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Cadmium Inhibition of Renal Endosomal Acidification

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Chronic exposure to cadmium (Cd) results in an inhibition of protein endocytosis in the renal proximal tubule, leading to proteinuria. In order to gain insight into the mechanism by which Cd impairs the protein endocytosis, we investigated the effect of Cd on the acidification of renal cortical endocytotic vesicles (endosomes). The endosomal acidification was assessed by measuring the pH gradient-dependent fluorescence change, using acridine orange or FITC-dextran as a probe. In renal endosomes isolated from Cd-intoxicated rats, the V_{max} of ATP-driven fluorescence quenching (H^+-ATPase dependent intravesicular acidification) was significantly attenuated with no substantial changes in the apparent K_m, indicating that the capacity of acidification was reduced. When endosomes from normal animals were directly exposed to free Cd *in vitro*, the V_{max} was slightly reduced, whereas the K_m was markedly increased, implying that the biochemical property of the H^+-ATPase was altered by Cd. In endosomes exposed to free Cd *in vitro*, the rate of dissipation of the transmembrane pH gradient after H^+-ATPase inhibition appeared to be significantly faster compared to that in normal endosomes, indicating that the H^+-conductance of the membrane was increased by Cd. These results suggest that in long-term Cd-exposed animals, free Cd ions liberated in the proximal tubular cytoplasm by lysosomal degradation of cadmium-metallothionein complex (CdMT) may impair endosomal acidification 1) by reducing the H^+-ATPase density in the endosomal membrane, 2) by suppressing the intrinsic H^+-ATPase activity, and 3) possibly by increasing the membrane conductance to H^+ ion. Such effects of Cd could be responsible for the alterations of proximal tubular endocytotic activities, protein reabsorption and various transporter distributions observed in Cd-exposed cells and animals.

Key Words: Cadmium, Kidney, Endosome, H^+-ATPase

INTRODUCTION

Cadmium (Cd) is an significant occupational and environmental pollutant. Chronic exposure to Cd via inhalation or ingestion may result in renal functional changes, such as proteinuria, glycosuria, aminoaciduria, and phosphaturia (Friberg, 1950; Kazantzis et al, 1963; Axelsson & Piscator, 1966; Piscator, 1966; Adams et al, 1969; Goyer et al, 1972; Nordberg & Piscator, 1972; Nomiyama et al, 1973, 1975, 1982; Gieske & Foulkes, 1974; Bernard et al, 1979, 1981; Iwao et al, 1980; Kim et al, 1988; Mason et al, 1988). The mechanisms underlying these changes have not been entirely elucidated.

Numerous previous studies have shown that Cd induces proteinuria of tubular type (Bernard et al, 1976, 1979, 1992; Lawerys et al, 1984; Caedenas et al, 1991). This indicates that Cd impairs the reabsorption of filtered proteins. Since the basic mechanism of protein reabsorption in the renal proximal tubule is a receptor-mediated endocytosis (Maack et al, 1992), we investigated in a previous study (Choi et al, 1999) the effect of Cd on the receptor-mediated endocytosis of albumin, using the opossum kidney cell line (OK cell) as a proximal tubular cell model. The results indicated that the albumin endocytosis was significantly inhibited in the cells treated with Cd. The mechanism of the inhibition was not ascertained, but the data implied that it was associated with a defect in the endosomal acidification. Endosomal acidification, which is achieved by the action

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of ATP-driven proton pump (vacuolar type or V-class H^+-ATPase) in the endosomal membrane, is essential for intracellular vesicle trafficking, hence endocytosis and exocytosis of various ligand and receptors (Mellman et al, 1986). Thus, an inhibition of endosomal acidification by H^+-ATPase inhibitors or by acidotropic agents impairs endocytosis and intracellular processing of various proteins (Mellman et al, 1986; Desbuquois et al, 1990; Manabe et al, 1993; Clague et al, 1994; Palokangas et al, 1994; Gekle et al, 1995).

The present study was, therefore, undertaken to systematically evaluate the effect of Cd on the endosomal acidification. The ATP-dependent vesicular acidification was determined in endosomes isolated from the renal cortex of Cd-intoxicated rats and in normal animal endosomes directly exposed to free Cd *in vitro*. The results suggest that Cd impairs endosomal acidification 1) by reducing the density of H^+-ATPase in the membrane, 2) by inhibiting the activity of residual H^+-ATPase, and 3) possibly by increasing the H^+ conductance of the endosome membrane.

METHODS

Animals

Male Sprague Dawley rats (200~300 g) were maintained under standard laboratory conditions with *ad libitum* access to food and water, unless otherwise mandated by experimental protocol. Cadmium intoxication was induced by daily subcutaneous injections of $CdCl_2$ at a dose of 2 mg Cd/kg body weight per day for 2~3 weeks, as described in a previous paper (Kim et al, 1988). Saline was injected into the control animals. At 1-week intervals, animals were kept in metabolic cages and denied food and water for 24 hrs. Urine was collected under the mineral oil and was analyzed for total protein (Bradford, 1976) and phosphorous (Fiske & SubbaRow, 1925).

