Cd²⁺ versus Zn²⁺ Uptake by the ZIP8 HCO_3^--Dependent Symporter: Kinetics, Electrogenicity and Trafficking

Zhiwei Liu, **Hong Li**[1], **Manoocher Soleimani**[1], **Kuppuswami Girijashanker**, **Jodie M. Reed**, **Lei He**, **Timothy P. Dalton**, and **Daniel W. Nebert**[*]

*Department of Environmental Health and Center for Environmental Genetics (**CEG**) University of Cincinnati Medical Center, Cincinnati OH 45267−0056*

1*Department of Internal Medicine, Division of Nephrology and Hypertension University Cincinnati Medical Center, Cincinnati OH 45267−0056*

Abstract

The mouse *Slc39a8* gene encodes the ZIP8 transporter, which has been shown to be a divalent cation/HCO_3^- symporter. Using ZIP8 cRNA-injected *Xenopus* oocyte cultures, we show herein that: **[a]** ZIP8-mediated cadmium (**Cd^{2+}**) and zinc (**Zn^{2+}**) uptake have Vmax values of 1.8 ± 0.08 and 1.0 ± 0.08 pmol/oocyte/hour, and Km values of 0.48 ± 0.08 and 0.26 ± 0.09 μM, respectively; **[b]** ZIP8-mediated Cd^{2+} uptake is most inhibited by Zn^{2+}, second-best inhibited by Cu^{2+}, Pb^{2+} and Hg^{2+}, and not inhibited by Mn^{2+} or Fe^{2+}; and **[c]** electrogenicity studies demonstrate an influx of two HCO_3^- anions per one Cd^{2+} (or one Zn^{2+}) cation, *i.e.* electroneutral complexes. Using Madin-Darby canine kidney (**MDCK**) polarized epithelial cells retrovirally-infected with ZIP8 cDNA and tagged with hemagglutinin at the C-terminus, we show that—similar to ZIP4—the ZIP8 eight-transmembrane protein is largely internalized during Zn^{2+} homeostasis, but moves predominantly to the cell surface membrane (trafficking) under conditions of Zn^{2+} depletion.

INTRODUCTION

Cadmium (**Cd**, **Cd^{2+}**,) is a non-essential metal. Due to the growth of industrialization, the amount of Cd in our environment has increased dramatically. In combination with longer life expectancy, the rising levels of environmental Cd have teamed up to enhance the body's Cd burden: for example, the average renal accumulation of Cd in a cigarette smoker who has smoked at least two packs a day for 40 years is close to the threshold (~30 μg/g wet weight of kidney) that is sufficient for causing overt renal failure (http://www.trace-elements.org.uk/cadmium.htm).

Cd has been classified by IARC as a "Category **I**" human lung carcinogen. Individuals at highest risk for Cd-induced lung cancer and chronic renal disease include cigarette smokers, those on a steady diet that is rich in high-fiber foods or contaminated shellfish, women having low body-iron stores, and malnourished populations [1-4].

In acute doses, Cd has been known for 80 years to cause damage to the central nervous system, lung, bone, gastrointestinal tract, liver, ovary, testis, placenta, and developing embryo [5;6].

*To whom correspondence should be addressed: Department of Environmental Health, University of Cincinnati Medical Center, P.O. Box 670056, Cincinnati OH 45267−0056, U.S.A. Tele. 513−558−0373; Fax 513−558−0974; email dan.nebert@uc.edu.

Chronic exposure to low Cd doses can cause renal proximal tubular metabolic acidosis and osteomalacia (renal Fanconi syndrome); Cd is eliminated very slowly from the body and thus accumulates as a total body burden—predominantly in the kidney—with age.

For many decades, Cd influx into mammalian cells has been assumed to take place before Cd-mediated disease occurs. Cd uptake by mammalian cultured cells has been shown to use Ca^{2+} channels [7-10]. SLC11A2 (**DMT1**) shows a preference for Fe^{2+}, but also transports Pb^{2+} and Cd^{2+} [11] with protons [11]. Using SLC11A2 knockdown in human intestinal Caco-2 cells [12], proton-dependent Cd transport was demonstrated. Additional reports point out that SLC11A2 participates in Cd transport in renal distal tubular cells [13;14] and in enterocytes [13;15;16]. Cd transport in *Xenopus* oocytes expressing human SLC11A2 shows Michaelis-Menten kinetics with a Km of 1.04 ± 0.13 μM [17].

