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Nanomolar concentrations of Cd^{2+} inhibit Ca^{2+} transport systems in plasma membranes and intracellular Ca^{2+} stores in intestinal epithelium

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The interactions of Cd^{2+} with active Ca^{2+} transport systems in rat intestinal epithelial cells have been investigated. ATP-driven Ca^{2+} transport in basolateral plasma membrane vesicles was inhibited by Cd^{2+} with an I_{50} value of 1.6 nM free Cd^{2+} at 1 μM free Ca^{2+}, using EGTA and HEEDTA to buffer Ca^{2+} and Cd^{2+} concentrations, respectively. The inhibition was competitive in nature since the K_m value of Ca^{2+} increased with increasing Cd^{2+} concentrations while the V_{max} remained constant. Cd^{2+} had similar effects on ATP-dependent Ca^{2+} uptake by permeabilized enterocytes, indicating that non-mitochondrial and mitochondrial Ca^{2+} stores are also inhibited by nanomolar concentrations of Cd^{2+}. We conclude that ATP-driven Ca^{2+} transport systems are the most sensitive elements so far reported in Cd^{2+} intoxication.

1. Introduction

Exposure to Cd^{2+} results in disturbances in Ca^{2+} homeostasis of the body. The most pronounced effect is skeletal deformation due to Ca^{2+} mobilization from bone as a consequence of decreased active Ca^{2+} absorption in the intestine [1–3]. Active transcellular Ca^{2+} transport consists in passive Ca^{2+} entry across the brush-border membrane, diffusion of Ca^{2+} through the cytosol mediated by a vitamin D-dependent Ca^{2+} binding protein (CaBP) and ATP-driven efflux across the basolateral plasma membrane (for review, see Ref. 4). Ca^{2+} influx into intestinal cells is inhibited by Cd^{2+} but a rather low Cd^{2+} sensitivity was observed [5,6]. It was also demonstrated that Cd^{2+} entered the enterocytes [6]. Binding of Cd^{2+} to Ca^{2+} binding protein with similar affinity as Ca^{2+} has been reported [7,8]. In addition, Cd^{2+} reduced Ca^{2+} binding protein concentrations in chick duodenum [8]. Information on Cd^{2+} interference with the Ca^{2+}-pumping ATPase in the basolateral membrane is not available. It is also unknown whether Cd^{2+} interacts with non-mitochondrial or mitochondrial Ca^{2+} stores in enterocytes. Therefore, the effect of Cd^{2+} on ATP-dependent Ca^{2+} transport in plasma membrane and intracellular stores was studied. Since Cd^{2+} is also very nephrotoxic the renal plasma membrane Ca^{2+} pump was included in our study. We report here an unanticipated high affinity for Cd^{2+} of ATP-dependent Ca^{2+} transport systems in both the intestine and kidney.

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; HEEDTA, *N*-(2-hydroxyethyl)-ethylenediamine-*N*,*N*′,*N*′-triacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DDT, dithiothreitol.

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Materials and Methods

2.1. Plasma membrane preparations

Male Wistar rats (180–200 g) were killed by cervical dislocation. The first 15 cm of the small intestine was removed and rinsed with ice-cold saline containing 1 mM dithiothreitol. Isolation of enterocytes and basolateral plasma membrane vesicles have been described in detail [9]. Kidneys from three male rats were removed and decapsulated. Cortical slices were homogenized and basolateral membranes were purified as previously described [10]. The purification factors for $(Na^+ + K^+)$-ATPase in basolateral membrane preparations of rat duodenum and renal cortex were similar to those previously reported [9,10].

2.2. Preparation of permeabilized enterocytes

Isolation and permeabilization of duodenal enterocytes was done as before with the following modifications [13]. Everted pieces of rat duodenum were tied onto rods and vibrated for 20 min in 150 mM NaCl containing 2.5 mM EDTA. Cell aggregates were collected at $200 \times g \times 5$ min and incubated for 30 min at 25 °C in a shaking waterbath in a medium containing (mM): 120 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 10 Hepes, 10 EGTA, 15 glucose, 1 dithiothreitol, 0.1% (w/w) bovine serum albumin and 1 mg/ml hyaluronidase. The suspension was gassed with 100% O_2. Saponin was used to permeabilize the isolated cells as previously described [13]. Trypan blue (0.5%) tests indicated 80% leaky cells after 10 min incubation at 25 °C with 30 μg/ml saponin.