Isolation of renal cortical endosomes and determination of proton pump activity

Rat renal cortical endocytotic vesicles (endosomes) were isolated by the method of Sabolic & Burkhardt (1990). Animals were killed by cervical dislocation. Kidneys were taken out and immediately immersed in ice-cold Ringer's solution. After removing the capsule, cortical slices of ~0.3 mm thick were cut off by razor blade. The pooled tissue (2~2.5 g wet) was put into 35 ml of homogenizing buffer (300 mM mannitol, 12 mM HEPES/Tris, pH 7.4) and homogenized with 20 strokes in a loose-fitting glass/Teflon Potter homogenizer (1,200 rpm). After adding another 35 ml of homogenizing buffer, the suspension was centrifuged at 2,500 *g* for 15 min. The pellet (P_1) was discarded and the supernatant (S_1) was centrifuged at 20,000 *g* for 20 min. Most of the resulting supernatant (S_2) was decanted and saved. The rest of the supernatant (about 2 ml) was used to disperse the fluffy upper part of the pellet (P_{2a}) by careful swirling of the tube. Therefore, care was taken not to disturb the hard, yellowish brown mitochondrial pellet (P_{2b}). Supernatant S_2 and the dispersed fluffy pellet P_{2a} were combined and centrifuged at 48,000 *g* for 30 min. The resulting supernatant (S_3) was siphoned off and discarded. The pellet (P_3) contained crude plasma membranes and endocytotic vesicles (endosomes).

Endosomes were separated from other membranes on a Percoll density gradient. Fraction P_3 was resuspended in 30 ml homogenizing buffer by 10 strokes (1200 rpm) in a tight-fitting glass/Teflon Potter homogenizer. Undiluted Percoll (6.1 g) was added to 32 g of vesicle suspension (16%, w/w, Percoll). The suspension was well mixed and centrifuged at 48,000 *g* for 30 min. The resulting self-orienting gradient was fractionated from top to bottom by pumping a 60% sucrose solution onto the bottom of the centrifuge tube via a steel cannula. The last 5 ml of the gradient contained the highest activity of proton pump was pooled (P_4) and diluted with 30 ml of cold potassium buffer (300 mM mannitol, 100 mM KCl, 5 mM $MgSO_4$, 5 mM HEPES/Tris, pH 7.0). The sample was kept on ice for 30 min and then centrifuged at 48,000 *g* for 30 min. The clear supernatant (S_5) was completely removed by suction. The fluffy white membrane pellet (P_5), which overlayed the hard glassy pellet of Percoll, was transferred into the Eppendorf tube and dispersed by vigorous vortexing in 1 ml of potassium buffer. The sample was centrifuged (by using a tube adapter) at 2,500 *g* for 15 min. The slightly opaque supernatant (S_6) was removed completely by careful suction using a syringe with a needle. The white-yellow pellet on the bottom of the tube (P_6) containing endocytotic vesicles was dispersed in 50 μl of potassium buffer and immediately transferred to a new Eppendorf tube.

Care was taken not to re-suspend the membranes adherent to the walls of Eppendorf containing a high amount of brush-border membranes. The protein concentration in the final vesicle preparation was adjusted to be ~10 mg/ml in a total volume of ~60 μl. The isolated endosomes were immediately used in experiment or kept frozen in liquid nitrogen and used within 2 weeks. The deposition of vesicles at 4°C overnight or longer is known to result in a time-dependent drop in ATPase activity and increase in proton and potassium conductance in the membranes.

The proton pump activity in the endosomal membrane was determined by measuring the ATP-dependent intravesicular acidification using a pH gradient (ΔpH)-sensitive fluorescent dye acridine orange (Sabolic et al, 1985; Sabolic & Burkhardt, 1986, 1990) or fluorescein isothiocyanate-labeled dextran (FITC-dextran) (Lencer et al, 1990). The weak base acridine orange accumulates in vesicles whenever intravesicular pH is acidic relative to the pH of the extravesicular medium. The fluorescence of accumulated dye is quenched. Thus, the degree of fluorescence quenching (decrease of absorption) is a measure of the magnitude of the pH gradient across the vesicular membrane.

Endosomes loaded with potassium buffer were incubated at 25°C in the same buffer containing valinomycin (final concentration of 2.5 μM) and acridine orange (final 6 μM). The H^+-ATPase reaction was started by addition of ATP (final 1.5 mM). The acridine orange fluorescence was continuously monitored at 25°C (excitation 510 nm; emission 548 nm). During the measurement, the samples were stirred.

