Potential mechanisms involved in the absorptive transport of cadmium in isolated perfused rabbit renal proximal tubules

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ABSTRACT

Lumen-to-cell transport, cellular accumulation, and toxicity of cadmium as ionic cadmium (Cd^{2+}) or as the L-cysteine (Cys) or D,L-homocysteine (Hcy) S-conjugate of cadmium (Cys-S-Cd-S-Cys, Hcy-S-Cd-S-Hcy) were studied in isolated, perfused rabbit proximal tubular segments. When Cd^{2+} (0.73 μM) or Cys-S-Cd-S-Cys (0.73 μM) was perfused through the lumen of S_2 segments of the proximal tubule, no visual evidence of cellular pathological changes was detected during 30 min of study. Cd^{2+}-transport was temperature-dependent and was inhibited by Fe^{2+}, Zn^{2+}, and elevated concentrations of Ca^{2+}. Luminal uptake of Cys-S-Cd-S-Cys was also temperature-dependent and was inhibited by the amino acids L-cystine and L-arginine, while stimulated by L-methionine. Neither L-aspartate, L-glutamate, the synthetic dipeptide, Gly-Sar nor Zn^{2+} had any effect on the rate of Cys-S-Cd-S-Cys transport. Conclusions: When delivered to the luminal compartment, Cd^{2+} appears to be capable of utilizing certain transporter(s) of Zn^{2+} and some transport systems sensitive to Ca^{2+} and Fe^{2+}. In addition, Cys-S-Cd-S-Cys and Hcy-S-Cd-S-Hcy appear to be transportable substrates of one or more amino acid transporters participating in luminal absorption of the amino acid L-cystine (such as system $b^{0,+}$). These findings indicate that multiple mechanisms could be involved in the luminal absorption of cadmium (Cd) in proximal tubular segments depending on its form. These findings provide a focus for future studies of Cd absorption in the proximal tubule.

1. Introduction

Cadmium (Cd) is a nephrotoxic heavy metal found in a number of occupational and environmental settings. After entering systemic circulation, Cd localizes primarily in the kidneys and the liver (Felley-Bosco and Diezi, 1987; Robinson et al., 1993; Zalups and Ahmad, 2003). At present, however, the mechanisms involved in the transport and handling of Cd in target cells are not well defined, especially in the kidneys. In order to focus and design future studies, this study was undertaken to eliminate or establish, if any of the known transport mechanisms in the luminal membrane of the proximal tubule could participate in the sequestering of Cd into the epithetical cells of this segment.

Notwithstanding this deficiency in our knowledge, several mechanisms for the uptake of Cd by renal tubular epithelial cells have been proposed. For example, it has been suggested that renal tubular uptake of Cd ions may involve transporters of the cationic species of the essential elements, such as Ca^{2+}, Fe^{2+} and Zn^{2+}. One transport-pathway implicated in the uptake of the divalent cationic form of Cd (Cd^{2+}) involves traversing Ca^{2+}-channels (Blazka and Shaikh, 1991; Zalups and Ahmad, 2003). It is well established that Cd^{2+} can serve as an effective Ca^{2+}-channel antagonist in excitable cells (Zalups and Ahmad, 2003). Even though both Cd^{2+} and Ca^{2+} have similar ionic radii, Cd^{2+} traverses Ca^{2+}-channels at much slower rates than Ca^{2+}, which is the functional basis of the antagonistic properties of Cd^{2+} in Ca^{2+}-channels.

Other transporters of divalent cations have also been implicated recently in the uptake of Cd^{2+} by selective epithelial cells in the kidneys, liver and intestines (Zalups and Ahmad, 2003). One such transporter is the divalent cation transporter 1 (DCT1), which is a proton-coupled transporter. In the kidneys, DCT1 has been localized in epithelial cells lining both proximal and distal segments of the nephron. Based on data from *Xenopus laevis* oocytes, it appears that DCT1 has a broad range of substrate specificity, with Fe^{2+}, Pb^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+} and perhaps Zn^{2+}, as potential transportable substrates (Gunshin et al., 1997; Okubo et al., 2003). Evidence for DCT1-mediated Cd^{2+}-uptake has been provided in an established renal epithelial cell line by Olivi et al. (2001), who used

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Madin–Darby canine kidney (MDCK) cells (which are derived from the distal nephron).

The localization of DCT1 in proximal tubular cells, however, is somewhat controversial. Using polyclonal antibodies, one group reported the presence of DCT1 in the apical membrane of proximal tubular cells (Canonne-Hergaux and Gros, 2002), while another group found evidence for DCT1 being present in the cytoplasm of proximal tubular cells only (Ferguson et al., 2001). Therefore, the role of DCT1 in Cd^{2+} transport along the proximal tubule remains uncertain.

A protective effect of zinc ions (Zn^{2+}) against the toxic effects of Cd^{2+} has been demonstrated in rat hepatocytes and porcine proximal tubular epithelial (LLC-PK(1)) cells (Ishido et al., 1999; Jacquillet et al., 2006). This protective effect may be related to influences on the activity of the selective Zn^{2+}-transporters. Possible Zn^{2+}-transporters involved, include the ZnT-like transporter 1 (ZTL1), which has been identified in the kidney (Cragg et al., 2002), and/or Zrt-and Irt-related proteins 8 and 14 (ZIP8 and ZIP14), which are also present on the apical membrane of the proximal tubule, and have been implicated in the transport of Cd^{2+} (Girijashanker et al., 2008; He et al., 2006; Wang et al., 2007).

Although Cd^{2+} can be taken up at the luminal membrane of proximal tubular cells under certain *in vitro* and *in vivo* conditions (Felley-Bosco and Diezi, 1987; Robinson et al., 1993; Zalups, 2000), it is highly unlikely that the Cd^{2+} present in blood (after exposure to a source of Cd) is filtered and delivered to the luminal membrane of proximal tubules in an unbound, ionic state. Due to its high affinity for reduced sulfur atoms, Cd^{2+} in blood is most likely bound to thiol-containing molecules, such as the amino acids L-cysteine (Cys), L-homocysteine (Hcy), *N*-acetylcysteine (NAC), peptides such as glutathione, or proteins such as albumin. Low-molecular-weight thiol *S*-conjugates of Cd have been hypothesized to be transportable species of Cd along the proximal tubule. Moreover, these conjugates are hypothesized to act as molecular homologues or mimics of L-cystine (Cys-S-S-Cys) and/or L-homocystine, which may allow them to be transported by constituent transporters of L-cystine and/or L-homocystine present in the plasma membrane of proximal tubular cells (Zalups, 2000).

