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Changes in Phosphate Transporter Activity Evaluated by Phosphonoformic Acid Binding in Cadmium-Exposed Renal Brush-Border Membranes

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Direct exposure of renal tubular brush-border membranes (BBM) to free cadmium (Cd) causes a reduction in phosphate (P*i*) transport capacity. Biochemical mechanism of this reduction was investigated in the present study. Renal proximal tubular brush-border membrane vesicles (BBMV) were isolated from rabbit kidney outer cortex by Mg precipitation method. Vesicles were exposed to 50~200 μM $CdCl_2$ for 30 min, then the phosphate transporter activity was determined. The range of Cd concentration employed in this study was comparable to that of the unbound Cd documented in renal cortical tissues of Cd-exposed animals at the time of onset of renal dysfunction. The rate of sodium-dependent phosphate transport (Na^+-P*i* cotransport) by BBMV was determined by ^{32}P-labeled inorganic phosphate uptake, and the number of Na^+-P*i* cotransporters in the BBM was assessed by P*i*-protectable ^{14}C-labeled phosphonoformic acid ([^{14}C]PFA) binding. The exposure of BBMV to Cd decreased the Na^+-P*i* cotransport activity in proportion to the Cd concentration in the preincubation medium, but it showed no apparent effect on the P*i*-protectable PFA binding. These results indicate that an interaction of renal BBM with free Cd induces a reduction in Na^+-P*i* cotransport activity without altering the carrier density in the membrane. This, in turn, suggest that the suppression of phosphate transport capacity (V_{max}) observed in Cd-treated renal BBM is due to a reduction in Na^+-P*i* translocation by existing carriers, possibly by Cd-induced fall in membrane fluidity.

Key Words: Phosphonoformic acid, Na^+-P*i* cotransport, Cadmium, Kidney, Brush-border membrane, Vesicle

INTRODUCTION

One of the typical renal functional changes documented in cadmium (Cd)-exposed humans and experimental animals is phosphaturia (Kazantzis et al, 1963; Adams et al, 1969; Iwao et al, 1980; Kim et al, 1988). The mechanisms by which Cd induces phosphaturia have not been fully elucidated. However, our studies using isolated renal plasma membrane vesicles (Ahn & Park, 1995) have indicated that the first step of the phosphate reabsorption in the proximal tubule is impaired. In the renal cortical brush-border membrane vesicles (BBMV) derived from Cd-intoxicated rats and rabbits, the Na^+-dependent transport system for inorganic phosphate (P*i*) was impaired with no changes in Na^+-independent transport system. Kinetic analysis (Park et al, 1997) indicated that the transport capacity (V_{max}) of specific carrier in the brush-border membrane (BBM) is reduced by Cd. Such a reduction in transport capacity may be attributed, in part, to a loss of carrier units in the membrane due to an inhibition of *de novo* protein synthesis, as reported for the type II Na^+-P*i* cotransporter (NaPi-2) in the renal BBM of Cd-treated rats (Herak-Kramberger et al, 1996). Direct exposure of normal renal BBMV to free Cd *in vitro* also induces alterations of Na^+-dependent transport activities similar to those observed in the BBMV of Cd-exposed animals (Ahn & Park, 1995). However, exposure of vesicles to CdMT has no such effect (Park et al, 1997). These facts suggest that during a long-term exposure, free Cd ions released by

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lysosomal degradation of CdMT may also interact with the BBM, thereby interfering with various Na^+-cotransport carrier activities. The precise nature of the interaction is not known.

The present study was undertaken to evaluate changes in Na^+-P*i* cotransporter density in the renal BBM directly exposed to free Cd, using phosphonoformic acid (PFA) as a probe to titrate the Na^+-P*i* cotransport carrier. Since PFA is a competitive inhibitor of the Na^+-P*i* cotransport in the renal BBM and specifically binds to the Na^+-P*i* cotransport carrier (Szczepanska-Konkel et al, 1986, 1987; Kempson, 1988), it may be used to quantify the Na^+-P*i* cotransporter density in the renal BBM (Hoppe et al, 1991).

METHODS

Chemicals

[^{14}C]-phophonoformic acid was purchased from Amersham (Buckinghamshir, England) and $NaH_2[^{32}P]O_4$ was obtained from NEN (Boston, MA, USA). Phosphonoformic acid, HEPES, Tris and other chemicals were obtained from Sigma Chemicals (St. Louis, MO, USA).