When FITC-dextran was used, endosomes were loaded with FITC-dextran *in vivo* via the process of fluid-phase endocytosis (Lencer et al, 1990). Rats were injected with a bolus of FITC-dextran (100 mg/ml) through a sublingual vein (0.4 ml/100 g body weight), and kidneys were excised out after 15 min. Superficial cortices of excised kidneys were dissected and endosomes were isolated as described above. The ATP-driven intravesicular acidification was determined by measuring the pH-dependent changes in fluorescein fluorescence at excitation 510 nm and emission 537 nm.

Chemicals

We purchased fluorescein isothiocyanate (FITC)-labeled dextran (9400 S), acridine orange, N-ethylmaleimide (NEM), nigericin, $CdCl_2$, Tris and HEPES from Sigma Chemicals (St. Louis, MO, USA) and Percoll from Pharmacia Fine Chemicals (Sweden). All other chemicals used were of analytical grade.

Statistical analysis

Statistical evaluation of data was done using Student's t-test (unpaired comparison) or Analysis of covariance.

RESULTS

Renal function in cadmium-exposed rats

Table 1 shows the change in renal function in Cd-exposed rats. Subcutaneous injections of $CdCl_2$ at a dose of 2 mg Cd/kg/day for 3 weeks resulted in a marked increase in urine flow and urinary excretions of protein and phosphorous, characteristics of chronic-cadmium intoxication (Kim et al, 1988).

Acidification of endosomes isolated from Cd-intoxicated rat renal cortices

Fig. 1 compares the time courses of ATP-driven intravesicular acidification in renal cortical endosomes isolated from the control and cadmium-intoxicated rats. The intravesicular acidification was

Table 1. Urine flow and urinary excretions of protein and phosphorus in control and cadmium-exposed rats

	Exposure	Control	Cadmium
Urine flow (ml/kg/day)	Pre	30.7±7.2	30.5±4.8
	Post	21.4±4.3	37.3±8.3*
Protein excretion (mg/kg/day)	Pre	15.8±2.4	18.7±4.1
	Post	28.2±6.4	44.5±11.0*
Phosphorous excretion (mg/kg/day)	Pre	16.5±10.9	10.8±3.9
	Post	14.0±7.6	42.8±9.2*

Cadmium group animals were subcutaneously injected with $CdCl_2$ at a dose of 2 mg Cd/kg/day for 3 weeks. The data represents the mean±SD of 7 rats in each group. *$P<0.05$ compared with the matched control value.

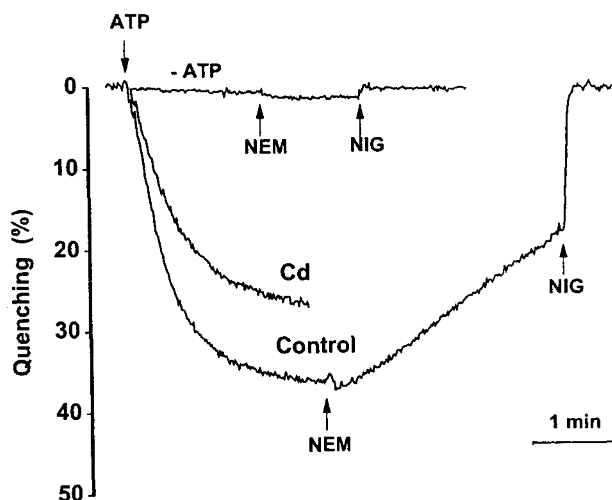

Fig. 1. ATP-driven intravesicular acidification in renal cortical endocytotic vesicles (endosomes) of control and cadmium-exposed rats as measured by FITC-dextran fluorescence quenching. The reaction was started by addition of ATP (final concentration 1 mM) to FITC-dextran labeled endocytotic vesicles in KCl-buffer (300 mM mannitol, 100 mM KCl, 5 mM $MgSO_4$, 5 mM HEPES/Tris, pH 7.0). Endocytotic vesicles labeled with FITC-dextran were prepared by intravenous infusion of 100 mg FITC-dextran and isolation as described in the MATERIALS AND METHODS. At the indicated time (arrow), proton gradients were dissipated by addition of N-ethylmaleimide (NEM, final 1 mM) and nigericin (NIG, final 10 μM). FITC fluorescence (excitation 510 nm, emission 537 nm) was monitored continuously in a stirred cuvette in an SLM 4800 C fluorimeter.

