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Kinetic characterization of zinc binding to brush border membranes from rat kidney cortex: interaction with cadmium

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Abstract

Extravesicular and intravesicular zinc bindings were evaluated in brush border membrane vesicles isolated from rat kidney cortex. The process was found to be time-, temperature- and substrate concentration-dependent and displayed saturability. Zn^{2+} influx measurements revealed a progressive uptake and massive accumulation at equilibrium which was 50 times higher than the amount that could have been accommodated by the intravesicular space calculated from the equilibrium uptake of D-glucose. Initial (5 s) and equilibrium uptakes (2 h) were found not to be osmotically sensitive as modified by adding mannitol to the medium. It was concluded from these results that the uptake involved massive binding of the Zn^{2+} to the brush border membranes components. The ionophore A23187 enhanced the rates of uptake and efflux of Zn^{2+} without affecting equilibrium values, suggesting binding of Zn^{2+} to interior sites of the membranes. Zn^{2+} flux measurements led to the conclusion that two vesicular pools of Zn^{2+} bindings existed: a small external pool, accessible to cation chelator (EGTA) or competitive cation cadmium and large intravesicular pool. Accumulated ^{65}Zn was quickly removed from its internal sites only after the membrane had been permeabilized by the cation ionophore A23187 in association with exchangeable ions like zinc and cadmium. Scatchard plot analysis revealed two distinct types of extravesicular binding sites. High affinity extravesicular zinc binding sites reached saturation at 1.6 mM zinc, had a K_d of 137 μM and the number of binding sites were 12 nmol/mg protein. Low affinity extravesicular zinc binding sites could not be saturated under experimental conditions up to 3.2 mM zinc. It had a K_d of 526 μM and the number of binding sites 28 nmol/mg protein. Interestingly intravesicular binding of zinc revealed only one type of high affinity binding sites (K_d of 104 μM and number of maximal binding sites 400 nmol/mg protein). Furthermore, kinetic analysis of inhibitory effect of Cd^{2+} on extravesicular zinc bindings showed an increase in K_d of both types of binding sites but there was no significant change in number of maximal binding sites. Extravesicular zinc binding was temperature-sensitive. Arrhenius plot showed the break point at 30°C. The apparent energies of activation were 13.36 Kcal/mol and 3.1 Kcal/mol below and above the break points respectively. The inhibitory effect of sulfhydryl blocking agents on extravesicular zinc binding suggest the involvement of -SH groups in zinc translocation. An increase in initial zinc uptake was observed in the presence of outwardly directed proton gradient. Intravesicular pool of ^{65}Zn was displaced by unlabelled 2 mM Zn^{2+} or 2 mM Cd^{2+} but not by calcium present in the bathing medium. It is inferred that intravesicular binding sites have a high affinity and are specific for zinc. It is concluded from the present study that in the first instance the binding of zinc to the exofacial zinc binding component and concomitantly its translocation across the membrane, and subsequently massive binding of zinc to interior sites of brush border membranes occurs.

Keywords: Zinc binding; Zinc binding site; Ionophore A23187; Cadmium; Brush border membrane (rat kidney cortex)

1. Introduction

Zinc plays a fundamental role in the expression of genetic potential, i.e., the synthesis, repair and structural integrity of nucleic acids. Thus zinc is indispensable for numerous physiological processes in humans, including growth, development, function of the endocrine, immune and nervous systems [1–4]. Zinc is also involved in stabilizing membrane structure and in protection at the cellular level by preventing lipid peroxidation and reducing free radical formation [5]. A number of clinical conditions predispose individuals to zinc deficiency [6]. Reduced serum zinc concentrations have been observed particularly in patients with severe renal failure and nephrotic syndrome [6,7]. There is also accumulating evidence that diabetes mellitus may lead to mild zinc deficiency [8]. In addition patients with stress, trauma and a variety of malignancies have low serum levels and high urinary zinc

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concentrations [9–11]. All these zinc deficiencies are currently thought to be associated with renal tubular zinc reabsorption defect.

