parameters were determined from nonlinear regression analysis of the data (GraphPad Prism). Points represent means of triplicate determinations (±S.D.) in a single experiment. The apparent K_m for Cd^{2+} uptake was 65 ± 0.32 nM, and the V_{max} was 4.9 ± 0.1 pmol mg^{-1} min^{-1}. B, MT(−/−) parental (○) and CdR1 (●) cell lines were incubated for 1 h at 37°C with Cd^{2+} (0.1 μM; 40 nCi) at the indicated pH. Symbols represent means of triplicate determinations (±S.D.) in a single experiment, and similar results were obtained in one additional experiment.

affected by Mn^{2+} or mibefradil, whereas it was inhibited modestly by Zn^{2+}(31% inhibition) (Fig. 7D).

Expression Levels of the T-Type Ca^{2+} Channel Cacnα_{1G} Suggest Potential Importance for Cd^{2+} Uptake. The relative expression levels of the Ca^{2+} channels Cacnα_{1G} and Cacnβ_3 were found to be decreased in CdR clones compared with the MT(−/−) parental cell line in preliminary microarray analysis [further genomic analysis will be reported in a separate article (E. M. Leslie, J. Liu, D. M. K. Ducharme, and M. P. Waalkes, manuscript in preparation)]. This prompted further characterization of Cacnα_{1G} and Cacnβ_3 expression in the CdR1-rev, CdR1, and MT(−/−) parental cell lines using real-time RT-PCR and immunoblot analysis (Fig. 8). Consistent with the intermediate function of the CdR1-rev cell line, the Cacnα_{1G} T-type Ca^{2+} channel was expressed at a level between that of the CdR1 and MT(−/−) parental cell lines. Thus, the Cacnα_{1G} T-type Ca^{2+} channel was expressed in CdR1 cells at 0.2% of the expression in the MT(−/−) parental cell line and at approximately 8% of the CdR1-rev (Fig. 8A). The protein expression of Cacnα_{1G} was also analyzed and confirmed the RNA expression level differences between the cell lines (Fig. 8B). Protein expression was not detectable in cell homogenate prepared from the CdR1 cell line, a faint band was visible in the CdR1-rev, whereas the MT(−/−) parental cell line contained a bright band at a molecular mass of ~240 kDa, consistent with the murine cerebellum-positive control. The CW53 antibody had been rigorously tested for Cacnα_{1G} specificity in a previous study (Yunker et al., 2003). The other Ca^{2+} channel subunit, Cacnβ_3 (Fig. 8C), did not differ in expression levels