2.3. ^{45}Ca uptake experiments

ATP-dependent Ca^{2+} uptake in basolateral membrane vesicles was done as described previously [9–11]. The final concentrations during uptake experiments were (mM): 150 KCl, 20 Hepes-Tris (pH 7.4), no ATP or 3 ATP, 0.5 EGTA, 0.5 HEEDTA, an amount of calculated $CaCl_2$ to bring the free Ca^{2+} concentration to the desired level (0.025 to 10 μM) and a calculated amount of $MgCl_2$ to keep the free Mg^{2+} concentration fixed at 1.5 mM. The free Ca^{2+} and Mg^{2+} concentrations were calculated as previously described [10]. The medium contained 3 μCi/ml ^{45}Ca. To study the effect of Cd^{2+} on ATP-dependent Ca^{2+} uptake the free Cd^{2+} concentration was varied between 10^{-10} and 10^{-8} M. The free Cd^{2+} concentration was calculated as described by Van Heeswijk et al. [10], using the following binding constants of Cd^{2+} for EGTA, HEEDTA, and ATP: 14.6, 13.0 [12] and 5.43 (log values). The binding constant of Cd^{2+} for ATP was determined by titration using a Cd^{2+} selectrode (Radiometer, F3000). The ^{45}Ca uptake was stopped by adding aliquots to 1 ml ice-cold stop solution (uptake medium + 0.1 mM $LaCl_3$). Membranes were collected by rapid filtration.

ATP-dependent Ca^{2+} uptake by permeabilized enterocytes was measured as recently described [13]. The final concentrations during uptake experiments were (mM): 120 KCl, 1.2 KH_2PO_4, 5 pyruvate, 5 succinate, 0.5 EGTA, 0.5 HEEDTA, zero or 10 ATP, 10 creatine phosphate, 10 U/ml creatine kinase, 5 μCi/ml ^{45}Ca and pH 7.4 (adjusted with KOH). The free Ca^{2+} concentrations studied were 0.1 and 1.0 μM. The free Mg^{2+} concentration was kept at 1.5 mM. The free Cd^{2+} concentration was varied between 10^{-10} and 10^{-8} M and calculated as above. The ^{45}Ca uptake was stopped by adding aliquots to 1 ml ice-cold stop solution (150 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes-Tris (pH 7.4) and 1 mM EGTA). Cells were collected by rapid filtration (ME25, 0.45 μm).

2.4. Materials

MgATP, oligomycin, antimycin, saponin, EGTA, HEEDTA, dithiothreitol were from Sigma (St. Louis, MO). $^{45}CaCl_2$ (±10 mCi/mg) was purchased from New England Nuclear (Dreieich, F.R.G.). All other chemicals were analytical grade and obtained from commercial suppliers.

3. Results

3.1. Effect of Cd^{2+} on the plasma membrane Ca^{2+}-pump

ATP-dependent Ca^{2+} uptake in basolateral membrane vesicles from rat duodenum was extremely sensitive to Cd^{2+} as shown in Fig. 1. An apparent I_{50} value of 1.6 nM free Cd^{2+} at 1 μM free Ca^{2+} can be derived from the data in Fig. 1. A further kinetic analysis of Cd^{2+} inhibition of ATP-dependent Ca^{2+} transport is shown in Fig. 2. The inhibition by Cd^{2+} is clearly competitive since

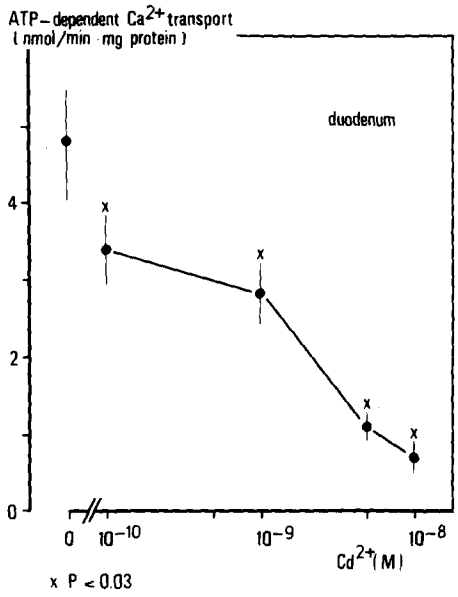

Fig. 1. Inhibition by Cd^{2+} of ATP-dependent Ca^{2+} transport in basolateral plasma membrane vesicles of rat duodenum (BLMV). The points represent mean values ± S.E. of 1 min uptakes at 1 μM free Ca^{2+} from at least five experiments in duplicate. Significance was tested with Mann-Whitney U-test ($P < 0.05$).

the affinity for Ca^{2+} decreased with increasing Cd^{2+} concentrations while the V_{max} is not influenced (Table I).