Specific amino acid transporters have been implicated recently in the absorptive transport of L-Cys and L-Hcy *S*-conjugates of inorganic mercury (e.g., Cys-*S*-Hg-*S*-Cys) in proximal tubular cells (Bridges et al., 2004; Bridges and Zalups, 2004; Cannon et al., 2001). Since both Cd and Hg are group IIB metals and both have a high binding affinity for sulfhydryl groups, it is possible that low-molecular-weight thiol *S*-conjugates of Cd^{2+} utilize one or more of the same transporters involved in the absorptive transport of low-molecular-weight thiol *S*-conjugates of Hg^{2+}.

In the present study, we first examined the effects of an excess of Ca^{2+}, Fe^{2+} or Zn^{2+} on the luminal uptake of Cd^{2+} in an attempt to implicate one or more divalent cation transporter(s) in the luminal uptake of Cd as Cd^{2+} in isolated perfused renal proximal tubular segments. The primary rationale for performing this aspect of our study is that we believe that Cd^{2+} can compete for specific transporters of the essentials elements Fe^{2+}, Zn^{2+}, and/or Ca^{2+} in the luminal membrane of proximal tubular segments. The basis of these assumptions comes from work performed in other systems (Barbier et al., 2004; Okubo et al., 2003; Olivi et al., 2001).

In a second series of experiments, we tested the hypothesis that when Cd^{2+} is delivered into the luminal compartment of proximal tubular epithelial cells as linear II coordinate covalent, Cys or Hcy *S*-conjugates, Cd can enter into proximal tubular epithelial cells by a mechanism of molecular mimicry at the site of certain amino acid carriers (such as system $b^{0,+}$) and/or dipeptide carriers (such as PepT2).

The isolated perfused tubule technique used in this study afforded us the most physiologically relevant conditions encoun-

tered by native proximal tubular epithelial cells for an intact non-cultured (primary) tubular epithelium (Schafer and Barfuss, 1980; Cannon et al., 2001).

2. Materials and methods

2.1. Animals

Female New Zealand rabbits (1–2 kg) were used in the present study. All animals were allowed at least two days of acclimation prior to any experimentation. Water and a commercial laboratory diet for rabbits were provided *ad libitum* during all phases of the study.

2.2. Composition of bathing and perfusing solutions

In all experiments, the solution bathing the perfused tubular segments consisted of a simple electrolyte solution. This bathing solution contained the following: 140 mM Na^+, 147.6 mM Cl^-, 5 mM K^+, 1.3 mM Ca^{2+}, 0.6 mM Mg^{2+}, 0.6 mM $SO_4{}^{2-}$, 2 mM NaH_2PO_4, 1 mM D-glucose, and 0.5 mM L-glutamine. The pH was adjusted to 7.4 by the addition of 1N NaOH. Final osmolality was adjusted to 290 mOsmol/kg of H_2O by the addition of either doubly distilled and deionized water or NaCl. To evaluate the net absorption of Cd or thiol conjugates of Cd, $^{109}Cd^{2+}$ (0.588 Ci/mg, GE Healthcare, Life Sciences, Amersham, USA) was added to the perfusate. The vital dye FD&C Green 3 (809 Da) was placed in the perfusate at a concentration of 250 nM to visually determine any toxic effects of the Cd^{2+} or Cd-conjugates. L-[^{3}H]-glucose (14.6 Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) was added to the perfusing solution as a volume marker and a leak indicator. The perfusate (perfusing solution) was identical to the bathing solution except that the 2 mM NaH_2PO_4 was replaced by 2 mM HEPES because it was found that $HPO_4{}^-$ or $HPO_4{}^{2-}$ precipitated $^{109}Cd^{2+}$. To investigate Cd luminal transport, 0.73 μM Cd was used in all experiments. This concentration was the lowest permissible concentration that could be used due to the low specific activity of the ^{109}Cd and it did not induce any visible functional toxicity. For experiments designed to determine if Fe^{2+} competes with Cd^{2+} at certain transporters, 10 μM $FeCl_2$ along with 100 μM ascorbic acid (to prevent oxidation of Fe^{2+}) were added to the perfusate (Olivi et al., 2001). In addition, this latter perfusate was adjusted to a pH of 6.8 to assure maximum DCT1 activity. For all other experiments Zn^{2+}, Ca^{2+}, L-cystine, L-Hcy, L-arginine, L-glutamate, L-aspartate, or Gly-Sar were added to the standard HEPES perfusion solution. To insure that all potential inhibitors would result in some degree of inhibition, if they were inhibitors, the ratio of all inhibitors to Cd was 10:1 except Zn^{2+}, which was 20:1. For experiments designed to investigate the luminal transport of thiol *S*-conjugates of Cd, a fresh perfusion fluid was made with a 2.1:1 ratio of the thiol-containing molecule (L-Cys or L-Hcy) to cadmium ion (Cd^{2+}). The 2.1:1 ratio of thiol to Cd^{2+} was used to ensure the formation of thiol *S*-conjugates of Cd consisting of a cadmium ion presumably binding to two molecules of the thiol-containing molecule in a linear II coordinate covalent manner (X-S-Cd-S-X). All perfusates were made fresh on the day of experiments.

2.3. Tubular dissection solution

The tubular dissection solution was a sucrose/phosphate buffer containing 125 mM sucrose, 13.3 mM anhydrous monosodium dihydrogen phosphate (NaH_2PO_4), and 56 mM anhydrous disodium monohydrogen phosphate (Na_2HPO_4). The pH was adjusted to 7.4 by the addition of either 1N NaOH or HCl. The osmolality was adjusted to 290 mOsm/Kg of water by the addition of either water or NaCl.

2.4. Procedure for obtaining segments of proximal tubules

On each day of experimentation, a rabbit was anesthetized with a combination of 33 mg/kg ketamine (Fort Dodge Animal Health, Livestock Division, Overland Park, KS, USA) and 33 mg/kg xylazine (LLOYD Laboratories, Shenandoah, IA, USA). After each rabbit had been anesthetized deeply (as determined by the corneal reflex) the abdomen was opened and the kidneys were removed rapidly. The kidneys were then placed in the cold (4 °C) aqueous sucrose–phosphate buffer solution (dissection solution). Once in solution, the kidneys were sliced quickly into 1–2 mm thick coronal sections using a single-edge razor blade. The sections were stored in the same sucrose–phosphate buffer solution on ice for up to next 8 h. The S_1, S_2, and S_3 segments of the proximal tubule were dissected from the coronal sections. The S_1 segments were identified as the larger diameter convoluted tubules dissected from the outer cortical regions of the kidney slice. The S_2 segments were identified as straight portions of the proximal tubule spanning the entire thickness of the cortex, while the S_3 segments were identified as the last 1 mm of the proximal straight tubule that was attached to the easily identifiable thin descending limb of Henle's loop and located in the outer stripe of the outer medulla.