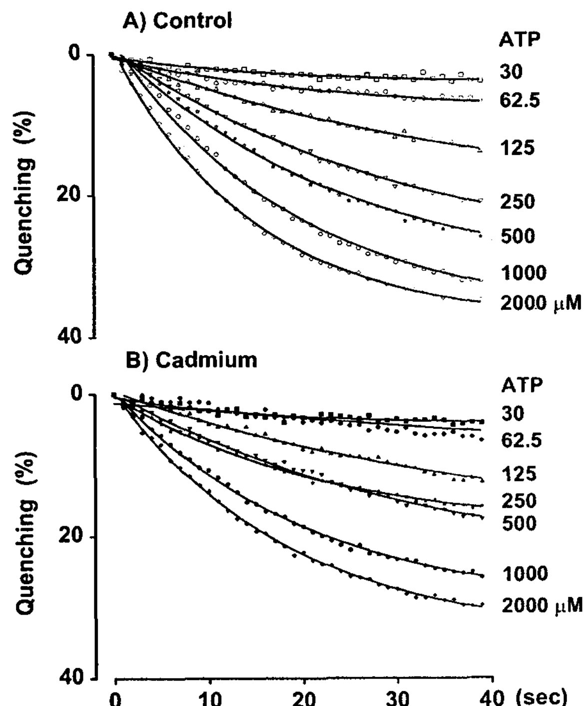

Fig. 2. Time courses of FITC-dextran fluorescence quenching (intravesicular acidification) in renal cortical endosomes of control and cadmium-exposed rats at varying ATP concentrations. Each curve was drawn by computer fitting of the data using the equation $f(t) = a + b \cdot e^{-ct}$. The initial rate of quenching could be obtained from derivative of the function $f(t)$ at $t = 0$.

assessed by pH-dependent fluorescence changes of FITC-dextran which had been loaded into endosomes in vivo by fluid-phase endocytosis. The endosomes containing FITC-dextran (25 mg protein/ml) were incubated at 25°C in KCl-buffer for 10 min. In both the control and Cd-group endosomes, addition of ATP (1 mM) to the extravesicular medium quickly induced fluorescence quenching (intravesicular acidification). Addition of NEM (1 mM), which inhibits the H^+-ATPase activity (Sabolic & Burkhardt, 1986), resulted in a spontaneous decay of the pH gradient. Addition of a protonophore nigericin (10 μM) to collapse the residual pH gradient caused an instantaneous and complete dissipation of the quenching. In the absence of ATP, no fluorescence quenching occurred. In the endosomes from Cd-exposed animals, the rate of fluorescence quenching appeared to be markedly attenuated as compared with that in the control endosomes. These results indicate that the intravesicular acidification (fluorescence quenching) is achieved by the action of proton pumps (H^+-ATPase) in the vesicle-limiting membrane, and the activity of the pump is attenuated in endosomes of cadmium-intoxicated animals.

Fig. 2 depicts the effect of ATP concentration on the fluorescence quenching. In both the control and Cd-group endosomes, the rate of quenching of intravesicular FITC-dextran increased as the ATP concentration increased. The initial rate of quenching, estimated by drawing the tangent to the initial part of the fluorescence recording (F/sec), represents the rate of acidification driven by the H^+-ATPase pump.

The initial rate of quenching increased with the ATP concentration, providing an evidence for saturability (Fig. 3, upper panel). Hofstee plot of the data (Fig. 3, lower panel) revealed that the quenching follows a simple Michaelis-Menten kinetics. In the Cd group endosomes, the V_{max} was reduced with no

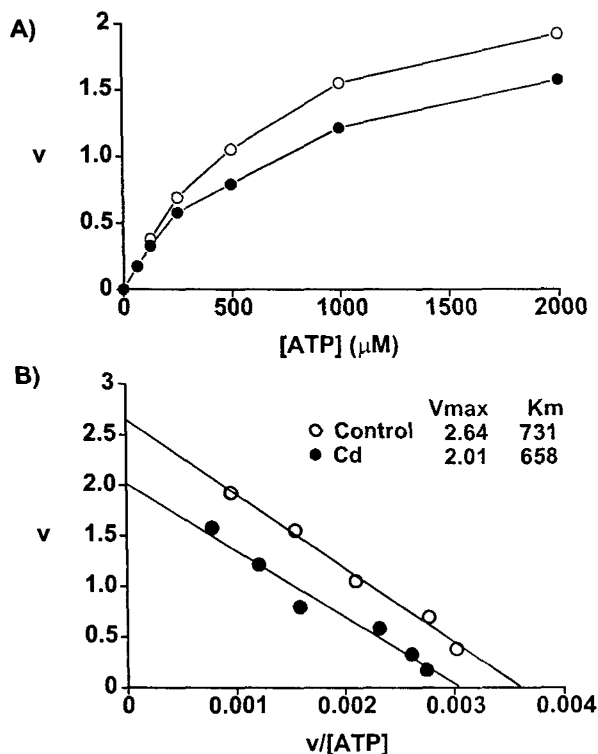

Fig. 3. Kinetics of intravesicular acidification in renal cortical endosomes of control and cadmium-exposed rats. A. Initial rates (v) of fluorescence quenching are plotted as a function of ATP concentration. Data are based on Fig. 2. B. Hofstee plots of the data in the panel A. Covariance analysis indicates that the two regression lines are significantly ($p<0.05$) different from each other in the y-intercept (V_{max}), but not in the slope (K_m).