Intestine, liver and kidney are of particular importance in maintaining the homeostasis of zinc. Intestine plays a role in zinc absorption, whereas liver plays a central role in the distribution and excretion of the metals. The sensitivity of the kidneys to metals is well documented [12]. In addition the extent of renal excretion of such metals plays a major part in determining their accumulation in the body. In spite of these facts, the nature of the reaction between kidney and trace metals remains unclear and relatively little is known about the mechanism of reabsorption and regulatory events which control movement of zinc across the renal epithelial cells.

Most of the knowledge concerning the transport and binding of zinc has been based on studies with intestinal brush border membrane vesicles [13–15]. However, very little is known about this process as it occurs in the renal BBM. We have previously reported the kinetics of zinc transport in monkey renal brush border membrane vesicles (BBMV) which showed that the uptake of Zn^{2+} was saturable, temperature-sensitive and competitively inhibited by cadmium [16]. A large degree of zinc binding to brush border membrane (BBM) was also observed at zero intravesicular space. In the present study, further attempts are made to characterize the phenomenon of zinc binding to extravesicular and intravesicular sites of BBMV from rat kidney cortex and possible interactions with cadmium and calcium.

2. Materials and methods

2.1. Chemicals

$^{65}Zn\ Cl_2$ (spec. act. 183 mCi/g Zn) and D-[U-^{14}C]glucose (specific activity 292 mCi/mmol) were purchased from Bhabha Atomic Research Centre, Trombay, Bombay. Ionophore A23187, N′-2-hydroxy-ethylpiperazine-N-2-ethanesulfonic acid (Hepes), sodium salt of 2-(N-morpholino) ethane sulfonic acid (MES) and ethylene glycol bis (ß aminoethyl ether)-N,N,N′,N′-tetracetic acid (EGTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Millipore filters (Pore size 0.65 μm) were obtained from Millipore Corporation (Bedford MA, USA). Glucose oxidase peroxidase kit was procured from Boehringer-Knoll, Germany. All other chemicals were analytical reagent grade compounds obtained from commercial sources.

2.2. Animals

Young male Wistar strain rats, weighing 150–200 g were obtained from Animal breeding colony of the Institute. The animals were acclimatised to laboratory conditions before commencement of the experiment for few days. All animals were housed individually in plastic cages with stainless steel lids, fed a standard rat pellet diet (Hindustan Lever Ltd, Bombay, India) and were given water ad libitum. The animals had a 12-h light–dark cycle (06.00–18.00 and 18.00–06.00 h), respectively.

2.2.1. Preparation of brush border membrane vesicles

The animals were sacrificed under anaesthetic ether. Rat renal cortical brush border membrane vesicles were prepared by differential centrifugation in the presence of 4 mM $MnCl_2$ [17,18]. The cortices from the kidneys were dissected and homogenized in 30 volumes (w/v) of ice-cold 50 mM mannitol buffered with 15 mM Hepes KOH (pH 7.0). All preparative steps were carried out at 4°C. $MnCl_2$ was added to a final concentration of 4 mM and the mixture was stirred in an ice bath for 10 min. The suspension was centrifuged at $4000 \times g$ for 15 min. The supernatant was taken and centrifuged at $43\,000 \times g$ for 20 min. The pellet material was suspended in 300 mM mannitol, 15 mM Hepes KOH (pH 7.5) and transferred to homogenizing tube and homogenized three times manually. The suspension was centrifuged again at $43\,000 \times g$ for 20 min. The resulting pellet was resuspended in 300 mM mannitol and 15 mM Hepes adjusted to pH 7.0 with KOH for Zn^{2+} binding studies. For glucose uptake the pellet was resuspended in 300 mM mannitol, 15 mM Hepes adjusted to pH 7.5 with KOH. Purity of the membrane was checked by measuring changes in the specific activities of marker enzymes of brush border membranes and in original homogenate. Alkaline phosphatase (EC 3.1 3.1) was assayed by monitoring the production of p-nitrophenol from p-nitrophenyl phosphate [19] and maltase (EC 3.2.1.20) activity was determined by measuring D-glucose liberated from the maltose using glucose oxidase-peroxidase system of Dahlqvist [20]. The contamination of basolateral membranes was checked by assaying Na^+-K^+-ATPase (EC 3.6.1.3) which was measured by the method of Quigley and Gotterer [21]. Protein content was determined by the method of Lowry et al. [22] after solubilization of the sample in 1% sodium lauryl sulfate as described earlier [16]. In the present study, the majority of vesicles were closed and spherical. Maltase and alkaline phosphatase, brush border marker enzymes, were enriched 14- to 16-fold in the isolated brush border membrane. The enrichment of Na^+-K^+-ATPase activity was 0.43-fold, indicating a negligible contamination of basolateral membranes in the preparation.