Recent studies have shown for the first time a relationship between a specific genotype (allelic differences in the mouse *Slc39a8* gene encoding the ZIP8 transporter) and specific phenotypes (risk of Cd-induced testicular necrosis; acute renal failure) [18;19]. The ZIP8 transporter, which can be hijacked by Cd, undoubtedly transports one or more essential divalent cation(s) important in carrying out critical life functions. In mammalian cell culture, manganese (**Mn**) was shown to be the best inhibitor of ZIP8-mediated Cd uptake and has a low Km (2.2 μM) for ZIP8-mediated uptake [20]; however, zinc (**Zn**) could not be ruled out as another essential divalent cation that uses ZIP8, because of multiple interfering receptors known to be on the surface of mammalian cells.

ZIP8 is a Cd^{2+} or Mn^{2+}/HCO_3^- symporter and has been localized to the apical surface of two cell types [20]: between the blood and vascular endothelial cells of the testis [18;19], and between the glomerular filtrate and proximal tubular epithelial cells of the kidney [19]. In the present study, we examine the kinetics and electrogenicity of ZIP8-mediated Cd^{2+}/HCO_3^- and Zn^{2+}/HCO_3^- uptake in *Xenopus* oocytes, as well as trafficking of the ZIP8 protein in MDCK cultured cells as a function of extracellular Zn^{2+} concentration.

MATERIALS and METHODS

Chemicals

All divalent cations were purchased as chloride or acetate salts from Fisher Scientific (Pittsburgh, PA). Tetramethylammonium (**TMA$^+$**) chloride, collagenase, and Chelex 100 were bought from Sigma (St. Louis, MO). $^{109}CdCl_2$ and $^{65}ZnCl_2$ have been described [20]. The ND-96 uptake medium (96 mM NaCl, 2.0 mM KCl, 1.0 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5.0 mM HEPES, pH 7.5) was prepared in our laboratory.

Cell cultures

Madin-Darby canine kidney (**MDCK**) cells cultured in Dulbecco's modified Eagle's medium (**DMEM**) has been described [18;20].

Preparation of the ZIP8 cDNA-containing vector

Generation of this vector has been described [18].

Generation of ZIP8 capped RNA (cRNA) from cDNA-containing vector

The ZIP8 cDNA described above was excised from the pBluescript vector, using *Bam* HI and *Sal* I sites and ligated into the multiple-cloning site of pXFRM [21;22], a specific *Xenopus* vector which was a generous gift of William F. Marzluff (University of North Carolina, Chapel Hill). For in vitro transcription, the plasmid was linearized with *BspQ* I. The cRNA was transcribed in vitro from the linearized cDNA template using the mMESSAGE mMACHINE

SP6 kit (Ambion, Austin, TX), according to manufacturer's instructions. The size of the purified transcription product and its quantity were evaluated by gel electrophoresis. The cRNA was dissolved in RNase-free water and stored at −80°C until use.

Microinjection of cRNA into Xenopus oocytes

All frog experiments were approved by, and conducted in accordance with, the National Institutes of Health standards for the care and use of experimental animals and the University Cincinnati Medical Center Institutional Animal Care and Use Committee. Preparation of the *Xenopus* oocytes and cRNA microinjection were carried out exactly as described [23]. All assays were carried out 3 days after cRNA microinjection. Incubation medium was maintained at 22°C (pH 7.5), with five to ten oocytes per time-point, or per concentration-point. Protein content varies so much among oocytes (range = 266 to 1250 μg/oocyte); thus, all kinetics data are expressed "per oocyte".

Divalent Cation Uptake and Km Determination

ND-96 medium always included 25 mM $NaHCO_3^-$ (pH 7.5). Oocytes grown in ND-96 were otherwise treated with radiolabeled Cd^{2+} or Zn^{2+} as previously described [20].

Inhibition of Cd uptake by other metal ions

The competing divalent cation was added to the ND-96 medium simultaneously with addition of 0.25 μM Cd, but at concentrations 3, 10 or 30 times greater than that of Cd. Due to precipitation problems, Hg^{2+} and Pb^{2+} studies were carried out in ND-96 in which Cl^- was replaced by gluconate ion. Fe^{2+} competition studies were performed in ND-96 having 1 mM ascorbic acid—in order to maintain reduced Fe^{2+}. Incubation of the oocytes was for 60 min, following which oocytes were washed, and radioactivity determined.

Electrogenicity

The modified ND-96 medium (containing 25 mM $NaHCO_3^-$) was adjusted with TMA^+ to maintain a constant 98-mM isosmolar replacement of monovalent cations: if K^+ = **60 mM**, then Na^+ = 38 mM; if K^+ = **20 mM**, then Na^+ = 38 mM and TMA^+ = 40 mM; if K^+ = **2 mM**, then Na^+ = 38 mM and TMA^+ = 58 mM; if K^+ = **1 mM**, then Na^+ = 38 mM and TMA^+ = 59 mM). Cd or Zn uptake for 30 min by ZIP8 cRNA-injected oocytes was compared with that by water-injected oocytes at 1, 2, 20 and 60 mM K^+ concentrations.