Animals

Adult male New Zealand white rabbits were used. They were maintained under standard laboratory condition with *ad libitum* access to food and water.

Preparation of renal cortical brush-border membrane vesicles

Renal outer cortical brush-border membrane vesicles (BBMV) were isolated by a procedure similar to that described by Sheikh et al (1982). Briefly, kidneys were perfused through the abdominal aorta with an ice-cold normal saline. The outer cortex was cut off, minced, and placed in 10 volumes of 300 mM mannitol, 10 mM HEPES/Tris, pH 7.4 (mannitol buffer). The cortical tissues from 2 animals were pooled and homogenized with 25 strokes in a Potter's homogenizer with a teflon pestle at 1,500 rpm. The tissue homogenate was centrifuged at 3,000 rpm for 10 min in a Sorvall refrigerated centrifuge (SS-34 Rotor). The supernatant was centrifuged at 10,000 rpm for 15 min; the resulting supernatant and the soft light portion of the pellet were isolated, and pooled. The pooled fraction was centrifuged again at 15,000 rpm for 1 h. The fluffy layer between the supernatant and dark pellet was collected and suspended in $MgCl_2$ solution. The final concentration of $MgCl_2$ was adjusted to be 5 mM. The suspension was stirred on ice for 30 min, then centrifuged at 3,500 rpm for 15 min. The supernatant was centrifuged again at 15,000 rpm for 1 h. The pellet was washed twice and suspended in loading buffer (100 mM mannitol, 100 mM KCl or NaCl, and 20 mM HEPES/10 mM Tris, pH 7.5) at protein concentration 7~9 mg/ml. The final suspension was passed several times through a 25 gauge needle for even dispersion of vesicles and stored at −70°C. Protein concentration was determined by the Bio-Rad protein assay kit.

The purity of BBMV fractions was evaluated by measuring the enrichment of marker enzymes. The enrichment was about 8-fold in alkaline phosphatase, a marker enzyme for brush-border membrane, but was less than 2-fold in Na^+-K^+-ATPase, a marker enzyme for basolateral membrane. Vesicles were thawed and equilibrated at 37°C for 30 min, prior to use.

Determination of substrate uptake by brush-border membrane vesicles

The uptake of a substrate by BBMV was determined by a rapid Millipore filtration technique (Hopfer et al, 1973) as described in detail in previous studies (Lee et al, 1990, 1991). In PFA uptake experiments, an aliquot of BBMV was incubated at 25°C in 9 volumes of incubation medium (100 mM mannitol, 100 mM NaCl or KCl and 20 mM HEPES/10 mM Tris, pH 7.5) containing appropriate concentrations of PFA (including 2~3 μCi [^{14}C]PFA/ml). At appropriate intervals, a 100 μl aliquot was removed and filtered through Millipore filter (HA, pore size 0.45 μm), which had been soaked overnight in distilled water. The filter was washed with 4 ml of cold stop solution (plain incubation medium). The radioactive materials retained in the filter were dissolved in 2 ml Lumagel (Lumac, AC Landggaf, the Netherlands) and counted on a liquid scintillation counter (Packard Tricarb 4530-C). Nonspecific binding of radioactive material to the filter paper was determined by filtering 100 μl incubation medium. The value of nonspecific binding was subtracted from the experimental value and the substrate uptake by

vesicles was expressed as nmol/mg protein for a given time.

When vesicular uptake of Pi was determined, the compositions of intra- and extra-vesicular media were adjusted appropriately.

To evaluate the effect of Cd, vesicles were first preincubated in the medium containing appropriate concentration of $CdCl_2$ for 30 min at 37°C, and then incubated with a substrate at 25°C for an appropriate time period.

Statistical analysis

All results were presented as the mean±SD and statistical evaluation of the data was done by unpaired Student's *t* test and covariance analysis.

RESULTS

The initial series of experiments were conducted to characterize PFA uptake by the renal cortical BBMV. The PFA binding was measured under equilibrium conditions (i.e., equal Na^+, K^+, and H^+ ion concentrations in intra- and extra-vesicular media) to avoid modulatory effects of these solutes. Fig. 1 depicts the time courses of PFA uptake by BBMV incubated in Na^+- or K^+-containing (Na^+ was replaced by K^+) media. In both cases, the PFA uptake increased rapidly during the initial period, then it gradually leveled off. At any time period, the amount of PFA uptake was much greater in Na^+-containing medium than in K^+-containing medium. The difference in the uptake between the two conditions represents the Na^+-dependent PFA uptake. This component has been shown to include the PFA accumulation inside the vesicle as well as the PFA binding to the membrane (Hoppe et al, 1991).