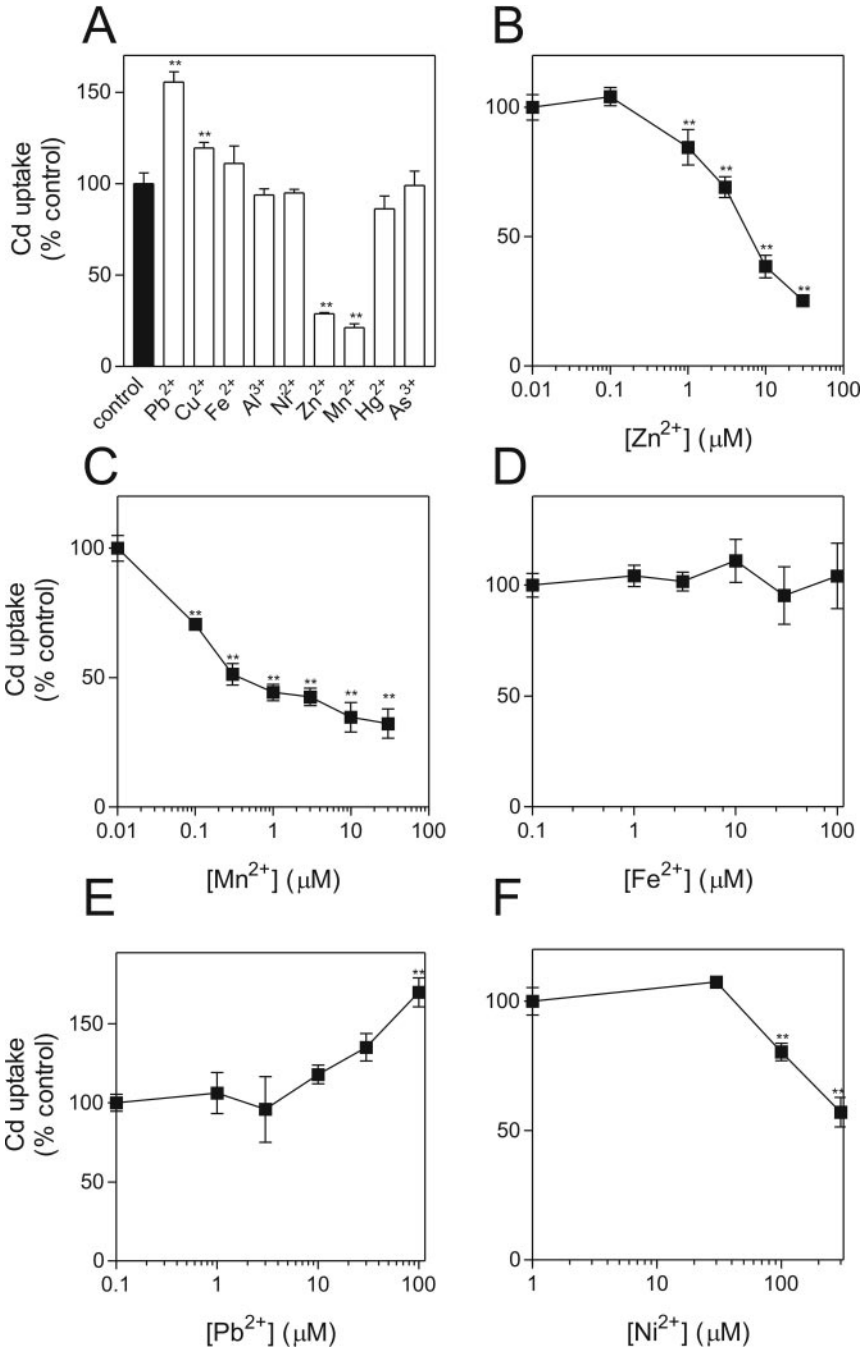

Fig. 5. Modulation of Cd^{2+} uptake by metals and metalloids. The MT(−/−) parental cell line was plated at 2×10^5 cells/well in a six-well plate and cultured 24 h before experiments. Cells were treated with ^{109}Cd (0.1 μM; 40 nCi) for 1 h at 37°C in the presence of 10 μM concentrations of the indicated metals/metalloids (A) or indicated concentrations of Zn^{2+} (B), Mn^{2+} (C), Fe^{2+} (D), Pb^{2+} (E), or Ni^{2+} (F). Points represent means of triplicate determinations (±S.D.) in a single experiment, and similar results were obtained in at least one additional experiment. **, statistically different from untreated control ($P < 0.01$) (analysis of variance followed by Newman-Keuls post hoc test).

between the CdR1 and CdR1-rev cell lines, indicating that this protein is unlikely to influence Cd^{2+} uptake.

Expression Levels of Dmt1/Slc11a2, AE4/Slc39a7, and Zip8/Slc39a8 Are Unchanged. The expression levels of two Mn^{2+} transporters, Dmt1/Slc11a2 and AE4/Slc39a7, were measured because of the proposed importance of Mn^{2+} transporters in Cd^{2+} uptake. Dmt1/Slc11a2 mRNA levels were not significantly different in the MT(−/−) cell line compared with the CdR1-rev or the CdR1 cell lines (Fig. 9A). AE4/Slc39a7 has been proposed to transport Mn^{2+} (Eide, 2004); however, expression was similar in the CdR1 versus MT(−/−) parental cell line (Fig. 9B) and therefore unlikely to be involved in the Cd^{2+} resistance observed. The expression of ZIP8/Slc39a8, a solute carrier protein that has recently been shown to facilitate uptake of Cd^{2+} in transfected cell lines and is thought to be associated with Cd^{2+} sensitivity (Dalton et al., 2005), was similar in the CdR1 versus the MT(−/−) parental cell line (Fig. 9C). Therefore, ZIP8/Slc39a8 is unlikely to be involved in the differential Cd^{2+} uptake and sensitivity in the CdR and MT(−/−) parental cell lines.

Expression of the Aquaporin 1 Channel Is Associated with Increased Cd^{2+} Uptake. In addition to changes in Ca^{2+} channel expression levels, microarray analysis indicated that expression of the zinc-iron regulated transporter-like (Zirtl/Slc39a1) and aquaporin 1 were decreased in the CdR1 and CdR2 clones compared with the MT(−/−) parental cell line (E. M. Leslie, J. Liu, D. M. K. Ducharme, and M. P. Waalkes, manuscript in preparation). This was confirmed by real-time RT-PCR analysis (Fig. 9, D and E). However, the expression of Zirtl/Slc39a1 was not significantly different in the CdR1 and CdR1-rev cell lines, suggesting that this transporter is not related to the observed functional changes in Cd^{2+} toxicokinetics. Aquaporin 1, an integral membrane protein that serves as a channel for the transfer of water across the cell membrane, was expressed in the CdR1 clone at 22% of the level found in the parental MT(−/−) cell line (Fig. 9E). Furthermore, the CdR1-rev cell line expressed aquaporin 1 at an intermediate level between the MT−/− parental (1.4-fold lower) and CdR1 (3.3-fold higher) cell lines (Fig. 9E), consistent with the intermediate phenotype of the revertant cell line (Fig. 7).