Delivery of the ZIP8 cDNA into MDCK Tet-off cells

Generation of the stable retrovirus-infected rvMDCK-LUC (which carries the non-ZIP luciferase cDNA control), rvMDCK-ZIP4ha, and rvMDCK-ZIP8ha cell lines has been described [20].

Detection of ZIPha protein levels on the cell surface

Antibody-detectable amounts of ZIP4ha and ZIP8ha on the cell surface were assessed by measuring the levels of anti-**ha** antibodies bound to the outside of rvMDCK-ZIP4ha and rvMDCK-ZIP8ha cells. These cells, plus rvMDCK-LUC control cells, were assessed by procedures detailed previously [24].

Statistical Analysis

Statistical significance between groups was determined a 4-way Student's *t* test. Statistical analyses were performed with the use of SAS statistical software (SAS Institute Inc., Cary, NC). The determinations of Km and Vmax values for ZIP-mediated metal uptake were determined using Sigma Plot (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Cd and Zn uptake kinetics

In *Xenopus* oocytes, we found that ZIP8-mediated Cd uptake was linear over 60 min of incubation time (Fig. 1A, *top*). We therefore used the 60-min time-point for examining Michaelis-Menten kinetics (Fig. 1A, *bottom*). Vmax values were estimated to be 1.8 ± 0.08 pmol/oocyte/hr, with a Km of 0.48 ± 0.08 μM. This Km value is ~25% lower than the Km value of 0.62 μM found in MFF cultures in Hank's balanced salt solution (**HBSS**) medium [20]; as is commonly the case, Km values in *Xenopus* oocytes are lower than those in mammalian cell cultures—due to fewer numbers of interfering transporters on the *Xenopus* cell surface. Moreover, this Km value of 0.48 μM for ZIP8-mediated Cd transport in frog oocytes is at least twice lower than that of 1.04 μM reported for Cd transport in *Xenopus* oocytes expressing human SLC11A2 [17]. Clearly, human populations are usually exposed to environmental Cd levels that are extremely low, and Cd most likely would be transported by the system having the lowest Km for that metal. We therefore conclude that the ZIP8 transporter is more relevant than SLC11A2 for the influx of Cd from our environment.

ZIP8-mediated Zn uptake was also linear over 60 min (Fig. 1B, *top*) and also demonstrated Michaelis-Menten kinetics (Fig. 1B, *bottom*), with a Vmax value of 1.0 ± 0.08 pmol/oocyte/hr and a Km of 0.26 ± 0.09 μM. Interestingly, Zn uptake in water-injected oocytes over the 90-min incubation period was about 4-fold greater than Cd uptake in water-injected oocytes; this finding suggests the existence of additional Zn transporters on the *Xenopus* oocyte cell surface that do cause some degree of interference. The Km value for Zn was almost twice lower than that for Cd, indicating that Zn is indeed an endogenous very-high-affinity substrate for ZIP8. As stated above, we were unable to determine a valid Km value for Zn in MFF cultures in HBSS medium [20]—most likely due to the excess numbers of interfering transporters on the mammalian cell surface.

Metal-mediated competitive inhibition of Cd uptake

Studying six metals in *Xenopus* oocytes (Fig. 2), we found that Zn was the best inhibitor of ZIP8-mediated Cd influx. Curiously, Mn had been found to be a strong competitor of ZIP8-mediated uptake in MFF cultures in HBSS [20], whereas it was not a significant inhibitor in *Xenopus* oocytes; we have no explanation for this discrepancy.

Fe^{2+} also showed no significant inhibition of ZIP8-mediated Cd influx in frog oocytes (Fig. 2). Evolutionarily, the nearest neighbor to the *Slc39a8* gene is the *Slc39a14* gene, encoding ZIP14; this transporter has also been demonstrated to be a Cd^{2+}/HCO_3^- symporter [25]. There was a recent study [26] indicating that ZIP14 mediates non-transferrin-bound Fe^{2+} into human HEK 293H cells, Sf9 insect cells, and AML12 mouse hepatocyte cultures. However, we did not find that Fe^{2+} competes for ZIP14-mediated Cd influx in MFF cultures [25].

Cu^{2+}, Pb^{2+} and Hg^{2+} were also significant inhibitors of Cd uptake by ZIP8. There were specific reasons for testing these three metals. Why Cu^{2+}? Whereas Zn was found to be the best competitive inhibitor of ZIP14-mediated Cd uptake, Cu^{2+} along with Mn^{2+} were tied for the second best inhibitor [25]. Why Hg^{2+} and Pb^{2+}? The ZIP8 protein has been demonstrated to be localized on the apical surface of renal proximal tubular epithelial cells, and to participate in Cd uptake and Cd-mediated renal failure [19]. Along with Cd—Hg and Pb are known to cause renal proximal tubular acidosis (human renal Fanconi syndrome); the only other metals known to cause this human disease are uranium and platinum [27].