2.5. Method for perfusing segments of proximal tubules

Each dissected tubule was transferred to a Lucite perfusion chamber and was suspended between two sets of pipettes. One set of pipettes was used to perfuse the suspended tubule, whereas the other set was used to collect the perfused fluid.

Each tubular segment was warmed from room temperature to 37 °C over 15 min prior to the beginning an experiment. The perfusion rate was maintained, on average, at 7–10 nl min^{-1}, with constant hydrostatic pressure. Because of differences in tip diameters of the perfusion pipettes used in the present study, the hydrostatic pressure needed to perfuse the segments of proximal tubules at 7–10 nl min^{-1} varied between 15 and 50 mmHg. Each perfused tubule was monitored for any changes in tubular diameter resulting from abnormally high intraluminal pressures. The perfused fluid (perfusate) was collected from the lumen into a constant-volume pipette (designed to accurately collect 30–50 nl). The bathing fluid surrounding the outside basolateral surface of the perfused tubule was pumped into the bathing chamber at a rate of approximately 0.3 ml min^{-1} and was aspirated continually and collected into a scintillation vial at 5-min intervals. The volume of the perfusion chamber was approximately 0.3 ml, thus the bathing solution was exchanged about once per minute.

2.6. Collection of samples

Three collectate (fluid exiting the perfused tubular segment) samples and three corresponding bathing solution samples were collected for each perfused tubule to measure the rates of lumen-to-cell flux of ^{109}Cd and the appearance of the volume marker (^{3}H-L-glucose) in the bathing solution. A constant-volume pipette was used to collect collectate samples, which were immediately added to 4 ml of scintillation fluid. The bathing solution samples were routinely collected (5 min intervals) in 8-ml scintillation vials that were configured with a vacuum trap. To each vial, 4 ml of scintillation fluid (Opti-Fluor; Packard Instrument Company, Downers Grove, IL) was added. The collectate and bathing fluid samples were then counted in a Beckman 5800 scintillation counter (Beckman Instruments, Fullerton, CA) to quantify the amount of ^{109}Cd and ^{3}H present in each sample using standard isotopic separation methods.

2.7. Harvesting of perfused tubular segments

To calculate the cellular content of Cd in the isolated perfused segments of proximal tubules, it was necessary to harvest the perfused tubule at the end of each experiment. The tubular segment was quickly harvested (<1 s) with the aid of a pair of fine forceps while it was being perfused. The tubule then was removed from the bathing solution and placed in 10 μl of 3% trichloroacetic acid (TCA). The TCA precipitated the larger proteins leaving the tubule opaque-white and rigid while releasing the cytosolic contents into the TCA solution. After a few minutes, the tubular segment (TCA-precipitable fraction) was removed from the TCA solution, placed into a vial with 4 ml of scintillation fluid and later analysed by scintillation counting with standard isotopic methods for the contents of ^{109}Cd and the volume marker, [^{3}H]-L-glucose. Like wise the TCA solution (TCA-soluble fraction, presumably cytosolic content) data was obtained. The TCA-soluble fraction permitted the approximate calculation of the cellular concentration of ^{109}Cd.

2.8. Calculations

Lumen-to-cell flux and cellular content values were calculated using the same equations previously described (Zalups and Barfuss, 1993).

2.9. Statistical analyses

A minimum of five tubules were perfused under each experimental condition, three samples per tubule. Moreover, data for each variable assessed was obtained from tubular segments isolated from at least two animals. For each perfused tubule, the three J_D measurements of ^{109}Cd were averaged. These averaged values for J_D were used to compute the overall mean and standard error of the mean for each experimental condition. The same analysis sequence was used for the cellular concentrations.

Each set of data was first analysed with the Smirnov–Kolmogorov's test for normality and Levene's test for homogeneity of variance to prove that the data met the conditions for performing parametric statistical analyses. A one-way analysis of variance (ANOVA) was first applied to all relevant data. When the statistically significant ($P < 0.05$) F-values were obtained with the ANOVA, all logical pairs of means were assessed to determine which means were statistically different ($P < 0.05$) from the others. Values were assumed to be significantly different at $P < 0.05$. All values expressed graphically represent the percent of the relevant control group ± SE.

3. Results

3.1. Effect of Fe^{2+} on the lumen-to-cell transport of Cd^{2+}

To determine if Fe^{2+} has an effect on the transport of Cd^{2+}, 10 μM FeCl$_2$ along with 100 μM ascorbic acid (to prevent oxidation of Fe^{2+}) was co-perfused with 0.73 μM ^{109}CdCl$_2$ (Fig. 1). The disappearance flux (J_D) and cellular concentration of ^{109}Cd were 0.60 ± 0.04 fmol min^{-1} (mm tubular length)$^{-1}$ and
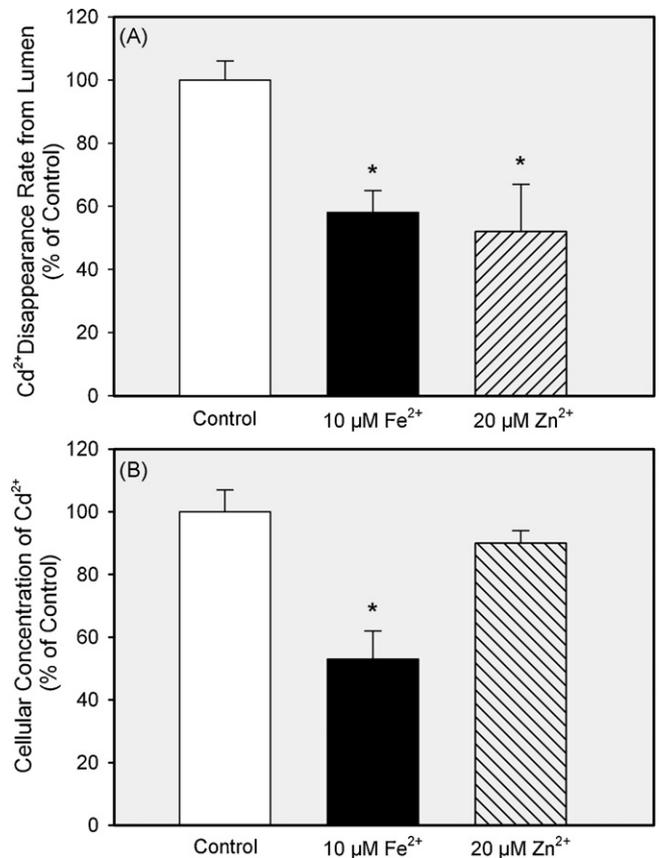
Fig. 1. The effect of 10 μM Fe^{2+} and 20 μM Zn^{2+} on the luminal disappearance rate (A) and cellular concentration (B) of Cd^{2+} in isolated S$_2$ segments of the proximal tubule of the rabbit perfused with 0.73 μM Cd^{2+} (at 37 °C). Each value represents the mean ± S.E.M. for a sample size of five or six. The "*" indicates a significant difference ($P < 0.05$) from control S$_2$ segments.