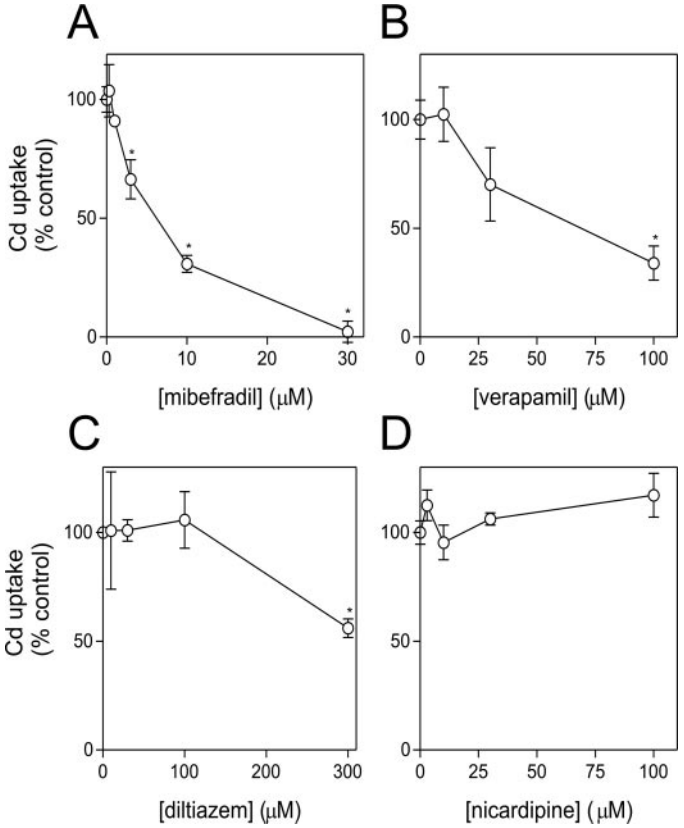

Fig. 6. Modulation of Cd^{2+} uptake by Ca^{2+} channel antagonists. The MT(−/−) parental cell line was plated at 2×10^5 cells/well in a six-well plate and cultured 24 h before experiments. Cells were incubated with ^{109}Cd (0.1 μM; 40 nCi) for 1 h at 37°C in the presence of mibefradil (A), verapamil (B), diltiazem (C), or nicardipine (D). Points represent means of triplicate determinations (±S.D.) in a single experiment, and similar results were obtained in at least one additional experiment. **, statistically different from untreated control ($P < 0.01$) (analysis of variance followed by Newman-Keuls post hoc test).

Discussion

In this work, a Cd^{2+}-resistant MT(−/−) cell line was established and characterized to investigate non–MT-mediated cellular protection mechanisms. In addition, a revertant cell line (CdR-rev) was developed and proved to be a powerful tool for the determination of gene expression changes relevant or incidental to development of the Cd^{2+} resistance phenotype. Through functional and molecular analysis of the differences between the revertant, resistant, and parental MT(−/−) cell lines, we have significantly extended previous work (Yanagiya et al., 1999, 2000) by more precisely defining the transport process involved in cellular Cd^{2+} uptake. Genetic, biochemical, and functional analyses suggest that the T-type Ca^{2+} channel, Cacnα_{1G}, is an important pathway for cellular entry of Cd^{2+}. Likewise, evidence implies that decreased expression of Cacnα_{1G} is a protective mechanism for cells exposed to Cd^{2+}. Decreased Cacnα_{1G} expression may be a key factor in the cellular response to Cd^{2+} exposure and may provide for acquired tolerance in cells that poorly produce MT. Given the frequently observed interindividual variability of MT expression seen in human subjects (Onosaka et al., 1985; Bem et al., 1988; Silevis-Smitt et al., 1992; Yoshida et al., 1998; Allan et al., 2000; Wu et al., 2000), knowledge of secondary factors in acquired Cd^{2+} tolerance could be very important.

Previous studies have suggested that Cd^{2+} can enter cells through L-type Ca^{2+} channels (Friedman and Gesek, 1994; Souza et al., 1997), and in the current study, uptake of Cd^{2+} was inhibited by verapamil, an L-type Ca^{2+} channel antagonist. However, verapamil has also been reported to inhibit T-type Ca^{2+} channels at concentrations as low as 10 μM, whereas more specific inhibition of L-type channels occurs at low micromolar concentrations (Heady et al., 2001). Consistent with the possibility of non–L-type channel inhibition by verapamil was the observation that two other L-type channel antagonists, diltiazem and nicardipine, had no effect on Cd^{2+} uptake. In contrast with verapamil, mibefradil blocks T-type Ca^{2+} channels 10 to 30 times more potently than L-type Ca^{2+} channels (Heady et al., 2001). Electrophysiological experiments using a variety of T-type Ca^{2+} currents have shown mibefradil IC_{50} values ranging from 0.1 to 4.7 μM (Heady et al., 2001). Thus, the ability of mibefradil to inhibit Cd^{2+} uptake by the MT(−/−) parental cell line with an IC_{50} of 5 μM is consistent with inhibition of a T-type Ca^{2+} channel.

Unlike other members of the T-type Ca^{2+} channel protein family, Cacna$_{1G}$, has been shown to be relatively insensitive to Ni^{2+} inhibition (Lacinová et al., 2000). Thus, it has been previously reported that Ni^{2+} inhibits Cacna$_{1G}$ with an IC_{50} value of 470 μM, comparable with the inhibitory potency of Ni^{2+} observed in the MT(−/−) parental cell line (IC_{50} = 300 μM) (Lacinová et al., 2000). Combined with these previous results, the present data implicate T-type Ca^{2+} channels as a critical component of Cd^{2+} entry into cells.

Uptake of Cd^{2+} was not affected by the metabolic inhibitors rotenone or sodium azide, suggesting a passive mechanism, consistent with a channel. T-type Ca^{2+} channels are "activated" or opened by low-voltage currents close to the resting membrane potential of the cell. High extracellular concentrations of K^+ are often used as a depolarizing stimulus to activate voltage-dependent Ca^{2+} channels and therefore increase Ca^{2+} influx into cells (Jagannathan et al., 2002). Thus, our observation that Cd^{2+} uptake by MT(−/−) parental cells was unchanged by increased K^+ concentrations is inconsistent with a voltage activation mechanism. However, previous studies have shown that recombinant Cacna$_{1G}$ channels expressed in human embryonic kidney 293 cells are insensitive to increased K^+ concentrations, unless cell membranes contain endogenous ion channels, such as Kir2.1, that stabilize resting membrane potential (Kim et al., 2004). Therefore, the uptake of Cd^{2+} in our cell system is possibly not affected by increased K^+ concentrations, because such endogenous ion channels are absent. Indeed, preliminary real-time RT-PCR analysis of Kir2.1 expression in the MT(−/−) cell line suggested that it is present at very low levels (data not shown).