To find out whether the high affinity inhibition of Ca^{2+}-pumping ATPase by Cd^{2+} is typical for the intestinal Ca^{2+}-pump, we tested the effect of Cd^{2+} on the Ca^{2+}-pump in renal basolateral membranes. The results are shown in Fig. 3. As in Fig. 1 the renal Ca^{2+}-pump is also inhibited by Cd^{2+} with an apparent I_{50} value of 1.8 nM at 1 μM free Ca^{2+}. Since one of us (P.M.V.) found an identical I_{50} value for Cd^{2+} inhibition of ATP-driven Ca^{2+} uptake in a plasma membrane preparation of trout gill, these results suggest that the ubiquitous plasma membrane Ca^{2+}-pump has an affinity for Cd^{2+} two orders of magnitude higher than for Ca^{2+}.

TABLE I

INFLUENCE OF Cd^{2+} ON KINETIC PARAMETERS OF ATP-DEPENDENT Ca^{2+} TRANSPORT

K_m and V_{max} values were derived from Eadie-Hofstee plots; free Ca^{2+}-concentrations were varied around the apparent K_m values.

Cd^{2+} concentration	K_m [a]	V_{max} [b]	n
0 (control)	0.07 ± 0.01	4.60 ± 0.40	12
10^{-9} M	0.36 ± 0.01 *	4.60 ± 0.57	5
$5 \cdot 10^{-9}$ M	2.25 ± 0.39 *	4.20 ± 0.96	5

[a] K_m in μM Ca^{2+}.
[b] V_{max} in nmol Ca^{2+}/min per mg protein.
* $P < 0.05$.

3.2. Cd^{2+} and intracellular Ca^{2+} stores

It was recently demonstrated that permeabi-

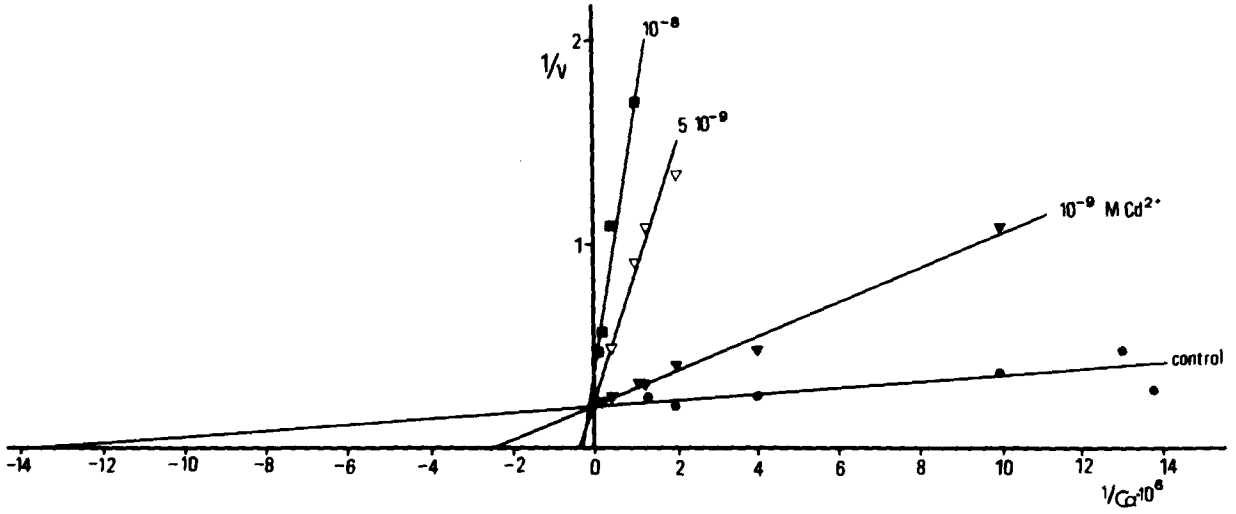

Fig. 2. Lineweaver-Burk plots of Ca^{2+} concentration dependence of ATP-dependent Ca^{2+} transport in rat duodenal BLM vesicles at different free Cd^{2+} concentrations. The points represent mean values of 1 min uptakes from five experiments.