12.78 ± 1.11 μM, respectively. Comparative (control) studies were performed with 100 μM ascorbic acid and ^{109}CdCl$_2$ alone and no Fe^{2+}. The disappearance flux (J_D) and cellular concentration of ^{109}Cd were 1.04 ± 0.06 fmol min^{-1} (mm tubular length)$^{-1}$ and 24.10 ± 1.58 μM, respectively. Compared to the control group, the presence of Fe^{2+} significantly reduced the J_D of Cd^{2+} by 42%, and cellular concentration of Cd^{2+} by 47%.

3.2. Effect of Zn^{2+} on the lumen-to-cell transport of Cd^{2+}

To investigate whether Zn^{2+} can affect absorption of Cd^{2+}, 20 μM ZnCl$_2$ was added to the perfusate with 0.73 μM ^{109}CdCl$_2$. The J_D of Cd^{2+} in the presence of Zn^{2+} is 1.62 ± 0.24 fmol min^{-1} (mm tubular length)$^{-1}$, and the cellular concentration was 125.60 ± 5.50 μM. The J_D of Cd^{2+} in the control group is 3.13 ± 0.14 fmol min^{-1} (mm tubular length)$^{-1}$, and the cellular concentration was 139.14 ± 11.72 μM. Compared to the control group, Zn^{2+} significantly decreased the J_D of Cd^{2+} by 48%, whereas the cellular concentration was not significantly changed (Fig. 1).

3.3. Effect of Ca^{2+} on the lumen-to-cell transport of Cd^{2+}

To determine whether Ca^{2+} and Cd^{2+} shared any transport mechanisms for entry into the renal epithelial cells at the luminal membrane, the concentration of Ca^{2+} in perfusate was adjusted from 1.3 (control, normal level) to 1.95 mM or 2.6 mM. The J_Ds at these concentrations were 3.13 ± 0.14, 2.28 ± 0.50 and 0.98 ± 0.17 fmol min^{-1} (mm tubular length)$^{-1}$ (Fig. 2). The presence
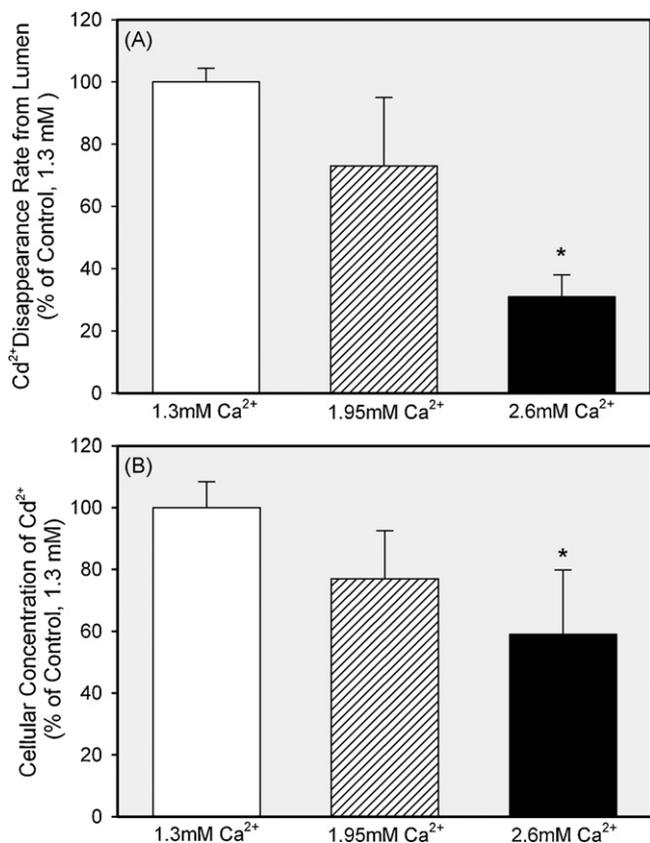
Fig. 2. The effect of increasing concentrations of Ca^{2+} on the luminal disappearance rate (A) and cellular concentration (B) of Cd^{2+} in isolated S_2 segment of the proximal tubule of the rabbit perfused with 0.73 μM Cd^{2+} (at 37 °C). Each value represents the mean ± S.E.M. for a sample size of five or six. The "*" indicates a significant difference ($P < 0.05$) from control S_2 segments.

of high concentrations of Ca^{2+} in the perfusate decreased the J_D of Cd^{2+} (27% reduction at 1.95 mM and 69% at 2.6 mM). The cellular concentrations of Cd^{2+} at these three concentrations were 139.14 ± 11.72, 106.46 ± 16.48 and 82.24 ± 17.11 μM, respectively. High concentrations of Ca^{2+} in the perfusate decreased the cellular concentration of Cd^{2+} (23.5% reduction at 1.95 mM and 40.9% at 2.6 mM). When the concentration of Ca^{2+} in perfusate was adjusted to be less than that in control perfusate (1.3 mM), the tubules expressed an acute visual toxicity, such as blebbing of the luminal membrane and cellular swelling (data not shown), consequently, transport data was not reliable.

3.4. Effect of L-cystine and L-arginine on lumen-to-cell transport of the L-cystine S-conjugate of Cd^{2+}

Fig. 3 shows that 10 μM L-cystine (Cannon et al., 2001) reduced the J_D of Cd-Cys conjugate (Cys-S-Cd-S-Cys, 0.73 μM) by about 55%, from 2.92 ± 0.31 to 1.31 ± 0.10 fmol min^{-1} (mm tubular length)$^{-1}$, and the cellular concentration by about 57.68% from 159.98 ± 16.76 to 67.70 ± 6.04 μM. 10 μM L-arginine also reduced the J_D of Cys-S-Cd-S-Cys by about 48.6%, from 2.92 ± 0.31 to 1.50 ± 0.15 fmol min^{-1} (mm tubular length)$^{-1}$, but the cellular concentration was not changed significantly.