In vivo, Cacna$_{1G}$ is expressed in many brain regions with especially high levels in amygdala, thalamus, subthalamic nuclei, and cerebellum (Perez-Reyes et al., 1998; Yunker et al., 2003). In addition, Cacna$_{1G}$ is expressed at lower levels in heart, placenta, lung, and kidney (Perez-Reyes et al., 1998; Andreasen et al., 2000). In the rat, Cacna$_{1G}$ mRNA is expressed in all regions of the kidney, including the distal and proximal tubules. Immunohistochemistry of rat kidney showed that Cacna$_{1G}$ is localized to the apical plasma membrane of distal convoluted tubule, connecting tubule principal cells and inner medullary collecting duct principal cells (Andreasen et al., 2000). Cd^{2+}-induced renal injury is dependent upon uptake and accumulation of this metal and although speculative, expression of Cacna$_{1G}$ in this tissue could have implications for Cd^{2+} toxicity (Friedman and Gesek, 1994).

Dmt1/Slc11a2 is a transporter expressed at the apical surface of cells in many mammalian tissues and at particularly high levels in the small intestine and kidney (Gunshin et al., 1997). Evidence strongly suggests that Dmt1/Slc11a2 plays an important role in the absorption of ferrous iron and potentially other divalent metals in the proximal duodenum (Canonne-Hergaux et al., 1999; Leazer et al., 2002). Although Dmt1/Slc11a2 is a candidate transporter of Cd^{2+}, uptake in the MT(−/−) parental cell line indicated that this process was not Dmt1/Slc11a2-mediated. For example, uptake of Cd^{2+} by the MT(−/−) cell line was potently inhibited by Zn^{2+}, maximal at slightly acidic to neutral pH, insensitive to

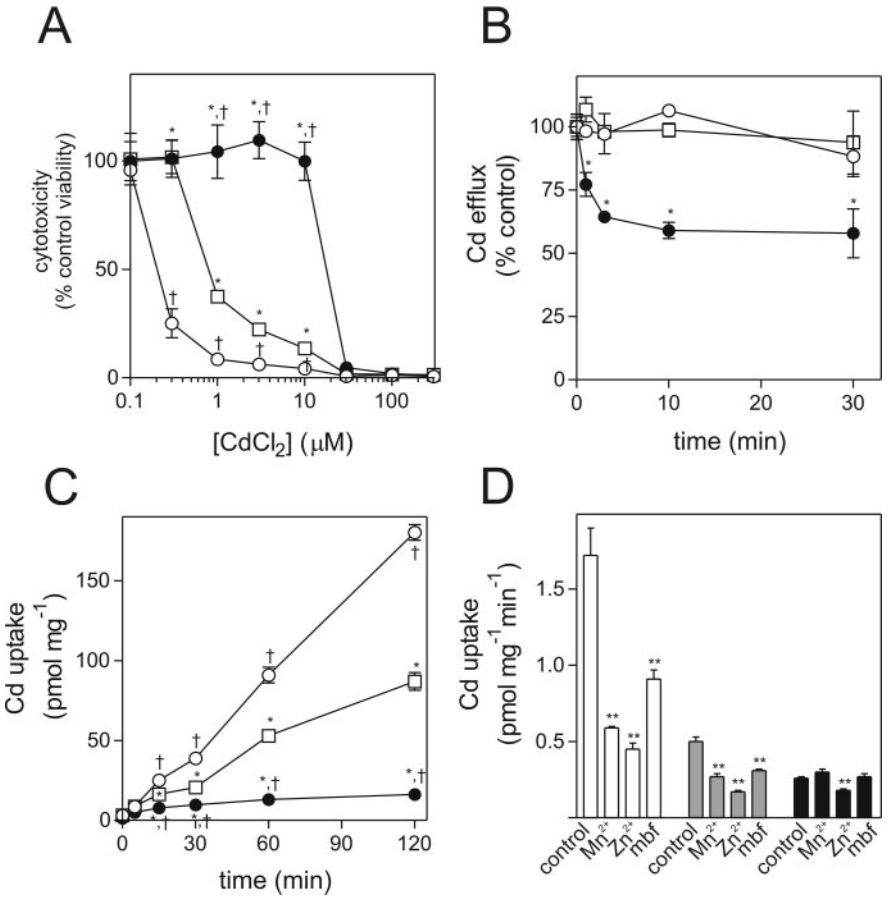

Fig. 7. Functional characterization of the CdR1-rev cell line. A, The CdR1 (■), CdR1-rev (□), and MT−/− parental (○) cell lines were plated at 1 × 10^4 cells/well in a 96-well plate, and 24 h later they were treated with Cd^{2+} for 72 h. A tetrazolium-based microtiter plate assay was used to measure acute cytotoxicity as described under *Materials and Methods*. Symbols represent means of quadruplicate determinations (±S.D.) in a single experiment, and similar results were obtained in two additional experiments. B, CdR1 (■), CdR1-rev (□), and MT−/− parental (○) cell lines were plated at 2 × 10^5 cells/well in a six-well plate and incubated 24 h before experiments. Cells were incubated with ^{109}Cd (0.1 μM; 40 nCi) for 6 h at 37°C, and then efflux was measured at the indicated time points (as described under *Materials and Methods*). Points represent means of triplicate determinations (±S.D.) in a single experiment, and similar results were obtained in one additional experiment. C, MT(−/−) parental (○), CdR1-rev (□), and CdR1 (■) were plated as described in B, and uptake of ^{109}Cd (0.1 μM; 40 nCi) was measured for the indicated time points at 37°C. Points represent means of triplicate determinations (±S.D.) in a single experiment, and similar results were obtained in one additional experiment. D, MT(−/−) parental (white columns), CdR1-rev (gray columns), and CdR1 (black columns) were plated as described in B and incubated with ^{109}Cd (0.1 μM; 40 nCi) ± Mn^{2+} (1 μM), Zn^{2+} (10 μM), or mibefradil (mbf) (10 μM) for 1 h at 37°C. Columns represent means of triplicate determinations (±S.D.) in a single experiment, and similar results were obtained in one additional experiment. *, statistically different from MT(−/−) ($P < 0.01$); †, statistically different from CdR1-rev ($P < 0.01$); **, statistically different from untreated cell line control (analysis of variance followed by Newman-Keuls post hoc test).