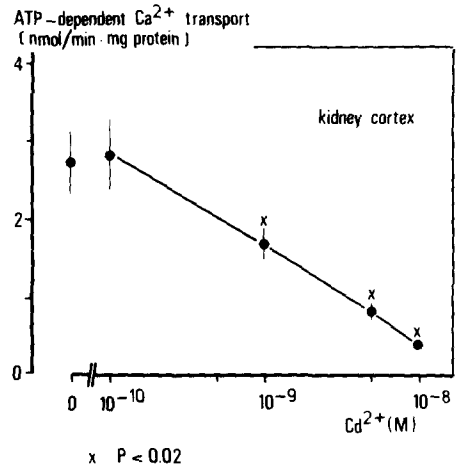

Fig. 3. Inhibition by Cd^{2+} of ATP-dependent Ca^{2+} transport in basolateral plasma membrane vesicles from rat renal cortex. Conditions are as in Fig. 1.

lized enterocytes accumulate Ca^{2+} when provided with ATP [13–15]. Discrimination between ATP-dependent Ca^{2+} uptake by mitochondrial and non-mitochondrial systems was made on the basis of the apparent half maximal activation Ca^{2+} concentrations of the respective systems. At 1.0 μM free Ca^{2+} mitochondrial Ca^{2+} uptake represented 95% of total ATP-dependent Ca^{2+} uptake as indicated by a 95% inhibition of uptake by mitochondrial inhibitors; non-mitochondrial uptake accounts for a minor portion of the total ATP-dependent Ca^{2+} uptake as indicated by a 22% inhibition of Ca^{2+} uptake by vanadate. At 0.1 μM free Ca^{2+} the ratio of the mitochondrial/non-mitochondrial Ca^{2+} uptake is exactly reversed. Kinetic analysis of non-mitochondrial Ca^{2+} uptake revealed a K_m value of 0.1 μM Ca^{2+} [13]. Mitochondria started to take up Ca^{2+} at 0.3 μM free Ca^{2+} [13]. Therefore, we used 0.1 μM and 1.0 μM free Ca^{2+} to study the effect of Cd^{2+} on non-mitochondrial and mitochondrial Ca^{2+} uptake, respectively. The effect of Cd^{2+} on ATP-dependent Ca^{2+} uptake by permeabilized enterocytes is shown in Fig. 4. Ca^{2+} accumulation into intracellular Ca^{2+} stores is strongly inhibited by Cd^{2+}. The apparent I_{50} values for the non-mitochondrial and mitochondrial Ca^{2+} stores seem to be 0.2 and 0.5 nM Cd^{2+}, measured at 0.1 and 1.0 μM Ca^{2+}, respectively. The 10-fold higher sensitivity of the intracellular Ca^{2+} stores for Cd^{2+} stems from the fact that Ca^{2+} uptake studies into non-mitochondrial and mitochondrial stores were carried out at Ca^{2+} concentrations below V_{max} conditions, whereas with the plasma membranes Cd^{2+} effects were studied under V_{max} conditions.

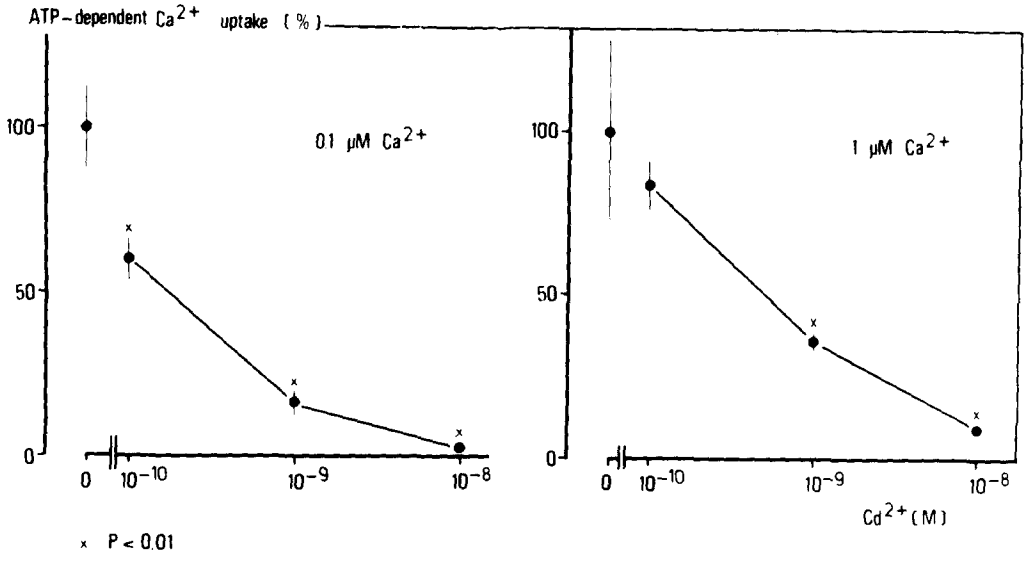

Fig. 4. Inhibition by Cd^{2+} of ATP-dependent Ca^{2+} uptake by permeabilized rat enterocytes. Uptake at 0.1 μM free Ca^{2+} represents uptake in non-mitochondrial stores. At 1.0 μM free Ca^{2+} uptake is predominantly into mitochondria. Points represent mean values ± S.E. of 1 min uptakes from at least four experiments.