Fig. 2 shows the effects of P*i* on PFA uptake by renal BBMV in Na^+- or K^+-containing media. The PFA uptake in K^+-containing medium was not affected by P*i*. On the other hand, the PFA uptake in Na^+-containing medium was progressively attenuated as the P*i* concentration increased from 0 to 10 mM, above which no further attenuation was observed. It seems that approximately half of the Na^+-dependent PFA uptake could be displaced by an excess of P*i*. This component, denoted as "P*i*-protectable Na^+-PFA binding", is known to represent the specific binding to the Na^+-P*i* cotransporter in the renal BBM (Hoppe et al, 1991).

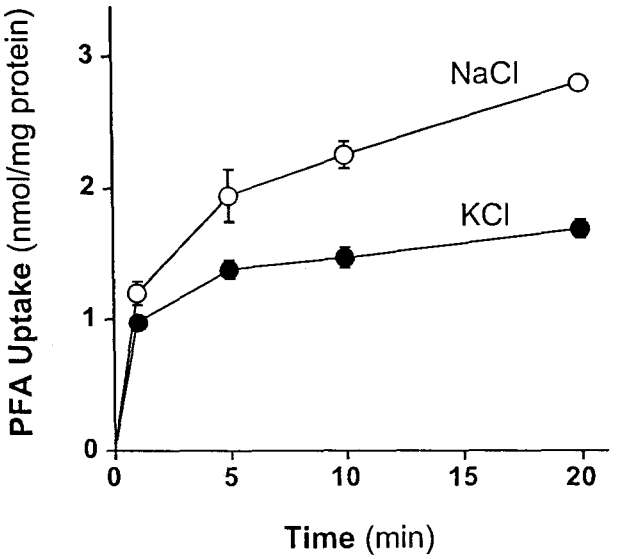

Fig. 1. Time course of PFA uptake by rabbit renal outer cortical BBMV. Vesicles were loaded with either KCl- or NaCl-containing medium and uptake of 0.52 mM PFA was measured in the same buffer. The extra- and intravesicular concentrations of Na^+, K^+, and H^+ were identical. Each point represents the mean±SD of three determinations.

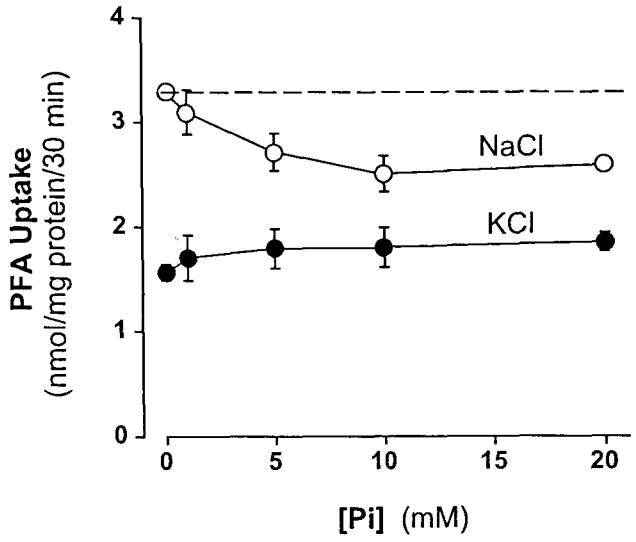

Fig. 2. Effect of P*i* on the PFA uptake by rabbit renal outer cortical BBMV. Vesicles were loaded as in Fig. 1 and the uptake of 0.52 mM PFA was determined in media containing 0-20 mM K_2HPO_4 or Na_2HPO_4. The Na^+ or K^+ concentrations in the medium were adjusted to be 100 mM. Each point is the mean±SD of three determinations.