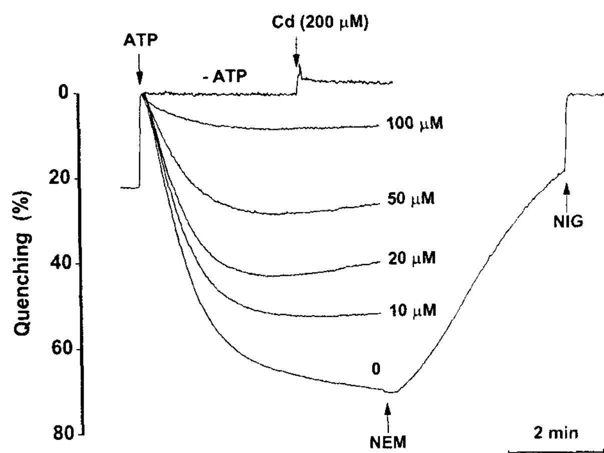

Fig. 4. Effects of *in vitro* exposure to cadmium ($CdCl_2$) on ATP-driven intravesicular acidification of renal cortical endosomes as measured by acridine orange fluorescence quenching. Vesicles were preincubated in acridine orange-containing KCl-buffer (contained 2.5 μM valinomycin, Ref. Fig. 1) without (0) or with indicated concentrations of $CdCl_2$ at 25°C for 10 minutes before intravesicular acidification (fluorescence quenching) was initiated by adding ATP. At the indicated time, N-ethylmaleimide (NEM, final 1 mM), Cd, or nigericin (NIG, final 10 μM) were added to the outside buffer in order to stop further acidification or dissipated the pH. Acridine orange fluorescence (excitation 510 nm, emission 548 nm) was monitored continuously in a stirred cuvette in an SLM 4800 C fluorimeter. Shown are representative curves for 3 similar experiments.

significant change in the K_m, as compared with the control. These results suggest that the capacity of endosomal acidification was reduced by cadmium intoxication.

Acidification of renal cortical endosomes directly exposed to free Cd in vitro

Fig. 4 presents the effect of free Cd directly applied to the renal cortical endosome on ATP-driven intravesicular acidification. The intravesicular acidification was assessed by the quench method of acridine orange fluorescence (Sabolic et al, 1985; Sabolic & Burkhardt, 1986, 1990). Vesicles were preincubated with various concentrations (0, 10, 20, 50, and 100 μM) of Cd ($CdCl_2$) at 25°C for 10 min before intravesicular acidification (fluorescence quenching) was initiated by adding ATP (1.5 mM). In the absence of Cd (0 mM), an ATP- and time-dependent quenching of acridine orange fluorescence was recorded. Addition of NEM (1 mM) caused a spontaneous decay of fluorescence quenching, and addition of nigericin (10 μM) resulted in an instantaneous and complete dissipation of the quenching. In the presence of $CdCl_2$, the ATP-driven fluorescence quenching was attenuated in a dose-dependent fashion. The initial rate of quenching was gradually reduced as the $CdCl_2$ concentration increased from 10 to 100 μM, showing a 50% reduction at ~50 μM (Fig. 5). For the same concentration range, Cd bound to metallothionein (CdMT) showed no significant effect on the fluorescence quenching. Thus, free (unbound) Cd appeared to be effective in the inhibition.

Fig. 6 illustrates the effect of free Cd on the kinetics of fluorescence quenching in renal cortical endosomes. The initial rates of ATP-driven fluorescence quenching (v) determined at various ATP concentrations ([ATP]) in the control and 20 μM

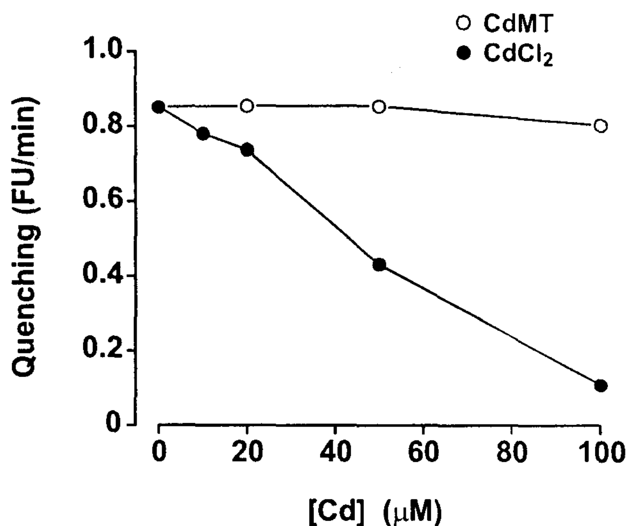

Fig. 5. Initial rates of intravesicular acidification in renal cortical endosomes directly exposed to free cadmium ($CdCl_2$) and cadmium bound to metallothionein (CdMT) as a function of cadmium concentration. Data represent the mean of 2~3 determinations.