3.5. Effect of various compounds on the lumen-to-cell transport of the L-cys S-conjugate of Cd

10 μM L-aspartic acid and 10 μM L-glutamate had no effect on the transport of Cys-S-Cd-S-Cys (Fig. 3). When 0.73 μM Cys-S-Cd-S-Cys was co-perfused with 10 μM L-methionine, the

J_D of Cys-S-Cd-S-Cys (0.73 μM) was increased by about 56%, from 0.93 ± 0.11 to 1.46 ± 0.14 fmol min^{-1} (mm tubular length)$^{-1}$, and the cellular concentration was increased by 58.28%, from 29.77 ± 3.45 to 47.12 ± 6.94 μM (Fig. 3). When 10 μM Gly-Sar was co-perfused with 0.73 μM Cys-S-Cd-S-Cys, both the J_D and the cellular concentration of Cys-S-Cd-S-Cys were not significantly changed (Fig. 3). In addition, when 20 μM Zn^{2+} was co-perfused with 0.73 μM Cys-S-Cd-S-Cys there was no significant change in either the J_D or the cellular concentration of Cys-S-Cd-S-Cys (Fig. 3).

### 3.6. Effect of L-cystine on the lumen-to-cell transport of Hcy S-conjugate of Cd^{2+}

When 10 μM L-cystine was added to the perfusate (Cannon et al., 2001), it reduced the J_D of the Hcy S-conjugate of Cd^{2+} (Hcy-S-Cd-S-Hcy, 0.73 μM) by about 58%, from 1.59 ± 0.21 to 0.66 ± 0.11 fmol min^{-1} (mm tubular length)$^{-1}$, and the cellular concentration by 41.16%, from 94.9 ± 7.35 to 55.84 ± 9.66 μM (Fig. 4).

3.7. Effect of temperature on the transport of Cd^{2+}

To assess whether the transport of Cd^{2+} is modulated by temperature, the S_2 segments of the proximal tubules were perfused with Cd^{2+} (0.73 μM) at 37, 22 and 11 °C (Fig. 5). The J_D of Cd^{2+} at these temperatures were 3.13 ± 0.14, 0.41 ± 0.11 and 0 fmol min^{-1} (mm tubular length)$^{-1}$. The corresponding cellular concentrations of Cd^{2+} were 139.14 ± 11.72, 52.23 ± 3.81 and
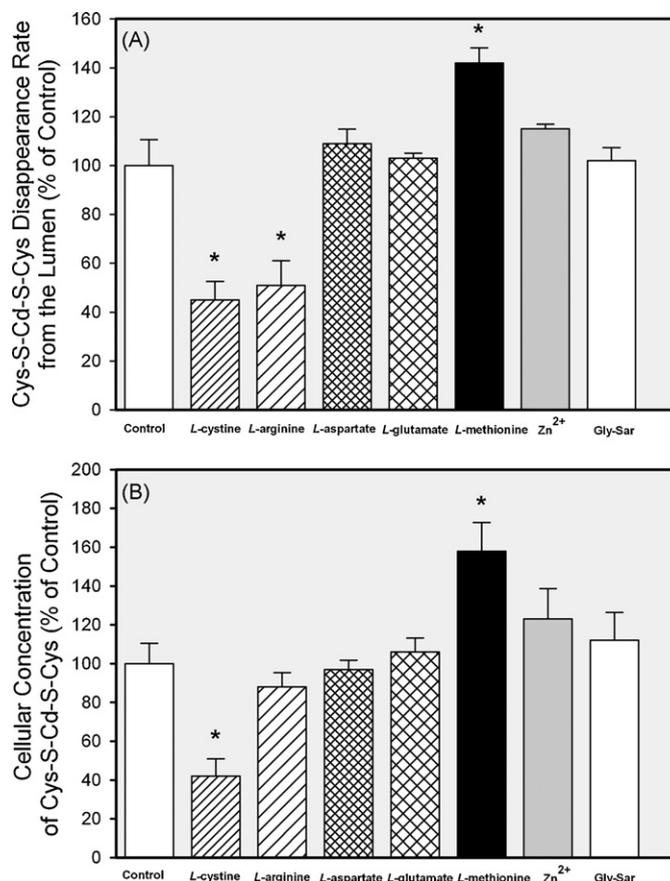
Fig. 3. The effect of 10 μM L-cystine, 10 μM L-arginine, 10 μM L-aspartate, 10 μM L-glutamate, 10 μM L-methionine, 20 μM Zn^{2+} and 10 μM Gly-Sar on the luminal disappearance rate (A) and cellular concentration (B) of Cd^{2+} in isolated S_2 segments of the proximal tubule perfused through the lumen with 0.73 μM Cys-S-Cd-S-Cys (at 37 °C). Each value represents the mean ± S.E.M. for a sample size of five or six. The "*" indicates a significant difference ($P < 0.05$) from control S_2 segments.
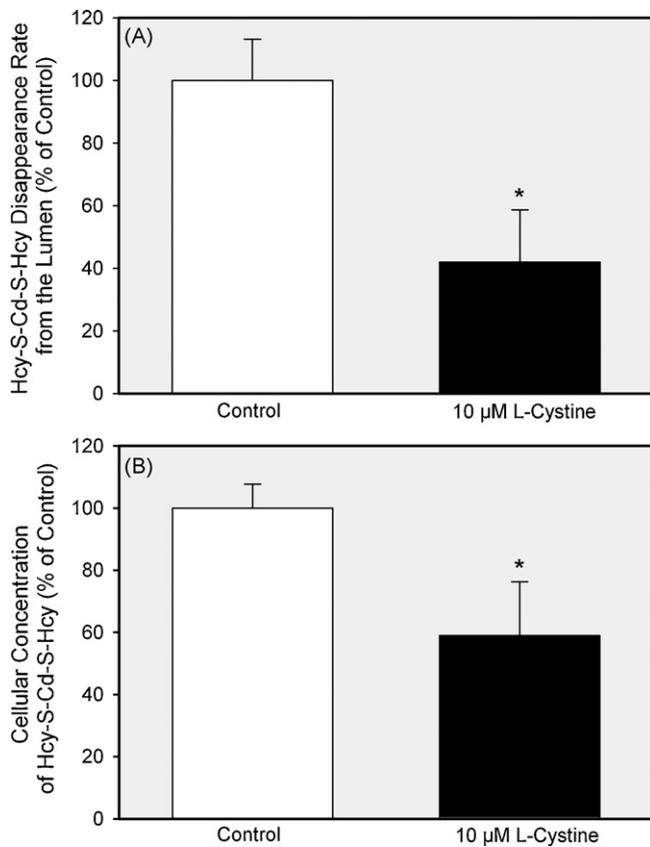
Fig. 4. The effect of 10 μM L-cystine on the luminal disappearance rate (A) and cellular concentration (B) of Cd^{2+} in isolated S_2 segments of the proximal tubule perfused through the lumen with 0.73 μM Hcy-S-Cd-S-Hcy (at 37 °C). Each value represents the mean ± S.E.M. for a sample size of five or six. The "*" indicates a significant difference ($P < 0.05$) from control S_2 segments.