2.3. Uptake and binding measurements

2.3.1. D-Glucose uptake

Uptake of D-glucose was measured by the rapid Millipore filtration technique described previously [16]. In the presence of sodium gradient $[Na^+]_o > [Na^+]_i$, D-glucose uptake showed a transient overshoot of the intravesicular

glucose concentration over its equilibrium uptake which is the best functional property of BBMV for uptake studies.

2.3.2. Time course of zinc uptake

10 μl of BBMV (80–140 μg protein) prepared in 300 mM Mannitol, 15 mM Hepes-KOH (pH 7.0) were incubated in 90 μl of uptake buffer containing 150 mM KCl, 15 mM Hepes-KOH (pH 7.0), 1 mM $^{65}ZnCl_2$. In another type of experiment, when the effect of ionophore A23187 on zinc uptake was tested, the ionophore was added to incubation medium at a final concentration of 10 μM. At various time intervals up to 2 h, the reaction was stopped by the addition of 3 ml of an ice-cold stop solution consisting of 150 mM KCl, 15 mM Hepes-KOH (pH 7.0) with or without 5 mM EGTA. Then the filters were rinsed twice with this solution as described above. Radioactivity of the filters was measured by autogamma scintillation counter (1282 Compugamma, Universal Gamma Counter).

2.3.3. Influence of medium osmolarity on zinc uptake

The effect of medium osmolarity on zinc uptake at 5 s and 2 h was studied by increasing the concentration of D-mannitol (100–800 mM) to give different osmolarity.

The osmolarity was calculated as the sum of contribution from all compounds assuming ideal behaviour. 10 μl of brush border membrane vesicles (80–140 μg protein) were incubated with uptake buffer of different osmolarity at 25°C, 5 s and 2 h uptake were terminated by stop solution with and without 5 mM EGTA respectively. The vesicles were washed twice and the radioactivity retained by the filters was determined.

2.3.4. pH effects on initial zinc uptake

Zinc uptake by BBMV in initial velocity conditions (5 s incubations) was measured as a function of pH. Uptake of 1 mM ^{65}Zn was measured at 25°C in the presence of different pH gradients. Four pH levels were tested (7.0, 6.0, 5.0 and 4.0). After reconstitution in different buffers, brush border membrane vesicles were kept on ice for 2 h for equilibration. pH of the intra or extravesicular medium was modified as follows:

(a) Inwardly directed proton gradient: 10 μl BBMV (100–150 μg protein) prepared in 300 mM mannitol, 15 mM Hepes-KOH pH (7.0) were incubated with 90 μl uptake buffers of different pH (7.0, 6.0, 5.0 and 4.0) at 25°C.

(b) Outwardly directed proton gradient: 10 μl BBMV (100–150 μg protein) prepared in the buffers of different pH (7.0, 6.0, 5.0, 4.0) were then incubated with 90 μl uptake buffer (pH 7.0), at 25°C.

Uptake buffers of pH 4.0, 5.0 and 6.0 were prepared with 15 mM 2(N-morpholino ethanesulphonic acid) (MES)-KOH and the uptake buffer of pH 7.0 was made in 15 mM Hepes-KOH. Uptakes of $^{65}ZnCl_2$ were performed as described above.