4. Discussion

The present in vitro study indicates that Ca^{2+} binding sites on active Ca^{2+} transport systems have an unprecedented high affinity for Cd^{2+}. The affinity for Ca^{2+} of Ca^{2+}-ATPases in plasma membranes and endoplasmic reticulum is around 100 nM (Refs. 9, 10 and 13, this study Table I), but the I_{50} value for Cd^{2+} is about 1 nM at 1 μM free Ca^{2+}. It is interesting to note that the voltage-dependent Ca^{2+} channel in synaptosomes has an affinity for Ca^{2+} of 0.3 mM and an I_{50} value for Cd^{2+} around 1 μM [16]. This comparison indicates that extracellular as well as intracellular Ca^{2+} binding sites on Ca^{2+} transport systems have affinities for Cd^{2+} two orders of magnitude higher than for Ca^{2+}. In contrast, Ca^{2+} binding sites on calmodulin and Ca^{2+} binding protein have equal affinities for Ca^{2+} and Cd^{2+} around 1 μM [17–19]. This difference in Cd^{2+} affinities of Ca^{2+} binding sites on calmodulin and Ca^{2+}-ATPase excludes the possibility that Cd^{2+} inhibition of ATP-driven Ca^{2+} transport is realized via Cd^{2+}-calmodulin. This difference also indicates that Ca^{2+} binding sites on calmodulin and Ca^{2+} binding protein are structurally different from those on Ca^{2+}-ATPases. In one study a non-competitive inhibition of calmodulin-dependent (Ca^{2+} + Mg^{2+})-ATPase activity of erythrocyte ghost was reported by μmolar concentrations of Cd^{2+} [20]. These authors noted that the free Cd^{2+} concentration in the assay medium must be significantly lower than the total concentration added, due to complex formation with various anions. In addition, these authors measured ATP-hydrolysis whereas we measured ^{45}Ca translocation. It is entirely possible that Cd^{2+} ions in the nmolar range are transported by the Ca^{2+}-ATPase, while inhibition of ATP-hydrolysis occurs at higher Cd^{2+} concentrations as reported by Åkerman et al. [20]. There is no information on the actual free Cd^{2+} concentration in intestinal and renal cells after Cd^{2+} exposure of rats. It is known that Cd^{2+} induces synthesis of metallothionein (MT) in these cells [7,21]. Metallothioneins are low molecular weight proteins with an exceptionally high content of SH-groups with high affinity for metal ions [22]. It was suggested that metallothioneins protect these cells against toxic effects of Cd^{2+} [22]. Binding constants for Cd^{2+} to metallothioneins have not been reported so far. Therefore, it remains to be demonstrated whether Ca^{2+}-transport ATPases are also inhibited by Cd^{2+} when metallothioneins are present in the cytosol.

The results of the present study suggest that the basolateral Ca^{2+} efflux pathway is the most sensitive element in the transcellular route for Ca^{2+} in connection with Cd^{2+} intoxication. It is therefore likely that inhibition of intestinal Ca^{2+} absorption is realized via competition between Ca^{2+} and Cd^{2+} for the Ca^{2+} binding site on the Ca^{2+}-pumping ATPase. At the same time Ca^{2+} uptake into intracellular Ca^{2+} stores is inhibited. Both events will eventually result in increased free cytosolic Ca^{2+} levels. Intestinal and renal cells employ Ca^{2+} as an intracellular messenger [4,23]. An increase in cytosolic Ca^{2+} reduces intestinal salt and water absorption [23]. Since Cd^{2+} also inhibits water transport in rat duodenum [6], it is possible that an increase in cell Ca^{2+} mediates Cd^{2+} inhibition of fluid absorption. For renal cells it was recently demonstrated that cell Ca^{2+} increased after Cd^{2+} administration to rats [24]. Also this effect of Cd^{2+} can be explained by Cd^{2+} interference with Ca^{2+}-pumping ATPases.

In conclusion, Cd^{2+} administration may upset intracellular Ca^{2+} homeostasis in view of the extreme sensitivity of the Ca^{2+}-pumping ATPases in plasma membranes and endoplasmic reticulum.

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