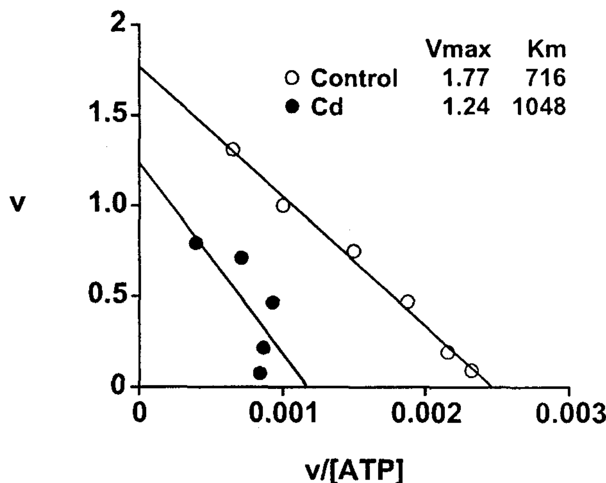

Fig. 6. Hofstee plots of the initial rates of intravesicular acidification in control and cadmium-treated renal cortical endosomes at various ATP concentrations. Vesicles were preincubated in acridine orange-containing KCl-buffer without (Control) or with 20 μM $CdCl_2$ (Cd) at 25°C for 10 minutes before intravesicular acidification was initiated by adding ATP. Covariance analysis indicates that the two regression lines are significantly ($p<0.05$) different from each other in the y-intercept (V_{max}) and the slope (K_m).

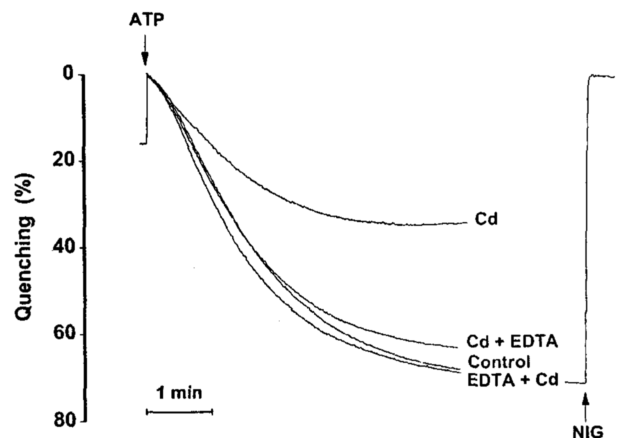

Fig. 7. Effects of EDTA on the cadmium ($CdCl_2$) inhibition of endosomal acidification. Normal renal cortical endosomes were preincubated in KCl-buffer containing acridine orange without (Control) or with 20 μM $CdCl_2$ (Cd) at 25°C for 10 minutes before intravesicular acidification was initiated by adding ATP. EDTA was added to the reaction mixture from a water stock solution at a final concentration of 0.2 mM either 5 min before adding Cd (EDTA+Cd) or 10 min after addition of Cd (Cd+EDTA). Nigericin (NIG, final 10 μM) was added later to dissipate the residual ΔpH. Shown are representative curves for 3 similar experiments.

Cd-treated endosomes were plotted against v/[ATP] (Hofstee plot). This analysis indicated that the Cd treatment caused a marked increase in K_m (1048 μM vs. 716 μM in control, $p<0.05$) and a decrease in V_{max} (1.24 vs. 1.77 in control, $p<0.05$). These results suggest that the biochemical property of the H^+-ATPase was changed in the Cd-treated endosomes.

In the next series of experiments, we tested the effect of EDTA, a divalent cation chelator, on the Cd inhibition of endosomal acidification. Addition of 0.2 mM EDTA to reaction mixture caused a complete recovery of the Cd-inhibition of fluorescence quenching (Fig. 7). This fact indicates that Cd reversibly acted upon the H^+-ATPase from the outside (cytoplasmic side) of the vesicle membrane and that Cd did not cause dissociation of functionally important H^+-ATPase subunits from the membrane.

Effect of Cd on the H^+ conductance of the renal cortical endosomal membrane

In order to assess the H^+ conductance of the endosomal membrane, we measured spontaneous dis-