2.4. Scatchard plot analysis

2.4.1. Extravesicular binding

In order to determine the kinetic constants, i.e., number, affinity and rate constant of the extravesicular zinc binding sites, the zinc uptakes under equilibrium condition (2 h incubation time) as a function of increasing $^{65}ZnCl_2$ concentrations (0.1–3.2 mM) were determined. The carrier-mediated uptake process is competitively inhibited by structurally related compounds. In view of this phenomenon and also the well known interaction between zinc and cadmium/calcium, uptake were carried out in the presence of 2 mM $CdCl_2$/2 mM $CaCl_2$. 2 h uptakes were stopped by stop solution with or without 5 mM EGTA. The difference between the uptake in the absence or presence of the EGTA corresponded to zinc bound to extravesicular sites.

2.4.2. Intravesicular binding

10 μl BBMV (100–150 μg protein) prepared in 300 mM mannitol, 15 mM Hepes-KOH (pH 7.0) were incubated in 90 μl of buffer containing increasing $^{65}ZnCl_2$ (0.1 to 3.0 mM) at 25°C. After 2 h incubation, when zinc uptake was at equilibrium, the uptake was stopped by 30-fold dilution with the ice-cold stop solution containing 5 mM EGTA. The vesicles were filtered and rinsed with the same solution and the radioactivity associated with the vesicles was measured at each $^{65}ZnCl_2$ concentration. This uptake reflected the intravesicular bound zinc.

2.5. Zinc efflux studies

To load the BBMV with ^{65}Zn, 10 μl of BBMV (80–120 μg protein) was incubated with buffer containing 150 mM KCl, 15 mM Hepes-KOH (pH 7.0), 0.3 mM $^{65}ZnCl_2$ at 25°C for 2 h. 100 μl suspension (80–120 μg protein) was then diluted in 2.5 ml of different efflux solutions (composition is detailed in the figure legends). At specified times, the mixture was filtered and then the filters were rinsed twice with ice-cold stop solution containing 5 mM EGTA. Zinc efflux rate was expressed as percent of equilibrium uptake. In another set of experiments, the effect of $CaCl_2$ or $CdCl_2$ or $ZnCl_2$ at a final concentration of 2 mM in the diluting medium was also studied. In these experiments, both the vesicles and efflux medium contained 10 μM ionophore A23187. The stop solution containing 5 mM EGTA was used.

2.6. Influence of temperature on extravesicular zinc binding

5 s extravesicular zinc binding was measured at 10°, 20°, 30°, 40° and 50°C in the presence of 1 mM $^{65}ZnCl_2$. The same procedure was used as described in the extravesicular zinc binding section. The results were plotted according to the Arrhenius plot. From the slope of lines, activation energy was calculated using the Arrhenius equation as described earlier [16].

2.7. Effect of sulfhydryl and carboxyl reacting reagents on extravesicular zinc binding

To assess the involvement of sulfhydryl and carboxyl groups in the extravesicular zinc binding, sulfhydryl reacting compounds, i.e., N-ethylmaleimide (5 mM), ß-mercaptoethanol (5 mM), iodoacetate (5 mM) and carboxylate reactive reagent N,N′-dicyclohexyl carbodiimide (DCCD) were added individually in the incubation medium. 5 s extravesicular zinc binding was measured as described earlier.

3. Statistical analysis

All values reported in this study are the means ± S.E. of at least six measurements on three or more separate membrane preparations.