Electrogenicity studies

No significant difference in Cd uptake was seen in water-injected *Xenopus* oocytes, comparing 2 mM with 20 mM K$^+$ in the incubation medium (Fig. 3A, *top*). Likewise, ZIP8-mediated Cd influx (Fig. 3A, *bottom*) was not statistically significantly different between 2 mM and 20 mM extracellular K$^+$. We also found no difference in Cd uptake by water-injected oocytes at 1 versus 60 mM K$^+$, nor did we find a difference in Cd uptake by ZIP8-injected oocytes at 1 versus 60 mM K$^+$ (data not illustrated). We believe the effects we saw at 1 mM K$^+$ and 60 mM K$^+$ are probably early signs of oocytes toxicity—caused by even as little as 30 min of incubation at such adverse K$^+$ concentrations.

For the membrane potential of a cell or a *Xenopus* oocyte, with regard to any cation,

$$E = E^{0'} + \frac{0.0591}{n} \log \frac{\left[K^+ \text{ cation outside cell} \right]}{\left[K^+ \text{ cation inside cell} \right]}$$

where **E** = electrode potential, **$E^{0'}$** = the standard electrode potential, **n** = number of electrons transferred in the half-reaction, and **0.0591** represents the universal gas constant **R** (8.31451 joules K^{-1} mol^{-1}) times **T** (temperature in °K) divided by the Faraday constant **F** (charge per one mole of electrons, which equals 9.6485309×10^4 coulombs mol^{-1}). Using this Nernst equation and knowing the electrogenic experiments were carried out at pH 7.5 and 22°C, we calculated the intracellular charge to be −132, −114, −53, and −24 mV at extracellular K$^+$ concentrations of 1, 2, 20 and 30 mM, respectively. Because Cd uptake is no more favorable with an electrochemical gradient of −53 mV instead of −114 mV (Fig. 3A), or even of the more extreme −132 mV instead of −24 mV (not shown), we are compelled to conclude that this complex is electroneutral, *i.e.* the cation/anion complex must include two HCO$_3^-$ anions and one Cd^{2+} cation. Therefore, the complex that moves through the ZIP8-microinjected *Xenopus* oocyte membranes (or, for that matter, any mammalian cell membranes) must be Cd^{2+}/[HCO$_3^-$]$_2$, *i.e.* an electroneutral complex that traverses the membrane no more efficiently when the electrochemical gradient is very negative such as −132 mV or −114 mV or much less negative such as −53 mV or even −24 mV. These findings are actually consistent with those described for ZIP-mediated Cd uptake in MFF cells: no differences in Cd uptake were observed at various extremes of extracellular K$^+$ concentrations [20].

If the rate of Cd uptake had been several-fold higher at −53 mV versus −114 mV, we would have concluded that the complex moving across the membrane is (Cd^{2+}/[HCO$_3^-$]$_3$)−, *i.e.* an electronegative complex. If the rate of Cd uptake had been several times lower at −53 mV than at −114 mV, we would have concluded that the complex moving across the membrane is [Cd^{2+}/HCO$_3^-$]$^+$, *i.e.* a positively-charged complex that moves across the membrane more readily when the electrochemical gradient is more negative such as −114 mV instead of −53 mV.

Just as with Cd, we found no statistically significant differences in Zn uptake in water-injected *Xenopus* oocytes (Fig. 3B, *top*), or in ZIP8-mediated Zn uptake (Fig. 3B, *bottom*), comparing 2 mM K$^+$ with 20 mM K$^+$ in the incubation medium. Likewise, no difference in ZIP8-mediated Zn influx was found at 1 mM versus 60 mM extracellular K$^+$ concentrations (not shown).

Evidence for "trafficking", dependent on extracellular Zn ion concentration

The ZIP4 transporter has previously been demonstrated to undergo trafficking—as a function of extracellular Zn concentrations [24]. Under Zn homeostasis, the majority of ZIP4 transmembrane-bound proteins was reported to be internalized; when the medium is depleted of Zn, a measurable increase was found in the amount of ZIP4 transmembrane-bound proteins on the cell surface [28-30]. Fig. 4 shows that we confirmed this effect on the ZIP4 transporter, and that the ZIP8 transporter also behaves similarly.