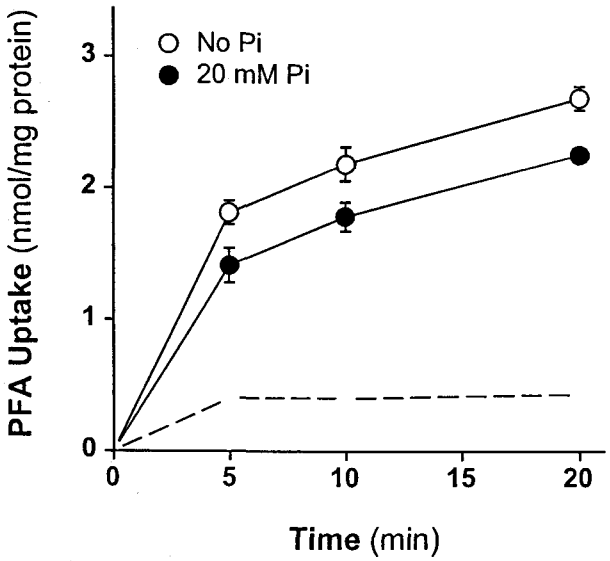

Fig. 3. Time course of PFA uptake by rabbit renal outer cortical BBMV in the presence and absence of 20 mM P*i*. Vesicles were loaded with Na^+-containing medium without P*i* and uptakes of 0.55 mM PFA were measured in 20 mM Na_2HPO_4-containing medium. Extravesicular Na^+ concentration was adjusted to be 100 mM. The dotted line representing the difference in uptake between P*i*-containing and P*i*-lacking media is equivalent to the P*i*-protectable Na^+-PFA binding. Each point represents the mean ± SD of three determinations.

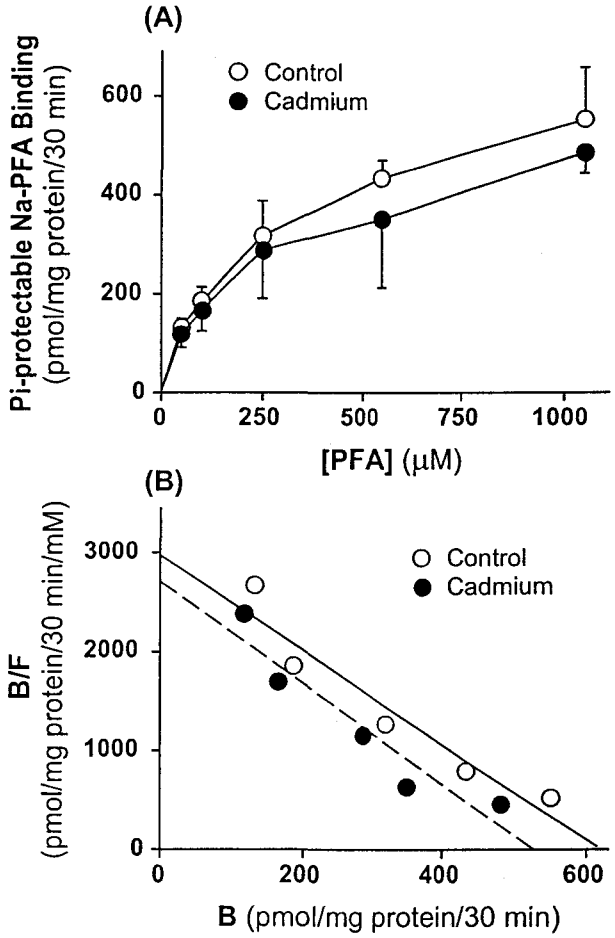

Fig. 4. (A) P*i*-protectable Na^+-PFA binding to the normal and cadmium-treated rabbit renal outer cortical BBMV as a function of PFA concentration. Vesicles were preincubated in either 0 or 50 μM $CdCl_2$-containing medium at 37°C for 30 min and the P*i*-protectable Na^+-PFA binding was determined for 30 min at 25°C in the presence and absence of 20 mM P*i* in the incubation medium. Each point represents the mean ± SD of three determinations. (B) Scatchard plots of P*i*-protectable Na^+-PFA binding to the normal and cadmium-treated rabbit renal outer cortical BBMV. Data are based on Fig. 4A. Covariance analysis indicated that the two regression lines are not significantly different in both the slop (K_d) (p=0.8) and the x-intercept (B_{max}) (p=0.07).

Fig. 3 presents the time courses of PFA uptakes by BBMV in Na^+-containing medium in the presence and absence of 20 mM P*i*. The difference between the two curves represents the P*i*-protectable Na^+-PFA binding (dotted curve). The P*i*-protectable Na^+-PFA binding reached the equilibrium level within 5 min.