4. Results

4.1. Zinc uptake as a function of time

Zinc uptake progressively increased with time and reached equilibrium level after 2 h of incubation (Fig. 1). Initial uptake of zinc was higher in the presence of 10 μM A23187, whereas the initial uptake was lower when vesicles were washed with stopping buffer containing 5 mM EGTA as compared to control. However, at equilibrium, zinc uptake (2 h) was similar either in the presence or absence of A23187. Uptake of zinc by BBMV was performed for short incubation periods (2 s, 5 s, 10 s) which was found to be time-dependent and linear. The effect of various concentrations of EGTA in the stop solution was examined to determine the amount of zinc reversibly bound to the outer surface of the vesicles. A substantial amount of zinc (75%) was released at all concentrations of EGTA (1–5 mM). This diminution of zinc binding to BBMV

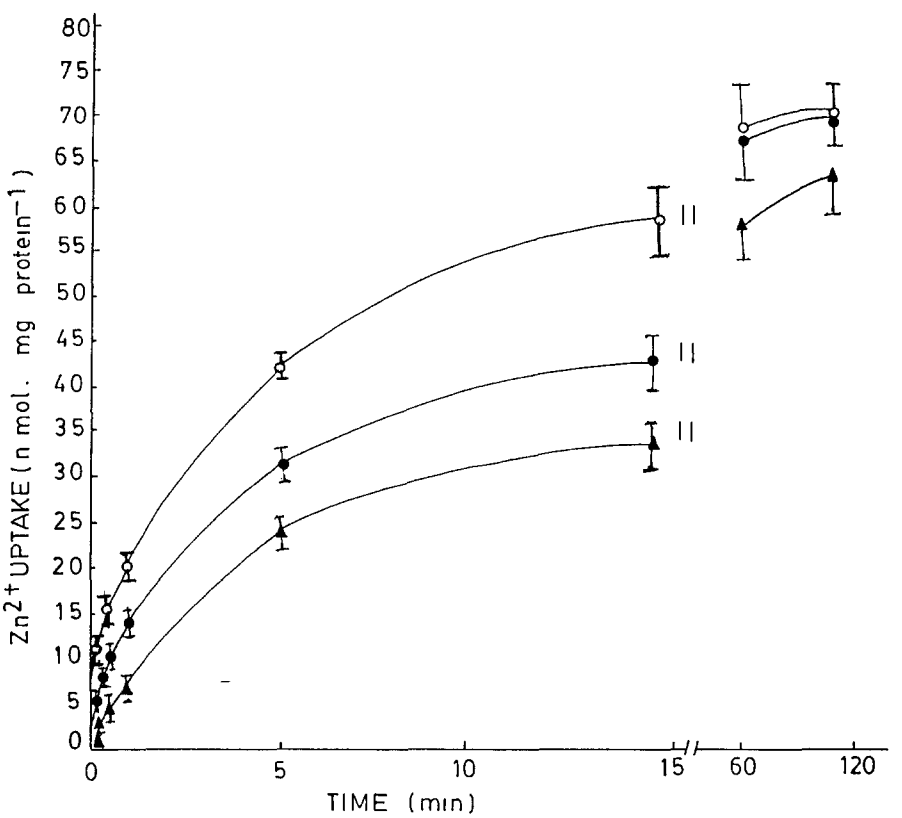

Fig. 1. Effect of ionophore A23187 and EGTA on zinc uptake: 10 μl BBMV (100–150 μg protein) prepared in 300 mM mannitol, 15 mM Hepes-KOH (pH 7.0) was incubated with 90 μl of uptake buffer containing 150 mM KCl, 15 mM Hepes KOH (pH 7.0), 1 mM $^{65}ZnCl_2$ with 10 μM A23187 (○) or without (●). The uptake was stopped by buffer containing 150 mM NaCl, 15 mM Hepes KOH (pH 7.0). In case of EGTA effect (▲), the uptake was stopped by stop solution also containing 5 mM EGTA. Vesicles were filtered and rinsed with respective stop solutions. Each data point represents the mean ± S.E. of three experiments each carried out in duplicate with three different membrane preparations.