This trafficking mechanism has biological significance: under comfortable conditions when extracellular Zn concentrations are physiological, there is no need for excess ZIP protein to be poised on the cell surface; under conditions of Zn depletion and therefore cell stress, there is much greater need for the ZIP protein to move to the cell surface so that larger amounts of Zn can be transported into the cell, if possible. The mechanism, or the "signal", which determines where these membrane-bound ZIP transporters are located in the cell, is not well understood at this time [23]. We believe that ZIP trafficking might involve participation by the trans-Golgi network [31] and/or perhaps by phosphoinositides [32] in such cell regulation and membrane dynamics; clearly, movement of these eight-transmembrane transporter proteins from inside the cell, to the cell surface, and back again, is an intriguing phenomenon.

Concluding remarks

ZIP8 functions efficiently in ZIP8 cRNA-injected *Xenopus* oocytes, as shown by Cd and Zn uptake (Fig. 1). The most effective inhibitor of Cd influx was Zn (Fig. 2), which was not possible to demonstrate in MFF cultures [20]. Curiously, Mn—which was an effective competitor of Cd uptake in MFF cultures [20]—did not block Cd influx in *Xenopus* oocytes; reasons for this difference are not understood. Carrying out electrogenic experiments in *Xenopus* oocytes (Fig. 3), we demonstrate that ZIP8-mediated divalent cation movement across the membrane occurs as the $Cd^{2+}/[HCO_3^-]_2$ and $Zn^{2+}/[HCO_3^-]_2$ electroneutral complexes.

Finally, trafficking experiments in MDCK cultures (Fig. 4) show that the ZIP8 transporter is internalized during normal Zn homeostasis and is predominantly on the cell surface during Zn depletion. It would therefore logically follow that Cd influx into cells should be enhanced under conditions of hypozincemia. As stated above, it is well known that Cd uptake is increased in anemic and malnourished populations [1-4]; both anemia and poor nutrition are associated with inflammation and hypozincemia and, thus, this phenomenon of trafficking might help explain the observations as to why enhanced Cd uptake occurs under such disease conditions..

ZIP14 is evolutionarily most closely related, and is highly similar in function, to ZIP8 as compared with the other 12 family members [25]. ZIP8 and ZIP14 are Cd^{2+}/HCO_3^- symporters, and in epithelial as well as endothelial cells, both these symporters are localized on the apical surface [20;25]. Hence, we predict that ZIP14 will also transport $Cd^{2+}/[HCO_3^-]_2$ and $Zn^{2+}/[HCO_3^-]_2$ electroneutral complexes. As illustrated in Fig. 4, we also expect that ZIP14 will participate in the trafficking of Cd and Zn, as a function of extracellular metal concentration.