8.52 ± 0.64 μM. Significant decreases in the J_D (87% reduction at 22 °C and 100% at 11 °C) and cellular concentration (62.5% reduction at 22 °C and 93.9% at 11 °C) of Cd^{2+} were observed in response to these reductions in temperature. In addition, the measured leak rate of the volume marker, presumable via the tight junction, was not altered with the changes of temperature (data not shown).

3.8. Effect of temperature on the transport of L-cys S-conjugate of Cd^{2+}

To assess whether the transport of Cys conjugate of Cd is modulated by temperature, the S_2 segments of the proximal tubules were perfused with 0.73 μM Cys-S-Cd-S-Cys at 37, 22 and 11 °C. The J_Ds of Cys-S-Cd-S-Cys at these temperatures were 2.92 ± 0.31, 0.70 ± 0.16 and 0 fmol min^{-1} (mm tubular length)$^{-1}$ while the corresponding cellular concentrations of Cys-S-Cd-S-Cys were 159 ± 16.8, 30 ± 5.01 and 8.16 ± 1.73 μM. Significant decreases in the J_D (76.0% reduction at 22 °C and 100% at 11 °C) and cellular concentrations (81.28% reduction at 22 °C and 94.90% at 11 °C) of Cys-S-Cd-S-Cys were observed at the reduction in temperature (Fig. 5). Additionally, the measured leak rate of the volume marker, presumable via the tight junction, was not altered with the changes of temperature (data not shown).

3.9. Comparison of transport of the L-cys S-conjugate of Cd^{2+} among S_1, S_2 and S_3 segments of the proximal tubule

Perfusate containing 0.73 μM Cys-S-Cd-S-Cys was perfused through the lumen of S_1, S_2, and S_3 proximal tubular

segments. In S_1 segments, the J_D of Cys-S-Cd-S-Cys was 1.23 ± 0.08 fmol min^{-1} (mm tubular length)$^{-1}$ and the cellular concentration was 35.72 ± 5.0 μM. In S_2 segments, the J_D of Cys-S-Cd-S-Cys was 2.92 ± 0.31 fmol min^{-1} (mm tubular length)$^{-1}$ while the cellular concentration was 159.98 ± 16.76 μM. In S_3 segments, the J_D of Cys-S-Cd-S-Cys was 3.09 ± 0.25 fmol min^{-1} (mm tubular length)$^{-1}$ while the cellular concentration was 95.31 ± 6.76 μM. The J_D of Cys-S-Cd-S-Cys in S_2 segments was comparable to that of the S_3 segments, but significantly more than that in S_1 segments (Fig. 6).

3.10. Comparison of L-cystine transport among S_1, S_2 and S_3 segments of the proximal tubule

Perfusate containing 20 μM ^{14}C-L-cystine was perfused through the lumen of S_1, S_2, and S_3 proximal tubular segments. In S_1 segments, the J_D of was 112 ± 3.8 fmol min^{-1} (mm tubular length)$^{-1}$ while the cellular concentration was 759 ± 52.8 μM. In S_2 segments, the J_D of was 138 ± 4.0 fmol min^{-1} (mm tubular length)$^{-1}$ while the cellular concentration was 2802 ± 201 μM. In S_3 segments, the J_D of L-cystine was 248 ± 14.7 fmol min^{-1} (mm tubular length)$^{-1}$ while the cellular concentration was 1945 ± 247 μM. The J_D of L-cystine transport showed axial progression, increasing from the S_1 to S_3 segment (Fig. 6).
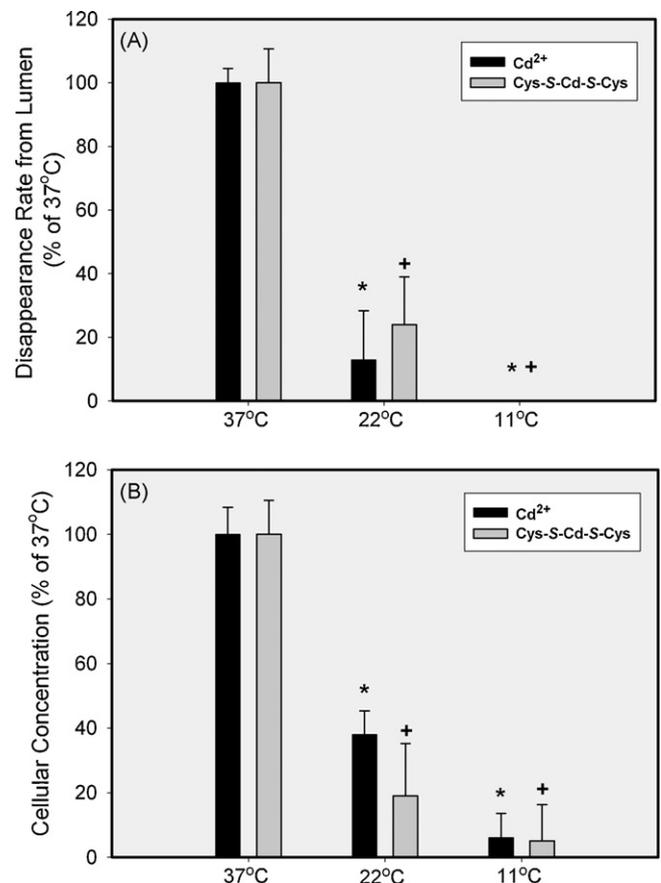
Fig. 5. The effect of temperature on the luminal disappearance rate (A) and cellular concentration (B) of Cd^{2+} and Cys-S-Cd-S-Cys in isolated S_2 segments of the proximal tubule of the rabbit perfused at 0.73 μM for each solute. Each value represents the mean ± S.E.M. for a sample size of five or six. The "*" and "+" indicate significantly different ($P < 0.05$) from the respective means obtained from the S_2 segments perfused at 37 °C.
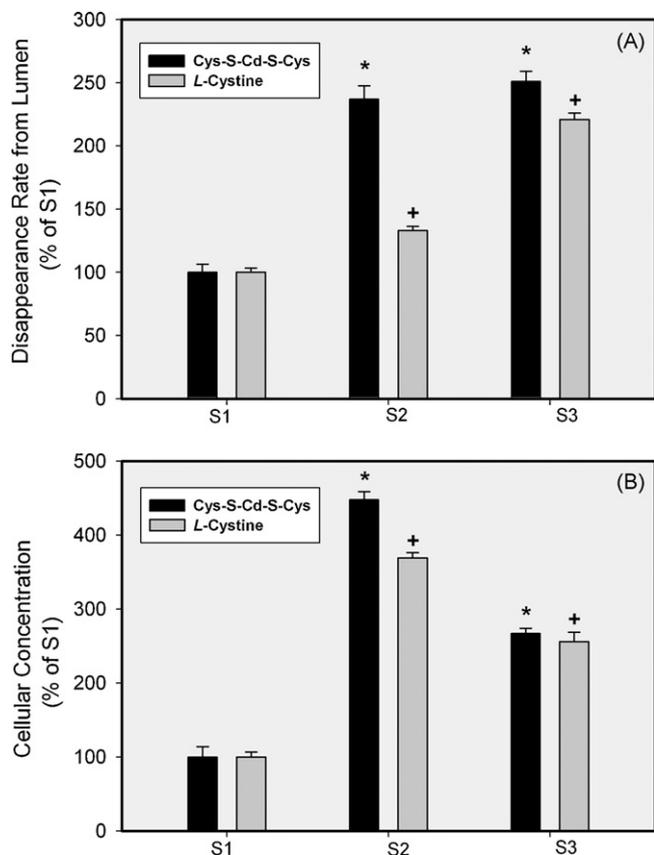
Fig. 6. Rate of luminal disappearance (A) and cellular concentration (B) of Cys-*S*-Cd-*S*-Cys and L-cystine in S_1, S_2, and S_3 segments of the proximal tubule of the rabbit. Each value represents the mean ± S.E.M. for a sample size of five or six. The "*" indicates statistically different ($P < 0.05$) from the companion group of Cys-*S*-Cd-*S*-Cys. The "+" indicates statistically different ($P < 0.05$) from the companion group of L-cystine.