Having established the basic experimental conditions for the steady-state P*i*-protectable Na^+-PFA binding to the rabbit renal BBMV, we next investigated the effect of Cd on the P*i*-protectable PFA binding. Vesicles were first treated with $CdCl_2$ for 30 min and tested for PFA binding. Fig. 4A presents the changes in steady-state P*i*-protectable PFA binding to the control and 50 μM Cd-treated BBMV as a function of PFA concentration in the medium. In both cases, the binding increased progressively as the PFA concentration increased, showing a tendency of saturation. The level of binding at a given substrate concentration was not significantly different between the two. Scatchard plots of the data (Fig. 4B) indicated that PFA binds to a single class of binding sites and that the Cd treatment does not alter the number of binding sites and their substrate affinity. The values of B_{max} (maximum binding,) and K_d (dissociation constant) in the control (B_{max}=620 pmol/mg/30 min; K_d= 0.207 mM) and Cd-treated BBMV (B_{max}=525; K_d= 0.191) were not significantly different.

Fig. 5 compares the effects of varying concentrations of Cd on P*i*-protectable Na^+-PFA binding

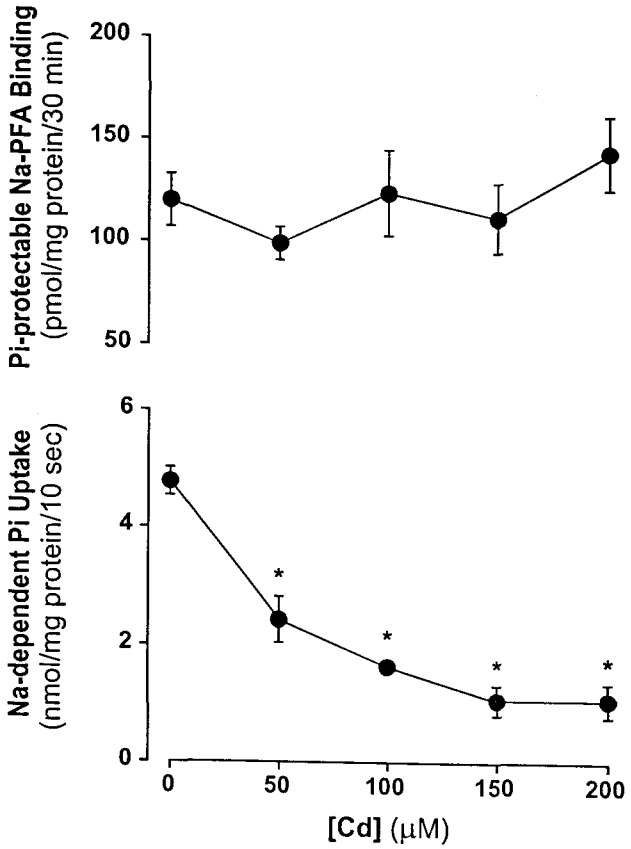

Fig. 5. Effect of increasing cadmium concentration on P*i*-protectable Na^+-PFA binding (upper panel) and Na^+-gradient-dependent P*i* transport (lower panel) to rabbit renal outer cortical BBMV. Vesicles were preincubated in $CdCl_2$-containing medium at 37°C for 30 min. P*i*-protectable Na^+-PFA binding was determined for 30 min at 25°C using 40 μM PFA and P*i* transport at 10 sec using 2 mM P*i*. Each point represents the mean ± SD of three determinations. * significantly different from the control (0 μM Cd).

(upper panel) and Na^+-dependent P*i* uptake (lower panel) by the renal BBMV. As the Cd concentration increased from 0 to 200 μM, the Na^+-dependent P*i* uptake was gradually suppressed, whereas the P*i*-protectable Na^+-PFA binding remained unchanged.

DISCUSSION

Previous studies on rabbit renal BBMV (Park et al, 1997) and *Xenopus laevis* oocytes transfectected by NaP*i*-3 (the human renal cotransporter for Na^+ and P*i*) (Wagner et al, 1996) have shown that the V_{max} of Na^+-P*i* cotransport is decreased in the membrane exposed to free Cd. Although these results suggested that the capacity for Na^+-P*i* cotransport was reduced by Cd interaction, the underlying mechanism was not identified.