Fe^{2+}, and mediated with very high affinity. In contrast, transport by Dmt1/Slc11a2 is insensitive to Zn^{2+}, inhibited by Fe^{2+}, maximal at pH 5.5, and mediated at lower affinity (K_m = 1 μM) (Gunshin et al., 1997; Okubo et al., 2003). Gene expression analysis indicated that there was no difference in the Dmt1/Slc11a2 mRNA expression in MT(−/−) parental versus the CdR or CdR-rev cell lines consistent with the non-Dmt1/Slc11a2 phenotype.

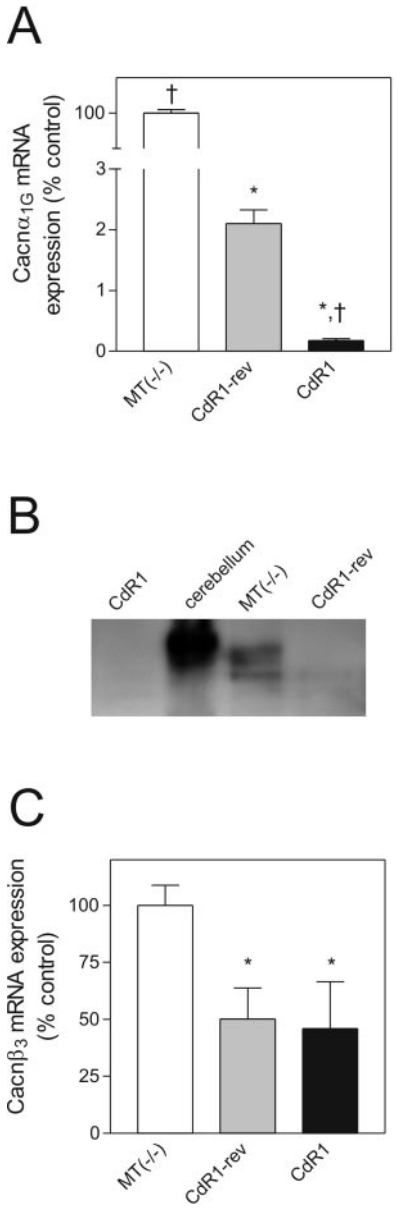

Fig. 8. Real-time RT-PCR and immunoblot analysis of Ca^{2+} channel expression. A, MT(−/−) parental (white columns), CdR1 (black columns), and CdR1-rev (gray columns) cell lines were grown in the absence of Cd^{2+} for 4 to 10 days, RNA was isolated, and expression levels of the Ca^{2+} channel Cacnα_{1G} were analyzed using real-time RT-PCR as described under *Materials and Methods*. Columns represent means of triplicate determinations, and similar results were obtained with RNA isolated from the CdR2 and CdR2-rev cell lines. B, immunoblot of cell (50 μg) and tissue (70 μg) homogenates prepared from CdR1 cell line, murine cerebellum (positive control), MT(−/−) parental, and CdR1-rev cell lines. Homogenates were resolved on a 6% acrylamide gel, transferred, and probed with the polyclonal CW53 as described under *Materials and Methods*. C, MT(−/−) parental (white columns), CdR1 (black columns), and CdR1-rev (gray columns) cell lines were grown in the absence of Cd^{2+} for 4 to 10 days, RNA was isolated, and expression levels of the Ca^{2+} channel Cacnβ_3 were analyzed using real-time RT-PCR as described under *Materials and Methods*. Columns represent means of triplicate determinations, and similar results were obtained with RNA isolated from the CdR2 and CdR2-rev cell lines. *, statistically different from MT(−/−) uptake ($P < 0.05$); †, statistically different from CdR1-rev uptake ($P < 0.05$) (analysis of variance followed by Newman-Keuls post hoc test).

Experiments conducted by Yanagiya et al. (2000) using an independently derived Cd^{2+}-resistant MT(−/−) cell line also suggested that a non-Dmt1/Slc11a2 Cd^{2+} uptake mechanism was present in the parental MT(−/−) cell line. Their Cd^{2+}-resistant cell line had a reduced ability to take up Mn^{2+}, and Mn^{2+} was a potent inhibitor of Cd^{2+} uptake (K_i = 0.14 μM) (Yanagiya et al., 2000). Thus, the authors concluded that the MT(−/−) cell line expresses a novel high-affinity Mn^{2+} transport system through which Cd^{2+} can enter the cell. In the current study, expression of AE4/Slc39a7, a member of the SLC39 family of metal ion transporters, a proposed Mn^{2+} transporter (Eide, 2004), was expressed at similar levels in CdR and MT(−/−) parental cell lines, suggesting it is not involved in the observed alterations in Cd^{2+} uptake. Very little is known about the molecular pathways by which Mn^{2+} enters mammalian cells. Although further experiments are required to be conclusive, there are examples of overlap in Ca^{2+} and Mn^{2+} uptake (Van Baelen et al., 2004); Mn^{2+} has been shown to permeate through a related T-type Ca^{2+} channel, Cacnα_{1H} (Kaku et al., 2003); and potentially a common mechanism exists (i.e., Cacnα_{1G}) for the passage of Cd^{2+}, Ca^{2+}, and Mn^{2+} into the MT(−/−) cell line.