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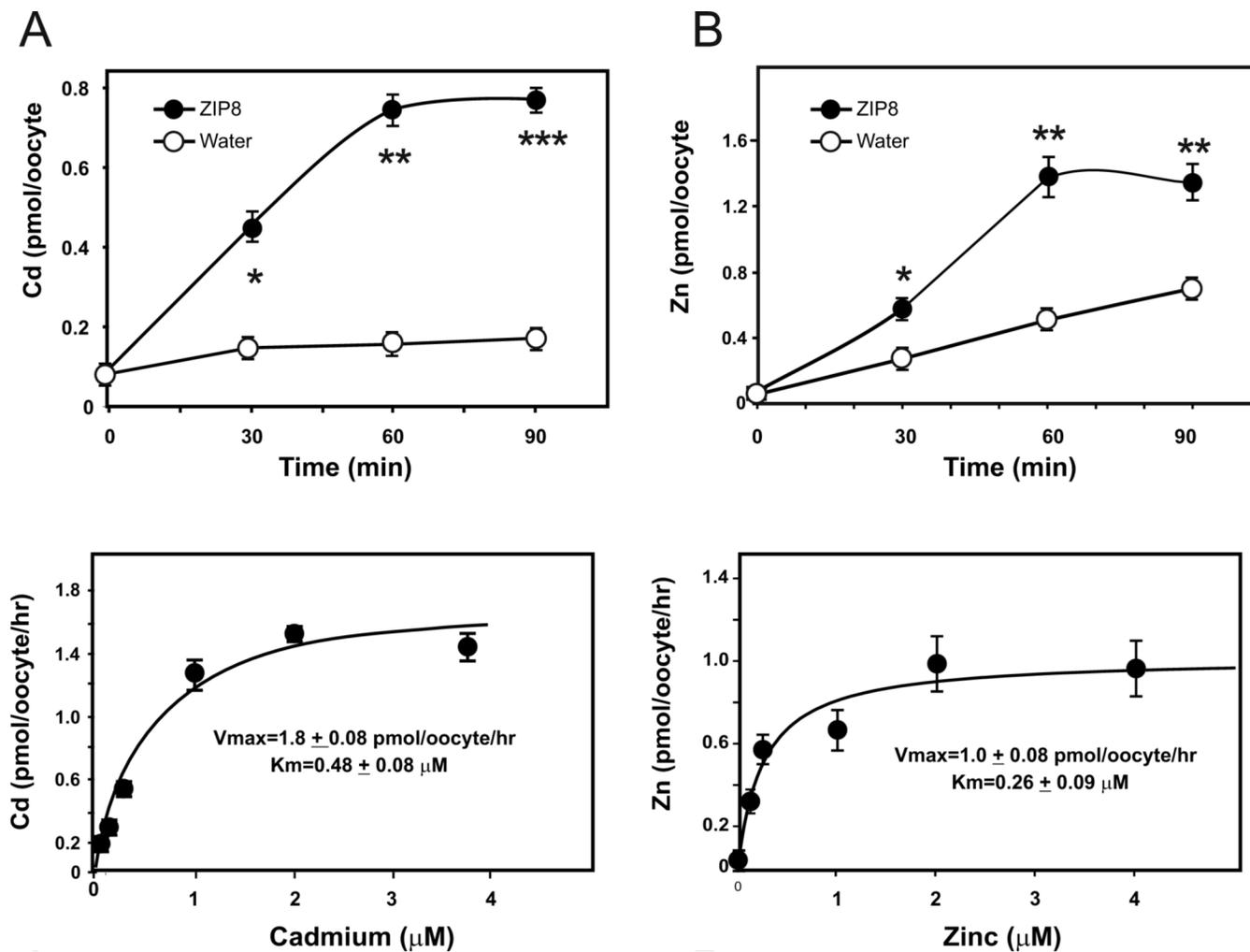
FIG. 1.
Kinetics of cation uptake in *Xenopus laevis* oocytes. *A, top*, Cd uptake as a function of time in ZIP8 cRNA-injected versus water-injected oocytes. *P <0.01. **P <0.001. At *bottom*, Cd uptake as a function of its concentration in ZIP8 cRNA-injected minus water-injected oocytes; incubation time was 60 min. *B, top*, Zn uptake in ZIP cRNA-injected versus water-injected oocytes. *P <0.05. **P <0.01. At *bottom*, Zn uptake, determined as described in part *A*. Circles and brackets at *top* denote