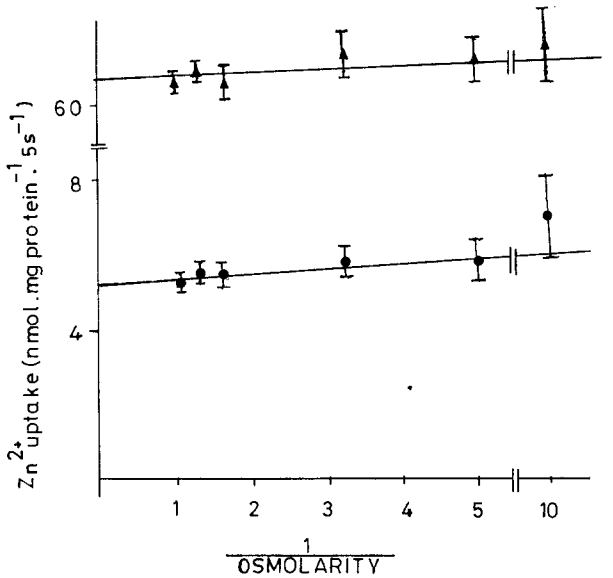

Fig. 2. Influence of increasing medium osmolarity: 10 μl BBMV (80–140 μg protein) prepared in 300 mM mannitol, 15 mM Hepes KOH (pH 7.0) were incubated for 5 s and 2 h at 25°C, in 90 μl of uptake buffer containing different concentrations of D-mannitol and 1 mM $^{65}ZnCl_2$. For 5-s uptakes (●), the vesicles were filtered and rinsed with stop solution with 5 mM EGTA or without EGTA respectively. The net value obtained by subtracting the value using stop solution with EGTA from without EGTA represented extravesicular 5-s bindings. 2-h uptakes (▲) were stopped by stop solution containing 5 mM EGTA represented intravesicular binding. Uptake values were expressed as mean ± S.E. of three experiments, each carried out in duplicate with three different membrane preparations and were plotted against the reciprocal of total osmolarity.

corresponded to displacement of zinc from its binding sites on the external surface by the chelator. EGTA complexes were then removed through the pores of the filters by washing. Thus 5 mM EGTA was used in stop solution for all transport measurements.

4.2. Influence of medium osmolarity on the zinc uptake

Mannitol cannot permeate across the brush border membrane. It has been used, therefore, to increase osmotic pressure and decrease the intravesicular space, which in turn decreases the uptake of substances not bound by the membrane and normally accumulated within the space. Results depicted in Fig. 2 revealed a complete lack of effect of mannitol on 5 s and 2 h zinc uptake, suggesting that the process represents mainly binding to the membrane. This conclusion is also substantiated by the fact that the intravesicular space is calculated to be 1.5 μl/mg protein on the basis of equilibrium uptake of D-glucose. Therefore, the vesicles can accommodate 1.5 nmol Zn^{2+}/mg protein at equilibrium. However, the equilibrium uptake noted under these conditions was approx. 75 ± 6.0 nmol/mg protein, reflecting a large degree of zinc in membrane-bound form.

4.3. Scatchard plot analysis

4.3.1. Extravesicular binding sites

The extravesicular binding of zinc was studied at equilibrium over a concentration range of 0.1 mM to 3.2 mM