The aquaporins are a family of membrane proteins that allow rapid transport of water across lipid membranes (Law and Sansom, 2002). Certain aquaporins, the aquaglyceroporins, will also allow the downhill movement of uncharged solutes such as glycerol and urea (Liu et al., 2004). It is noteworthy that several aquaglyceroporins, including human AQP7 and AQP9, have been shown to be important for arsenic passage into cells (Liu et al., 2004). Analysis of CdR1, CdR2, and MT(−/−) parental cell lines indicated that the expression of aquaporin 1 was decreased in the CdR versus the parental cell line and that aquaporin 1 expression was intermediate in the CdR-rev cell line, consistent with the intermediate Cd^{2+} resistance phenotype. The functional implications of the decreased RNA expression of aquaporin 1 are not clear. Unlike the aquaglyceroporins, aquaporin 1 transport has been shown to be highly selective for water molecules (Law and Sansom, 2002). The high-resolution X-ray structure of bovine aquaporin 1 (Sui et al., 2001) indicates that the channel pore is not wide enough to allow the passage of a hydrated ion, such as Cd^{2+}, and not energetically favorable for dehydration to occur before entering the channel (Law and Sansom, 2002). Therefore, it is highly unlikely that aquaporin 1 is directly involved in translocation of Cd^{2+} across the plasma membrane, but it could have a different function such as in osmoregulation. Indeed, aquaporin 1 is lost from the brush-border membrane of kidney proximal tubules of rats treated with Cd^{2+}, suggesting that this channel is affected by the metal in vivo (Sabolic et al., 2002).

In conclusion, understanding non–MT-mediated cellular protection mechanisms is important because of the interindividual variability in MT expression in humans. In addition, such mechanisms could work in synergy with MT to provide protection from this toxic metal. This work clearly indicates that decreased cellular uptake of Cd^{2+} is a very important

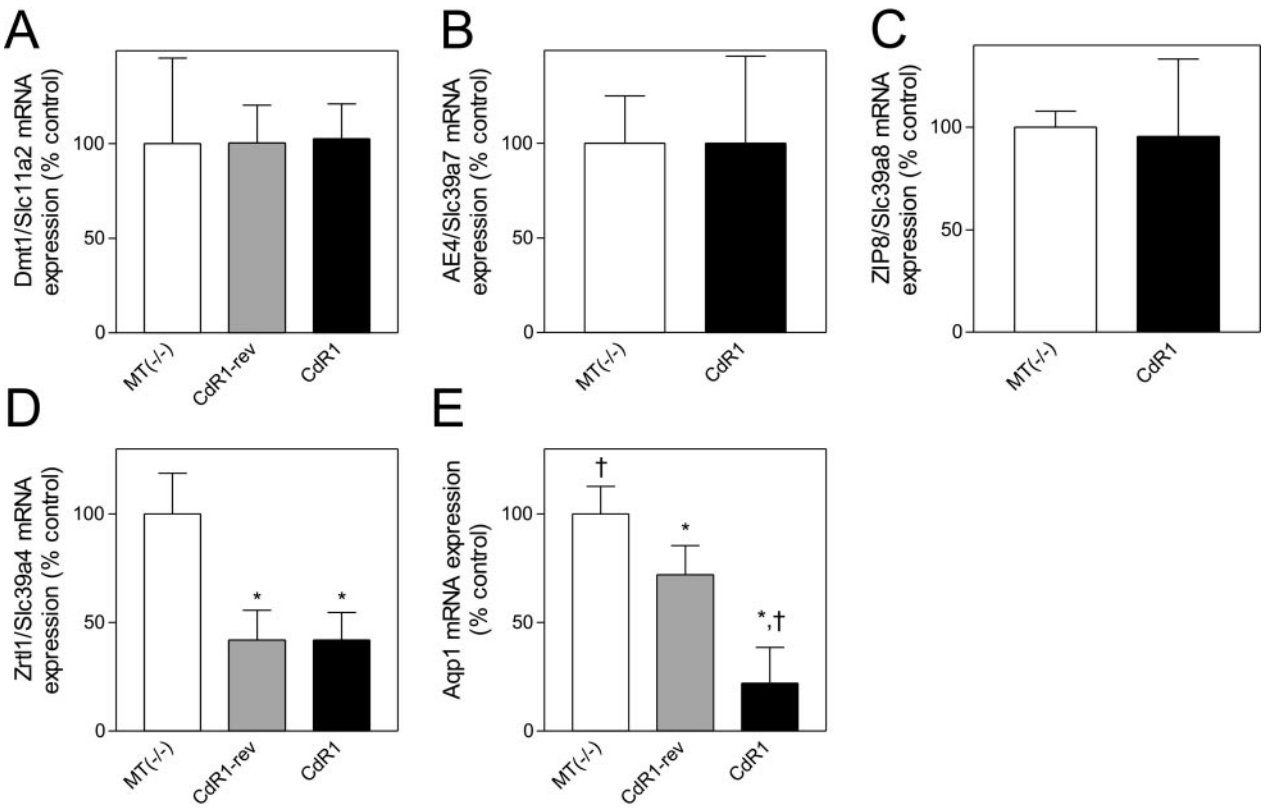

Fig. 9. Real-time RT-PCR analysis of Dmt1/Slc11a2, AE4/Slc39a7, ZIP8/Slc39a8, Zrtl1/Slc39a4, and aquaporin 1 (Aqp1) expression in MT(−/−) parental, CdR1, and CdR1-rev cell lines. Cell lines were grown in the absence of Cd^{2+} for 4 to 10 days, RNA was isolated, and expression levels of Dmt1/Slc11a2 (A), AE4/Slc39a7 (B), ZIP8/Slc39a8 (C), Zrtl1/Slc39a4 (D), and aquaporin 1 (E) were analyzed in MT(−/−) parental (white columns), CdR1-rev (gray columns), and/or CdR1 (black columns) cell lines using real-time RT-PCR as described under *Materials and Methods*. Columns represent means of triplicate determinations, and similar results were obtained with RNA isolated from the CdR2 and CdR2-rev cell lines. *, statistically different from MT(−/−) uptake ($P < 0.05$); †, statistically different from CdR1-rev uptake ($P < 0.05$) (analysis of variance followed by Newman-Keuls post hoc test).