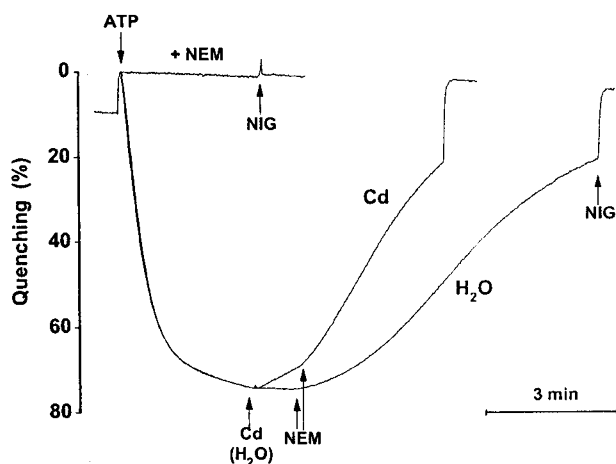

Fig. 8. Effect of cadmium ($CdCl_2$) exposure (*in vitro*) on dissipation of the transmembrane pH gradient in renal cortical endosomes. Normal renal cortical endosomes loaded with KCl-buffer were diluted in the same buffer containing acridine orange. ATP was then added to initiate intravesicular acidification (fluorescence quenching). At the indicated time, $CdCl_2$ (final 50 μM) or water (H_2O) was added in the outside buffer, followed by N-ethylmaleimide (NEM, final 1 mM), and spontaneous dissipation of the ΔpH was recorded. Nigericin (NIG, final 10 μM) was added later to dissipate the residual ΔpH. Shown are representative curves for 3 similar experiments.

sipation of pH gradient (ΔpH) after H^+-ATPase inhibition (Fig. 8). Vesicles loaded with K^+-buffer were incubated in the same buffer containing valinomycin (2.5 μM) and acridine orange. ATP (1.5 mM) was then added to initiate intravesicular acidification (fluorescence quenching). When ΔpH reached a steady-state (after about 3 min) Cd (50 μM) or H_2O was added in the outside buffer, followed by NEM (1 mM), and spontaneous dissipation of ΔpH was recorded. This ΔpH dissipation would reflect the H^+ conductance as it occurs in electroneutral conditions. Nigericin (10 μM) was added later to dissipate the residual ΔpH. In the presence of Cd, the ΔpH dissipation was much faster than that in the absence of Cd (H_2O addition), which indicates that the H^+ conductance of the endosome membrane was increased.

DISCUSSION

Although a considerable amount of plasma proteins are normally filtered through renal glomeruli, the protein loss into urine is prevented by the reabsorption of filtered proteins in the renal tubule (Maack et al, 1992; Rosenberg & Hostetter, 1992). Micropuncture studies showed that protein reabsorption takes place exclusively in the proximal tubule (Carone et al, 1979; Tojo & Endou, 1992). The basic mechanism of the reabsorption is a specific receptor-mediated endocytosis (Maack et al, 1992; Gekle, 1998).

A receptor-mediated endocytosis proceeds with the following sequence (Mellman, 1987; Lodish et al, 1995). The ligand binds to a specific receptor on the plasma membrane. The receptor-ligand complex is internalized in a clathrin-coated pit that pinches off to become a coated vesicle. The clathrin coat is then depolymerized to triskelions, resulting in an early endosome. This endosome fuses with a sorting vesicle, known as the late endosome, which contains V-class H^+-ATPase (Mellman et al, 1986), where the low pH causes the ligand particle to dissociate from the receptor. A receptor-rich region buds off to form a separate vesicle that recycles the receptors back to the plasma membrane. The remaining portion of the vesicle containing ligand particles (transport vesicle) ultimately fuses with a lysosome to form a large lysosome where the endocytosed molecule is hydrolyzed. The V-class H^+-ATPase plays a crucial role in intracellular vesicle trafficking by maintaining acidic environment within the lumen of endo- and exocytotic organelles in various mammalian cells (Wall & Maack, 1985; Mellman et al, 1986). Thus, inhibition of endosomal acidification by H^+-ATPase inhibitors or by acidotropic agents leads to a significant reduction in endocytosis and intracellular processing of various proteins (Mellman et al, 1986; Desbuquois et al, 1990; Manabe et al, 1993; Clague et al, 1994; Palokangas et al, 1994; Gekle et al, 1995).

Our previous study (Choi et al, 1999) in OK renal epithelial cells suggested that the receptor-mediated endocytosis of albumin is significantly inhibited in the Cd-treated cells. The kinetic nature of this inhibition was similar to that induced by NH_4Cl, a compound that interferes with vesicular acidification (Gekle et al, 1995; Batuman & Guan, 1997). We, therefore, suspected that the cadmium inhibition of albumin endocytosis was associated with an alteration of endosomal acidification.

The present study clearly demonstrated that the

mechanism of renal endosomal acidification is impaired by Cd exposure. In endosomes isolated from the Cd-intoxicated rat renal cortex, the ATP-dependent intravesicular acidification was significantly attenuated as compared with that in normal endosomes (Fig. 1). Kinetic analysis indicated that the V_{max}, but not the K_m, of endosomal acidification was reduced by Cd-intoxication (Fig. 3). These results suggest that the density of H^+-ATPase was reduced in the endosomes of Cd-intoxicated animals. Whether this was due to an inhibition of *de novo* synthesis of H^+-ATPase protein or to an inhibition of recycling of the proteins via intracellular vesicle trafficking is not certain. In any event, a reduction of functional H^+-ATPase units in the membrane would reduce the capacity of endosomal acidification, as manifested by a reduction in the V_{max}.