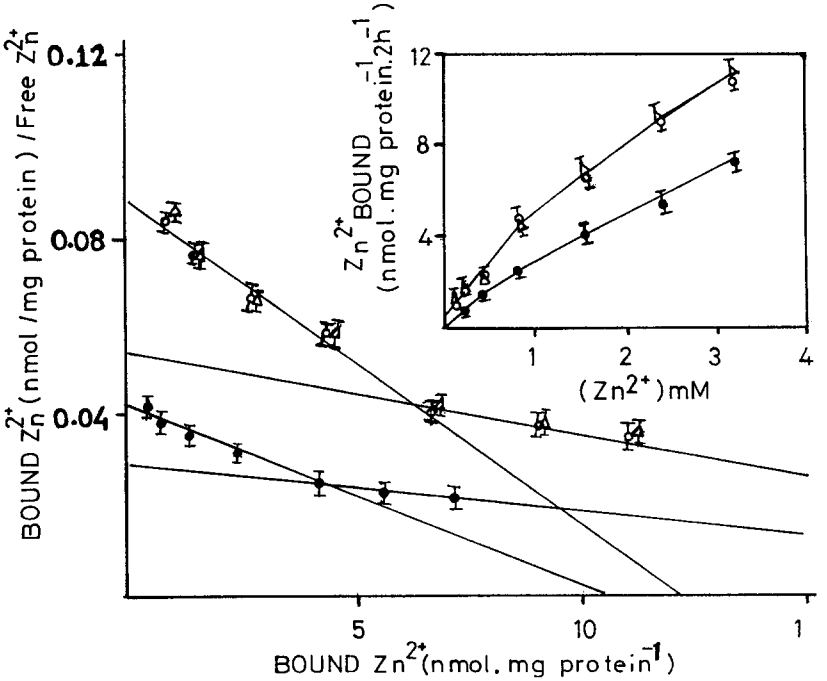

Fig. 3. Scatchard plot of Zn^{2+} extravesicular binding: effect of divalent ions Cd^{2+} and Ca^{2+}. 10 μl of BBMV (80–140 μg protein) were added to 90 μl of uptake buffer with varying concentrations of $^{65}ZnCl_2$ (0.1 mM–3.2 mM) in the presence of 2 mM $CdCl_2$(●), 2 mM $CaCl_2$ (△), absence as control (○). At 2 h, the reaction was stopped and vesicles were filtered and rinsed with stop solution with or without 5 mM EGTA. The difference of binding in the absence or presence of the chelator represented the Zn^{2+} bound to the external surface of vesicles. Inset shows extravesicular Zn^{2+} binding plotted against the corresponding zinc concentration in the absence or presence of 2 mM $CdCl_2$/2 mM $CaCl_2$. Each data point represents the mean ± S.E. of three experiments, each carried out in triplicate with different membrane preparations.

Table 1
Kinetic parameters of extravesicular zinc binding components of renal brush border membrane

	High affinity zinc binding sites		r	Low affinity zinc binding sites		r
	K_d (μM)	n (nmol · mg protein^{-1})		K_d (μM)	n (nmol · mg protein^{-1})	
Control	137	12	−0.97	526	28	−0.97
Cadmium	232	10	−0.99	800	26	−0.98
Calcium	137	12	−0.98	526	28	−0.98

Dissociation constant (K_d), maximal binding (n) and correlation coefficient (r) for different slopes were calculated from Scatchard plot (Fig. 3).

for 2 h [as shown in Fig. 3 (inset)]. Extravesicular zinc binding component is biphasic in nature. One site reached saturation at 1.6 mM of zinc with a half saturation concentration of 0.5 mM. Complete saturation of second zinc binding component was not obtained experimentally even at 3.2 mM zinc concentration. These two extravesicular zinc binding components were quantitated for kinetic parameters by Scatchard plot analysis [23] by plotting the ratio of bound zinc to free zinc against the amount of zinc bound per mg protein (Fig. 3). The curve comprised of two components, each with a different slope, indicating the presence of at least two affinity sites for zinc on the extravesicular surface. Calculated kinetic constants from Scatchard plot are given in Table 1. The high affinity site that was saturated at low concentrations of zinc, had a K_d of 137 μM while 12 nmol of zinc could interact with 1 mg protein. At the other, non-saturable low affinity site, K_d was 526 μM, while 28 nmol of zinc could interact with 1 mg of protein. In order to examine both specificity and affinity of the zinc binding sites, 2 h extravesicular zinc binding was measured in the presence of 2 mM $CdCl_2$/2 mM $CaCl_2$. Cadmium had a significant inhibitory effect on 2 h zinc binding while calcium had no effect at all. Kinetic analysis of Scatchard plot revealed that cadmium led to a significant increment in K_d of both high and low affinity sites, although no effect was observed on number of maximal binding sites of either the high affinity component or the low affinity component.