3.11. Acute cellular toxicity of Cd^{2+} and thiol S-conjugates of Cd

Both Cd^{2+} and Cd-sulfhydryl conjugates were transported into the epithelial cells, which resulted in substantial Cd accumulation in the cytosol of the epithelial cells as shown by the cellular concentration in all experiments when perfusate Cd^{2+} concentration was 0.73 μM. No visual evidence of acute cellular toxicity such as cellular swelling, blebbing of the luminal membrane, and cellular vital dye uptake, was noted in S_2 segments. However, perfused with 20 μM Cd^{2+} there was slight cellular swelling at the perfusion end of the tubule for approximately 40 μm along the tubule but there was no vital dye uptake and only an occasional bleb from the luminal membrane of this affected region (data not shown).

4. Discussion

Based on data from previous studies (Barbier et al., 2004; Blazka and Shaikh, 1991; Gunshin et al., 1997; Ishido et al., 1999; Okubo et al., 2003; Zalups and Ahmad, 2003), it appears Cd^{2+} can gain entry into proximal tubular cells, from the luminal compartment, via transport systems involved in the absorptive transport of certain essential elements. Our data tend to implicate transporters of, or sensitive to, Ca^{2+}, Fe^{2+}, and Zn^{2+} in the luminal uptake of Cd^{2+} along the proximal tubule.

DCT1 has recently been shown to transport a broad range of divalent metal ions, especially Fe^{2+} (Olivi et al., 2001). Our data indicate that ferrous iron (Fe^{2+}) (Fig. 1) inhibits the luminal uptake of Cd^{2+} in isolated perfused rabbit proximal tubular segments. These data are consistent with recent polyclonal antibody findings indicating that DCT1 is expressed in the luminal plasma membrane of proximal tubular cells (Canonne-Hergaux and Gros, 2002). By contrast, our findings are not consistent with those of Ferguson et al. (2001), who localized DCT1 only in the cytosolic compartment of proximal tubular cells. On the surface, the inhibitory effects of Fe^{2+} on the luminal uptake of Cd^{2+} detected in the present study would seem to implicate DCT1 as a participant in the translocation of Cd^{2+} from the luminal compartment into the epithelial cells of the proximal tubule. However, further studies are clearly needed to resolve the apparent conflict pertaining to the precise localization of DCT1 proximal tubular epithelial cells. If it turns out that DCT1 is not expressed in the luminal plasma membrane of these cells, then there is likely another Fe^{2+}-sensitive mechanism responsible for the findings detected in our study.

We also demonstrate that Zn^{2+} inhibited luminal uptake of Cd^{2+} in isolated perfused proximal tubules (Fig. 1). Since Zn^{2+} has a low binding affinity for DCT1, specific Zn-transporters are most likely responsible for the luminal absorption of Cd^{2+}. Interestingly, recent findings of Girijashanker et al. (2008) and He et al. (2006) have implicated the Zn^{2+} transporters, ZIP8 and ZIP14, in the proximal tubular uptake and toxicity of Cd^{2+}. Based on our data, it appears that Cd^{2+} may compete with Zn^{2+} to gain access to the translocation sites of one or more Zn^{2+}-transporters. The lack of change in the cellular content of Cd^{2+} in the experiments utilizing Zn^{2+} as an inhibitor of Cd^{2+}-transport, however, may be due to Zn^{2+} inhibiting the outward transport of Cd^{2+}.

Although HCO_3^- (an apparent requirement for ZIP-dependent transport) was not present in the perfusing or bathing solutions used in the present study, HCO_3^- was likely present in these solutions because of dissolved CO_2 from room air. "HCO_3^--free" medium has been estimated to contain as much as 171 μM HCO_3^- at 37 °C and pH 7.5 (Girijashanker et al., 2008; He et al., 2006). This concentration of HCO_3^- appears to be adequate to drive Cd^{2+} transport via the ZIP8 and ZIP14 transporters (He et al., 2006). Consequently, the inhibitory effects of low HCO_3^- on ZIP transporters was not considered problematic.

There are also findings suggesting that Ca^{2+} can diffuse from the luminal compartment of the proximal tubule via a paracellular pathway (Fromter and Gessner, 1974). Interestingly, however, paracellular movement does not appear to be a mechanism involved in the absorption of Cd^{2+} under the present conditions, inasmuch as very little Cd^{2+} disappeared from the luminal compartment of the perfused tubules at low temperatures (Fig. 5) and the measured intercellular leak of the volume marker through tight junctions was not temperature-dependent. Therefore, it is likely that increasing Ca^{2+} inhibited Cd^{2+} transport (Fig. 2) via an apical calcium channel or some transport mechanism, such as the protein kinase C-regulated Ca^{2+} channel (Zhang and O'Neil, 1996) and/or the transient receptor potential canonical 1 (TRPC1) channel (Beech, 2005;). These two channels are non-voltage gated channels for Ca^{2+} and other ions, and both are localized to the apical membrane of the epithelial cells lining the renal proximal tubule. However, the current data do not exclude the possibility that the increased intraluminal concentrations of Ca^{2+} used may have inhibited the Cd^{2+} transport via some unknown Ca^{2+} transport mechanism.