This, however, may not be the sole mechanism for the defect in renal endosomal acidification in Cd-intoxicated animals. During chronic exposure, the Cd ingested into the body is bound to metallothionein (MT) in the liver. The cadmium-metallothionein complex (CdMT) released into the blood circulation is transferred to the kidney, filtered through glomeruli, and taken up by cells in S_1 and S_2 segments of the proximal tubule (Dorian et al, 1992a, b). These segments are the sites of the most intensive endocytosis (Wall & Maack, 1985; Brown, 1989) and the highest V-class H^+-ATPase activity (Brown et al, 1988), and are the principal sites of nephrotoxicity (Dorian et al, 1992a, b). Recent studies have indicated that, following endocytosis by the proximal tubule, CdMT is rapidly degraded within a few hours, and the released Cd is retained in the cell (Dorian et al, 1992b). This degradation may take place in lysosomes (Squibb et al, 1979) and possibly in endosomes as well. In addition to the inhibition of specific mRNA expression (Herak-Kramberger et al, 1996), Cd released from this degradation may directly interact with various enzymes and membrane transporters, such as H^+-ATPase in the endosome. The experiments on the *in vitro* Cd effect strongly favor this possibility. Direct exposure of renal endosomes to free Cd ($CdCl_2$) at concentrations $10 \sim 100$ μM resulted in a dose-dependent inhibition of intravesicular acidification (Figs. 4 & 5). Similar results have been observed by others in rat renal endosomes (Herak-Kramberger et al, 1998). The concentrations of Cd used in the present study were within the range of unbound (free) Cd (13 μg/g, $\sim$115 μM) observed in renal cortical tissues of Cd-exposed rabbits at the time of onset of proteinuria (Nomiyama & Nomiyama, 1986). It is, therefore, likely that a direct inhibition of endosomal acidification by free Cd also occur *in vivo* in Cd-intoxicated animals. The exposure to CdMT had no effect on the endosomal acidification (Fig. 5), indicating that only the free Cd interacts with the endosome.

The molecular nature of the Cd-endosome interaction is not entirely certain. The Cd-induced inhibition of intravesicular acidification was completely recovered in the medium containing a divalent cation chelator EDTA (Fig. 7), which indicates that the Cd interaction did not cause dissociation of H^+-ATPase units from the membrane. This, in turn, suggests that the activity of the intrinsic H^+-ATPase was inhibited by the Cd interaction. In the kinetic analysis, the V_{max} of acidification was reduced, while the K_m was increased in Cd-treated endosomes (Fig. 6). These results imply that the biochemical property of the H^+-ATPase molecule was altered, such that the substrate-H^+-ATPase interaction was perturbed. The molecular mechanism by which Cd triggers these changes remains to be elucidated.

Herak-Kramberger et al (1998) have shown that in renal endosomes Cd facilitates the dissipation of the pH gradient generated by the H^+-ATPase action. We have confirmed this in the present study. The rate of dissipation of transmembrane pH gradient (pH) measured after H^+-ATPase inhibition (by NEM) appeared to be significantly increased by Cd (Fig. 8). Since these experiments were conducted in electroneutral conditions by having equal concentration of K^+ on both sides of the vesicle membrane and valinomycin in the outside medium, spontaneous dissipation of pH would be limited by the intrinsic H^+ conductance of the vesicle membrane. We, therefore, presume that an interaction of free Cd with endosome may cause an increase in the H^+ conductance of the membrane, leading to a faster dissipation of the pH. This may be another potential mechanism for the impaired endosomal acidification in Cd-intoxicated animals.

Proximal tubule cell endocytosis is not only responsible for the reabsorption of filtered proteins, but also is a part of vigorous recycling mechanism that is of pivotal importance for trafficking of membrane proteins between the brush border and intracellular membranes (Brown, 1989). Thus, an inhibition of this vesicle recycling process by altering the endosomal acidification by Cd may not only impair the re-

absorption of filtered proteins, but also selectively diminish the abundance of various transporters in the brush-border membrane.

In summary, the present study clearly demonstrated that the capacity of intravesicular acidification is impaired in renal endosomes of Cd-intoxicated rats and in normal endosomes directly exposed to free Cd *in vitro*. These results suggest that in long-term Cd-exposed animals, free Cd ions released in the proximal tubular cytoplasm may impair the endosomal acidification by 1) reducing the H^+-ATPase density in the endosomal membrane, 2) by suppressing the intrinsic H^+-ATPase activity, and 3) by increasing the membrane conductance to H^+ ion. Such effects of Cd could be responsible for the alterations of proximal tubular endocytotic activities, protein reabsorption and various transporter distributions observed in Cd-intoxicated animals.

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