means ± S.E. Vmax and Km values are expressed as means ± S.E. Circles and brackets at *bottom* denote means ± S.D.
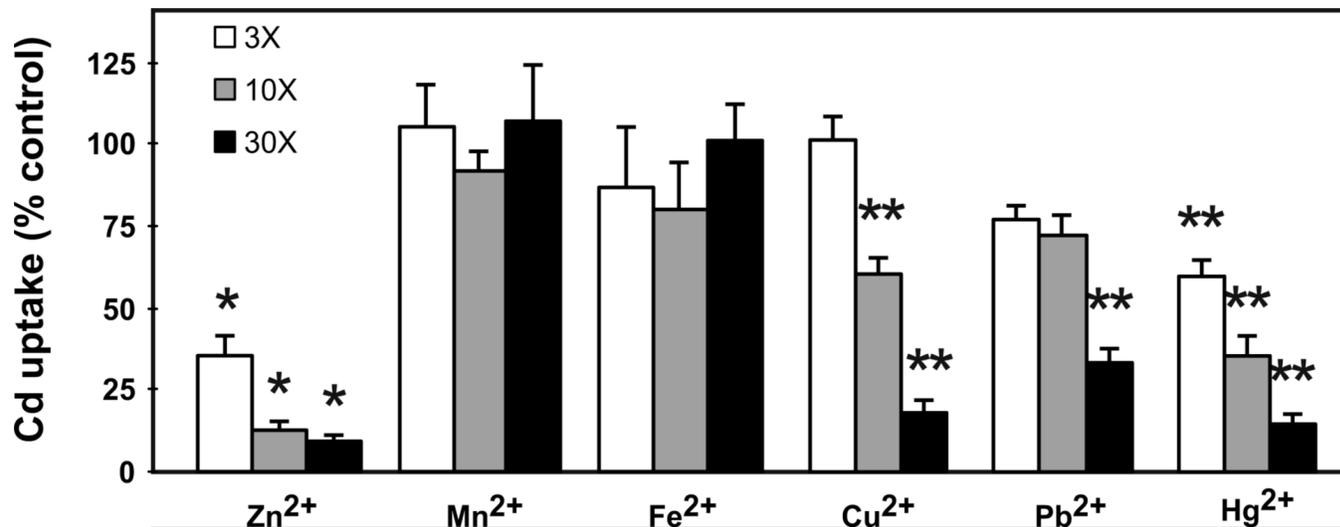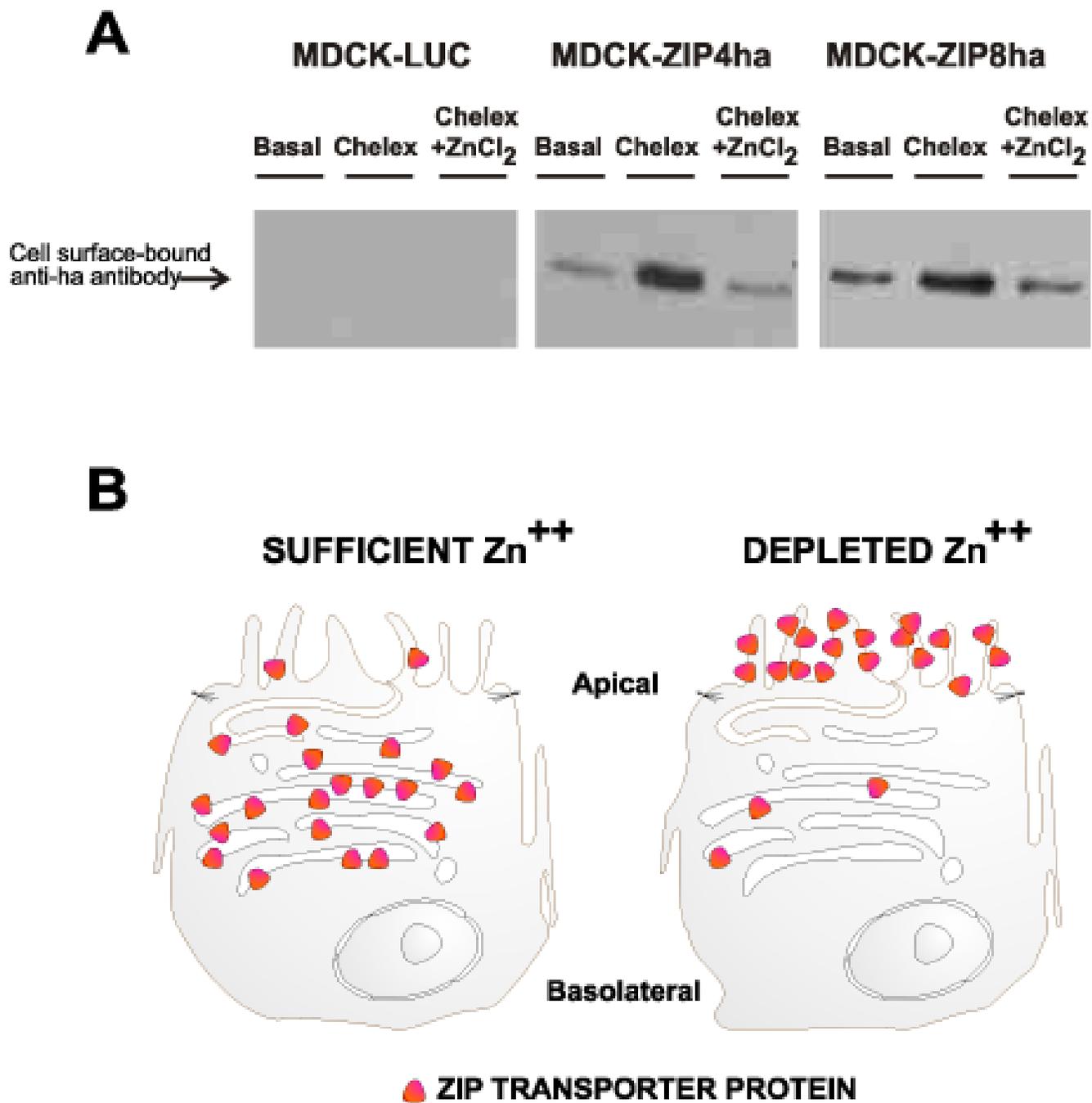
FIG. 2.
Metal cation competition for Cd uptake in *Xenopus* oocytes. Non-labeled Cd was spiked with $^{109}CdCl_2$ to make a final Cd concentration of 0.25 μM; the competing metal cations at concentrations of 0, 0.75, 2.5 or 7.5 μM were added at the same time as the Cd, and the oocytes were incubated at 20°C for 60 min, following which Cd accumulation was determined. *P <0.001. **$P < 0.01$).