Realizing the low probability of Cd being in a state not bound to some thiol-containing molecule in the extracellular fluid, we also studied the lumen-to-cell transport of the L-Cys and L-Hcy *S*-conjugates of Cd (Cys-*S*-Cd-*S*-Cys and Hcy-*S*-Cd-*S*-Hcy). The molecular structure of these two conjugates is very similar to that of the amino acid L-cystine (Cys-*S*-*S*-Cys). Therefore, these thiol *S*-conjugates of Cd may mutually compete with L-cystine at the site of one or more transporters for this amino acid. Our data show that Cd is indeed taken up from the luminal compartment by prox-

imal tubular epithelial cells when it is in the form of the thiol S-conjugates, Cys-S-Cd-S-Cys or Hcy-S-Cd-S-Hcy (Figs. 3 and 4). A likely L-cystine transporter involved in the luminal uptake of these thiol S-conjugates of Cd^{2+} is the amino acid transporter system $b^{0,+}$. In the kidneys this L-cystine transporter is present exclusively in the luminal membrane of proximal tubular epithelial cells (Furriols et al., 1993; Mora et al., 1996). Additionally, the absorption of L-cystine is aided by the metabolic reduction of L-cystine to L-Cys in the cytosol, which aids in maintaining an extracellular-to-intracellular concentration-gradient favoring the absorption of L-cystine.

It should also be mentioned that system $b^{0,+}$ has also been recently implicated in the renal cellular uptake of Cys-S-Hg-S-Cys (Bridges et al., 2004). To provide additional support for the hypothesis that Cys-S-Cd-S-Cys is a competitive substrate of selective amino acid transporters, we studied the transport of the aforementioned thiol S-conjugates of Cd^{2+} in the presence of L-cystine, L-arginine, L-aspartate, L-glutamate or L-methionine (Fig. 3). Both L-cystine and L-arginine, which are substrates of system $b^{0,+}$, inhibited significantly the absorptive transport of Cd^{2+} when delivered to the luminal membrane of proximal tubular cells as Cys-S-Cd-S-Cys. No significant decrease in the cellular concentration was observed due to L-arginine, this effect may be explained by inhibition at the basolateral transport of Cys-S-Cd-S-Cys out of the cells into the bathing solution as has been observed for other amino acids (Schafer and Barfuss, 1980).

By contrast, L-aspartate and L-glutamate, which are not substrates of system $b^{0,+}$, had no effect on the absorptive transport of Cd (Fig. 3). Interestingly, the neutral amino acid, L-methionine, enhanced the transport of Cys-S-Cd-S-Cys, presumably by utilizing the trans-stimulation characteristic of the $b^{0,+}$. These results provide the most direct evidence implicating a mechanism involving molecular mimicry, where the L-Cys or L-Hcy S-conjugates of Cd serve as molecular homologs of L-cystine to system $b^{0,+}$.

The molecular structure of Cys-S-Cd-S-Cys also resembles that of certain dipeptides. Thus, we tested the hypothesis that peptide transporter 2 (PepT2), which is localized in the luminal membrane of proximal tubular epithelial cells (Palacin et al., 2005), may serve as a carrier of the two thiol S-conjugates of Cd^{2+} studied. A previous report provides evidence that PepT2 is capable of transporting the conjugate histidine–Zn–histidine (Piersol et al., 2007). When Gly-Sar was co-perfused with Cys-S-Cd-S-Cys, no effect on the rate of absorption of Cd was detected (Fig. 3). These data suggest that PepT2 is likely not involved in transporting Cys-S-Cd-S-Cys in proximal tubular epithelial cells.

It is of particular interest that the presence of Zn^{2+} in the luminal compartment did not affect significantly the luminal uptake of Cd when it was delivered into the lumen as Cys-S-Cd-S-Cys (Fig. 3). These data suggest that the molecules of Cys-S-Cd-S-Cys remained intact during the luminal perfusing process. However, there must be circumstances where Cd^{2+} is capable of dissociating from organic ligands in the lumen of proximal tubules, inasmuch as the renal uptake and toxicity of Cd have been shown to be enhance in vivo in mice over-expressing of ZIP8 (Lucis and Lucis, 1969; Wang et al., 2007).

Our temperature-dependent experiments indicate further that the luminal uptake of Cd^{2+} and the L-Cys and L-Hcy S-conjugates of Cd^{2+} involves specific transport mechanisms, and is not due to non-specific binding and/or paracellular leakage (Fig. 5). We demonstrate that significant reduction in temperature practically eliminates the uptake of Cd^{2+} or Cys-S-Cd-S-Cys without altering the leak properties of tight junctions, which indicates that the disappearance of Cd from the luminal compartment and the cellular content of Cd in the perfused proximal tubular segments are not due to significant non-specific binding or paracellular leakage.

We also evaluated the transport of Cys-S-Cd-S-Cys in all three segments of the proximal tubule, i.e. S_1, S_2, and S_3 segments, to assess potential axial heterogeneity in transport (Fig. 6). Of particular interest, the apparent transport rate of Cys-S-Cd-S-Cys in S_2 and S_3 segments was comparable, but significantly greater than that in S_1 segment. This pattern of axial heterogeneity of transport follows the pattern for distribution of system $b^{0,+}$ and the transport of L-cystine. These observations add credence to the hypothesis that L-Cys S-conjugates of Cd can serve as substrates for the transport mechanism(s) involved in the luminal absorption of L-cystine.

In summary, our current findings clearly demonstrate that multiple mechanisms are involved in the luminal absorption of Cd when it is in the form of Cd^{2+} or Cys or Hcy S-conjugates in the luminal compartment of proximal tubules. Cd^{2+} appears to gain entry into proximal tubular cells at the luminal membrane by utilizing transporters sensitive to Zn^{2+}, Fe^{2+}, and Ca^{2+}. In addition, Cd appears to enter the proximal tubular cells as conjugates of sulfhydryl-containing compounds by one or more luminal transporters of the amino acid L-cystine. We feel the findings from the present study will help greatly in designing future studies to determine the precise nature for the luminal absorption of Cd along the proximal tubule.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

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