mechanism of acquired Cd^{2+} resistance. The thorough characterization of Cd^{2+} transport in MT(−/−) cells has lead to the identification of Cacnaα_{1G} as a potential pathway for cellular uptake of Cd^{2+}. Although further research is required to understand the physiological relevance of our observations, down-regulation of Cacnaα_{1G} expression could be very important for cellular protection from Cd^{2+} under normal circumstances and for persons with MT(−/−) deficiencies.

Acknowledgments

We thank Dr. John S. Lazo (University of Pittsburgh, Pittsburgh, PA) for the kind gifts of the MT(−/−) and MT(+/+) cell lines. We thank Dr. Maureen W. McEnery (Case Western Reserve University, Cleveland, OH) for the generous gifts of the CW53 antibody and murine cerebellum homogenates. We also thank Drs. Larry Keefer, Wei Qiu, and Jean Francois Coppin for critical review of the manuscript.

References

Allan KA, Hawksworth GM, Woodhouse LR, Sutherland B, King J, and Beattie JH (2000) Lymphocyte metallothionein mRNA responds to marginal zinc intake in human volunteers. *Br J Nutr* **84:**747–756.

Andreasen D, Jensen BL, Hansen PB, Kwon T, Nielsen S, and Skott O (2000) The α_{1G}-subunit of a voltage-dependent Ca^{2+} channel is localized in rat distal nephron and collecting duct. *Am J Physiol* **279:**F997–F1005.

Bem EM, Piotrowski JK, Sobczak-Kozlowska M, and Dmuchowski C (1988) Cadmium, zinc, copper and metallothionein levels in human liver. *Int Arch Occup Environ Health* **60:**413–417.

Bressler JP, Olivi L, Cheong JH, Kim Y, and Bannona D (2004) Divalent metal transporter 1 in lead and cadmium transport. *Ann NY Acad Sci* **1012:**142–152.

Canonne-Hergaux F, Gruenheid S, Ponka P, and Gros P (1999) Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulation by dietary iron. *Blood* **93:**4406–4417.

Dalton TP, He L, Wang B, Miller ML, Jin L, Stringer KF, Chang X, Baxter CS, and Nebert DW (2005) Identification of mouse SLC39A8 as the transporter responsible for cadmium-induced toxicity in the testis. *Proc Natl Acad Sci USA* **102:**3401–3406.

Dijkstra M, Havinga R, Vonk RJ, and Kuipers F (1996) Bile secretion of cadmium, silver, zinc and copper in the rat. Involvement of various transport systems. *Life Sci* **59:**1237–1246.

Eide DJ (2004) The SLC39 family of metal ion transporters. *Pflueg Arch Eur J Physiol* **447:**796–800.

Friedman PA and Gesek FA (1994) Cadmium uptake by kidney distal convoluted tubule cells. *Toxicol Appl Pharmacol* **128:**257–263.

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, and Hediger MA (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature (Lond)* **388:**482–488.

Heady TN, Gomora JC, Macdonald TL, and Perez-Reyes E (2001) Molecular pharmacology of T-type Ca2+ channels. *Jpn J Pharmacol* **85:**339–350.

Himeno S (2002) Application of metallothionein null cells to investigation of cadmium transport. *J Inorg Biochem* **88:**207–212.

Himeno S, Yanagiya T, Enomoto S, Kondo Y, and Imura N (2002) Cellular cadmium uptake mediated by the transport system for manganese. *Tohoku J Exp Med* **196:**43–50.

Jagannathan S, Publicover SJ, and Barratt CLR (2002) Voltage-operated calcium channels in male germ cells. *Reproduction* **123:**203–215.

Kaku T, Lee T, Arita M, Hadama T, and Ono K (2003) The gating and conductance properties of $Ca_v3.2$ low-voltage-activated T-type calcium channels. *Jpn J Physiol* **53:**165–172.

Kim T, Choi J, Kim S, Kwon O, Nah SY, Han YS, and Rhim H (2004) The biochemical activation of T-type Ca2+ channels in HEK293 cells stably expressing alpha1G and Kir2.1 subunits. *Biochem Biophys Res Commun* **324:**401–408.

Klaassen CD, Liu J, and Choudhuri S (1999) Metallothionein: an intracellular protein to protect against cadmium toxicity. *Annu Rev Pharmacol Toxicol* **39:**267–294.

Kondo Y, Yanagiya T, Himeno S, Yamabe Y, Schwartz D, Akimotoa M, Lazo JS, and Imura N (1999) Simian virus 40-transformed metallothionein null cells showed increased sensitivity to cadmium but not to zinc, copper, mercury or nickel. *Life Sci* **64:**PL145–PL150.