In theory, the V_{max} change could represent a change in the number of transporter molecules (carriers) or it could represent a change in the rate of Na^+-P*i* translocation (i.e., the turnover rate) by existing carriers. In the present study, utilizing PFA as a probe, we investigated whether the Cd-induced impairment of Na^+-P*i* cotransport is due to a change in the number of transporter molecules in the BBM. Our results suggest that the number of Na^+-P*i* cotransporters is not likely to be changed by the Cd interaction. In renal BBMV exposed to Cd, the B_{max} and the K_d of P*i*-protectable PFA binding did not undergo significant variations (Fig. 4), although the Na^+-P*i* cotransport activity was markedly attenuated (Fig. 5). As far as the P*i*-protectable PFA binding represents the specific binding to the Na^+-P*i* cotransporter molecule (Hoppe et al, 1991), these results would indicate that the reduction of Na^+-P*i* cotransport in Cd-treated membrane is not due to a loss of carrier units from the membrane. This, in turn, suggest that the Cd-induced inhibition of Na^+-P*i* cotransport is associated with a suppression of the carrier turnover across the BBM.

The molecular mechanism by which Cd induces this change is not certain at present. It is unlikely that Cd interacts directly with the transporter molecule *per se*, as suggested by the lack of changes in kinetic parameters of Na^+-P*i* cotransport, such as the K_m for P*i*, K_m for Na^+, and Na^+: P*i* stoichiometry in Cd-exposed BBMV (Park et al, 1997). The activity of Na^+-P*i* cotransporter is sensitive to the changes in membrane fluidity, as in many other integral membrane proteins (De Smedt & Kinne, 1981; Moritolis et al, 1985; Levi et al, 1989, 1990). Since Cd induces a fall in membrane fluidity, as observed in brain synaptosomal (Fasitsas et al, 1991; Kumar et al, 1996) and placental microvillus (Boadi et al, 1992) membranes, and as indirectly suggested by an increase in lipid peroxides in Cd-treated renal cell membranes (Oner et al, 1996), Cd binding to the renal BBM could impair the Na^+-P*i* cotransporter activity possibly by altering the membrane fluidity. In fact, it has been shown that several agents that affect the membrane fluidity alter the Na^+-P*i* cotransport activity without affecting PFA binding. For instance, the exposure of renal BBMV to benzyl alcohol, which increases

membrane fluidity, enhances Na^+-P*i* cotransport with no change in PFA binding (Yusufi et al, 1989), and the exposure of BBMV to cholesterol results in decreased membrane fluidity and a subsequent fall in Na^+-P*i* cotransport without any change in the PFA binding (Yusufi et al, 1989; Levi et al, 1990). Thus, we presume that the reduction in Na^+-P*i* cotransport capacity in Cd-exposed BBMV is due to an impaired turnover of the existing carriers across the membrane, a phenomenon most likely associated with a reduction in membrane fluidity. Further studies are required to delineate the direct relationship between the membrane fluidity and the Na^+-P*i* cotransport kinetics in Cd-exposed BBMV. Whether Cd alters carrier turnover by mechanisms other than membrane fluidity change is not known.

Regardless of the mechanism, the inhibition of Na^+-P*i* cotransporter activity induced by a direct interaction of Cd with BBM may partly account for the development of phosphaturia in Cd-intoxicated animals. During chronic exposure, Cd is gradually accumulated in the proximal tubular cell as CdMT complex, which is then hydrolyzed by lysosomal enzymes, releasing free Cd ions into cytoplasm (Webb & Etienne, 1977; Fowler & Nordberg, 1978; Squibb & Fowler, 1984; Min et al, 1986; Dorian et al, 1992). These Cd may interact with the BBM as well as intracellular binding sites. Some of the intracellular bindings may lead to an inhibition of *de novo* synthesis of Na^+-P*i* cotransporter protein, causing a fall of the carrier density in the membrane (Herak-Kramberger et al, 1996). On the other hand, the interaction of Cd with BBM may suppress the activity of the existing carriers, perhaps through alterations of membrane fluidity. We believe that such interaction may occur *in vivo* in Cd-intoxicated animals, as the Cd concentrations altering the Na^+-P*i* cotransport activity *in vitro* (Ahn & Park, 1995; Park et al, 1997; Ahn et al, 1999) are comparable to the range (13 μg/g cortex, ~115 μM) of the unbound Cd observed in renal cortical tissues of Cd-exposed animals at the time of onset of renal dysfunction (Nomiyama & Nomiyama, 1986).

In summary, the results of the present study indicate that in renal cortical BBMV directly exposed to free Cd, the Na^+-P*i* cotransport activity is attenuated with no significant changes in PFA binding. These results suggest that the impaired Na^+-P*i* cotransport in Cd-exposed BBMV is not due to a loss of transport carriers, but is associated with a reduction in the carrier turnover rate. This may be one of the mechanisms inducing phosphaturia in long-term Cd exposed humans and animals.

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