A

B
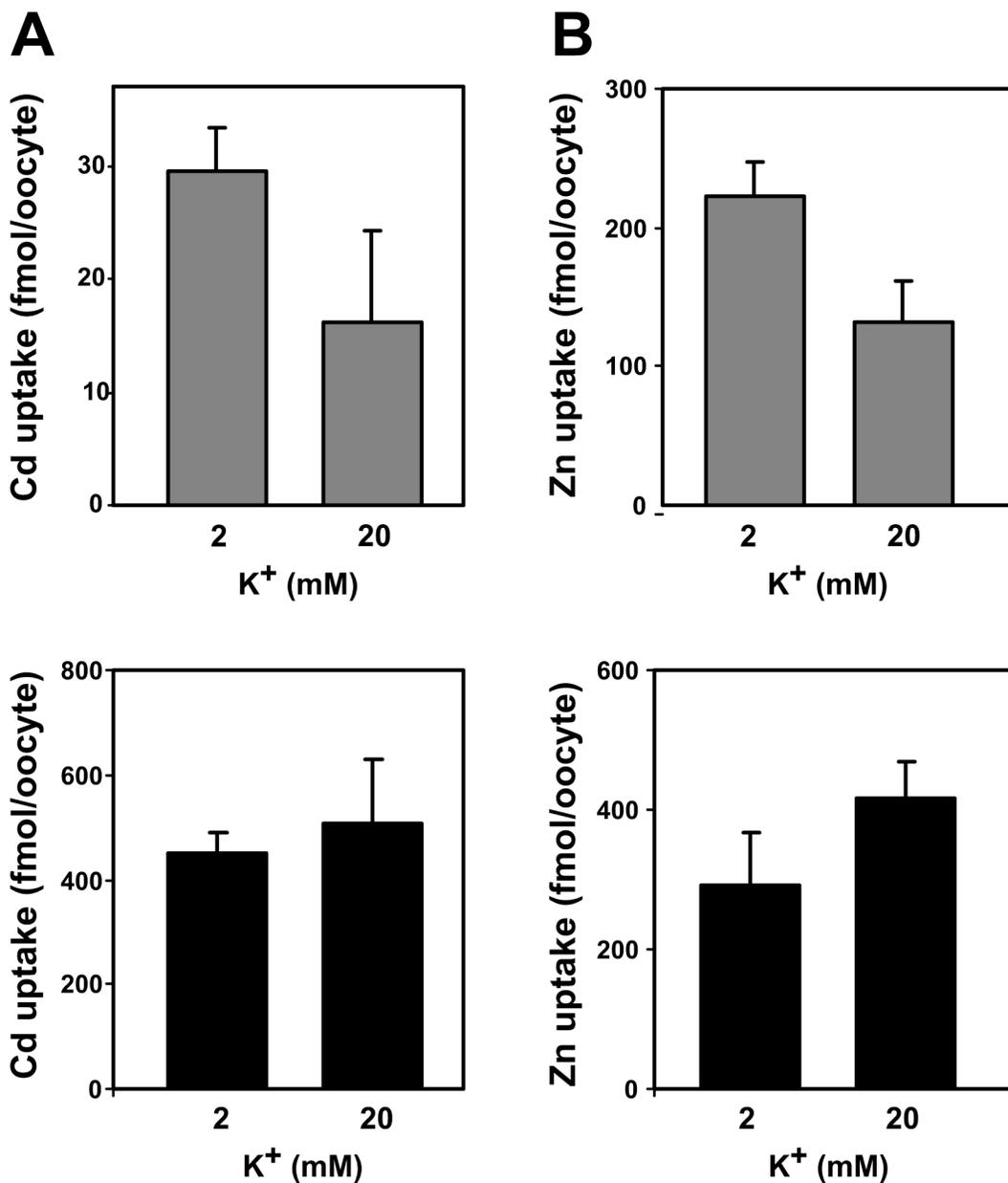
FIG. 3.
Electrogenicity studies. *A, top*, Cd (0.5 μM) uptake by water-injected oocytes. At *bottom*, Cd uptake by ZIP8 cRNA-injected, minus water-injected oocytes. *B,* Zn (1.0 μM) uptake by water-injected oocytes (*top*) and by ZIP8 cRNA-injected, minus water-injected oocytes (*bottom*). Note the ordinates for each of the four panels are different. Incubation time 30 min.

A

MDCK-LUC   MDCK-ZIP4ha   MDCK-ZIP8ha

| | Chelex | | | Chelex | | | Chelex |
| Basal | Chelex | +ZnCl$_2$ | Basal | Chelex | +ZnCl$_2$ | Basal | Chelex | +ZnCl$_2$ |

Cell surface-bound
anti-ha antibody⟶

B

SUFFICIENT Zn^{++} DEPLETED Zn^{++}

Apical

Basolateral

🔴 ZIP TRANSPORTER PROTEIN

FIG. 4.
A, Western immunoblot to detect the relative amounts of ZIP4ha versus ZIP8ha protein on the surface of MDCK cells, carried out identically to that previously described [24]. Equal loading was confirmed by Coomassie staining (not shown). **B,** Schematic diagram showing location of the membrane-bound ZIP8 transporter under normal Zn homeostasis (*left*), compared with that under low zinc conditions (*right*).