Lacinová L, Klugbauer N, and Hofmann F (2000) Regulation of the calcium channel

alpha(1G) subunit by divalent cations and organic blockers. *Neuropharmacology* **39:**1254–1266.
Law RJ and Sansom MSP (2002) Water transporters: how so fast yet so selective? *Curr Biol* **12:**R250–R252.
Leazer TM, Liu Y, and Klaassen (2002) Cadmium absorption and its relationship to divalent metal transporter-1 in the pregnant rat. *Toxicol Appl Pharmacol* **185:**18–24.
Leslie EM, Deeley RG, and Cole SP (2005) Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2 and BCRP (ABCG2) in tissue defense. *Toxicol Appl Pharmacol* **204:**216–237.
Liu Z, Carbrey JM, Agre P, and Rosen BP (2004) Arsenic trioxide uptake by human and rat aquaglyceroporins. *Biochem Biophys Res Commun* **316:**1178–1185.
Okubo M, Yamada K, Hosoyamada M, Shibasaki T, and Endou H (2003) Cadmium transport by human Nramp 2 expressed in *Xenopus laevis* oocytes. *Toxicol Appl Pharmacol* **187:**162–167.
Onosaka S, Min K, Fukuhara C, Tanaka K, Tashiro S, Shimizu I, Furuta M, and Yasutomi T (1985) Concentration of metallothionein in human tissues. *Eisei Kagaku* **31:**352–355.
Palmiter RD (1998) The elusive function of metallothioneins. *Proc Natl Acad Sci USA* **95:**8428–8430.
Paulusma CC and Oude Elferink RP (1997) The canalicular multispecific organic anion transporter and conjugated hyperbilirubinemia in rat and man. *J Mol Med* **75:**420–428.
Perez-Reyes E, Cribbs LL, Daud A, Lacerda AE, Barclay J, Williamson MP, Fox M, Rees M, and Lee J (1998) Molecular characterization of a neuronal low-voltage-activated T-type calcium channel. *Nature (Lond)* **391:**896–900.
Sabolic I, Ljubojevic M, Herak-Kramberger CM, and Brown D (2002) Cd-MT causes endocytosis of brush-border transporters in rat renal proximal tubules. *Am J Physiol* **283:**F1389–F1402.
Satarug S and Moore MR (2004) Adverse health effects of chronic exposure to low-level cadmium in foodstuffs and cigarette smoke. *Environ Health Perspect* **112:**1099–1103.
Silevis-Smitt PAE, van Beek H, Baars A-J, Troost D, Louwerse ES, Krops-Hermus ACM, de Wolf FA, and de Jong JMB (1992) Increased metallothionein in the liver and kidney of patients with amyotrophic lateral sclerosis. *Arch Neurol* **49:**721–724.
Souza V, Bucio L, and Gutierrez-Ruiz MC (1997) Cadmium uptake by a human hepatic cell line (WRL-68 cells). *Toxicology* **120:**215–220.
Sui HX, Han BG, Lee JK, Walian P, and Jap BK (2001) Structural basis of water-specific transport through the AQP1 water channel. *Nature (Lond)* **414:**872–878.
Van Baelen K, Dode L, Vanoevelen J, Callewaert G, De Smedt H, Missiaen L, Parys JB, Raeymaekers L, and Wuytack F (2004) The Ca2+/Mn2+ pumps in the Golgi apparatus. *Biochim Biophys Acta* **1742:**103–112.
Waalkes MP (2003) Cadmium carcinogenesis. *Mutat Res* **533:**107–120.
Waalkes MP and Pérez-Ollé (2000) Role of metallothionein in metabolism, transport and toxicity of metals, in *Molecular Biology and Toxicology of Metals* (Koropatnick DJ and Zalups R eds) pp 414–55, Taylor & Francis, London.
Waalkes MP and Poirier LA (1985) Interactions of cadmium with interstitial tissue of the rat testes. Uptake of cadmium by isolated interstitial cells. *Biochem Pharmacol* **34:**2513–2518.
Waisberg M, Joseph P, Hale B, and Beyersmann D (2003) Molecular and cellular mechanisms of cadmium carcinogenesis. *Toxicology* **192:**95–117.
Walker NJ (2001) Real-time and quantitative PCR: applications to mechanism-based toxicology. *J Biochem Mol Toxicol* **15:**121–127.
Wu M-T, Demple B, Bennett RAO, Christiani DC, Fan R, and Hu H (2000) Individual variability in the zinc inducibility of metallothionein-IIA mRNA in human lymphocytes. *J Toxicol Environ Health* **61:**553–567.
Yanagiya T, Imura N, Enomoto S, Kondo Y, and Himeno S (2000) Suppression of a high-affinity transport system for manganese in cadmium-resistant metallothionein-null cells. *J Pharmacol Exp Ther* **292:**1080–1086.
Yanagiya T, Imura N, Kondo Y, and Himeno S (1999) Reduced uptake and enhanced release of cadmium in cadmium-resistant metallothionein null fibroblasts. *Life Sci* **65:**PL177–PL182.
Yoshida M, Ohta H, Yamauchi Y, Seki Y, Sagi M, Yamazaki K, and Sumi Y (1998) Age-dependent changes in metallothionein levels in liver and kidney of the Japanese. *Biol Trace Elem Res* **63:**167–175.
Yunker AM, Sharp AH, Sundarraj S, Ranganathan V, Copeland TD, and McEnery MW (2003) Immunological characterization of T-type voltage-dependent calcium channel $Ca_v3.1$ (alpha1g) and $Ca_v3.3$ (alpha1I) isoforms reveal differences in their localization, expression and neural development. *Neuroscience* **117:**321–335.
Zalups RK and Ahmad S (2003) Molecular handling of cadmium in transporting epithelia. *Toxicol Appl Pharmacol* **186:**163–188.

Address correspondence to: Dr. Michael P. Waalkes, Inorganic Carcinogenesis Section, National Cancer Institute, National Institute of Environmental Health Sciences, P.O. Box 12233, Mail Drop F0-09, 111 Alexander Dr., Research Triangle Park, NC 27709. E-mail: waalkes@niehs.nih.gov