4.3.2. *Intravesicular zinc binding sites*

The Scatchard plot of intravesicular zinc binding is shown in Fig. 4. The data fitted with a single type of

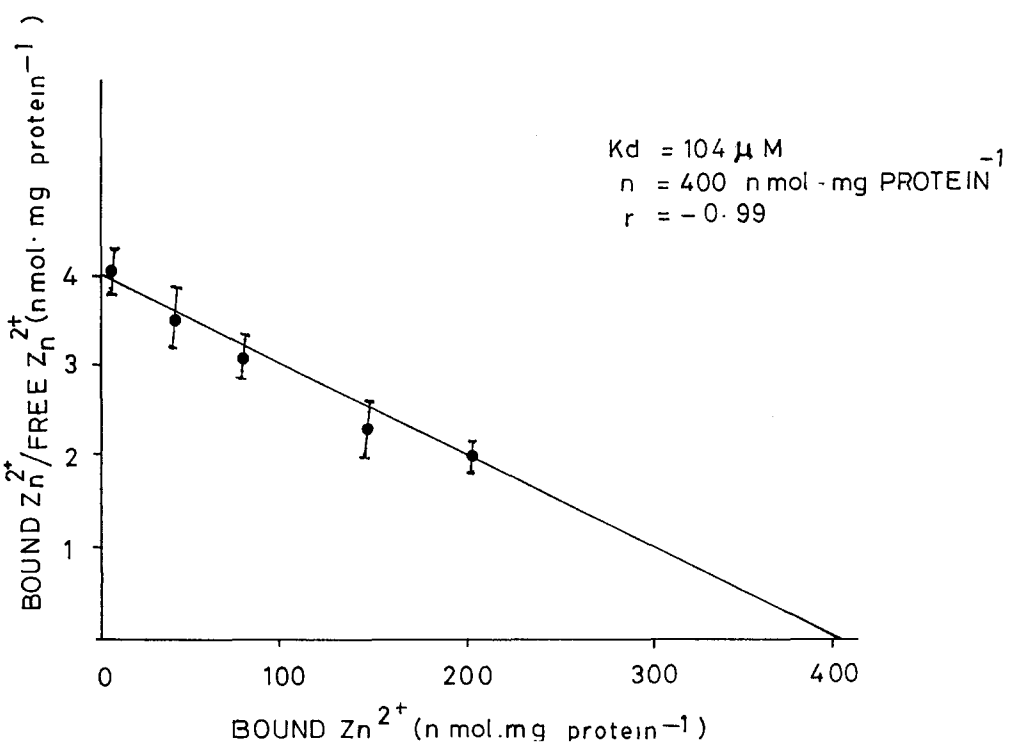

Fig. 4. Scatchard plot of Zn^{2+} intravesicular binding: 10 μl of BBMV (100–150 μg protein) were loaded with various $^{65}ZnCl_2$ concentrations (0.1–3.0 mM) in total assay volume of 100 μl for 2 h at 25°C. Then the vesicles were diluted with the ice-cold stop solution containing 5 mM EGTA, filtered and rinsed with the same solution and the radioactivity associated with the vesicles was measured. Kinetic constants were calculated from the plot which are mentioned in text. Data represents the mean ± S.E. of 3 experiments each carried out in triplicate with different membrane preparations.

intravesicular zinc binding site whose K_d was 104 μM and which bound 400 nmol Zn^{2+} per mg protein. Major differences between extravesicular and intravesicular sites revealed that intravesicular sites were much more numerous than extravesicular sites and their zinc binding affinity was even greater.

4.4. Effect of A23187 and EGTA on zinc efflux measurements

Results illustrated in Fig. 5 showed that efflux from vesicles equilibrated in the presence of 0.3 mM Zn^{2+} and diluted in media containing EGTA/A23187 or EGTA + A23187 was greatly accelerated by the presence of the ionophore. At 1 min, 50% increase in efflux due to ionophore in the presence of EGTA was seen as compared to control. These studies suggest the instantaneous removal of Zn^{2+} by chelator from interior sites of BBMV and concomitant translocation of Zn^{2+} by ionophore from inside of the vesicle to the bathing medium.

4.5. Effect of calcium, cadmium and zinc on zinc efflux

In order to determine both specificity and interactions of cadmium and calcium with zinc binding sites, we followed the displacement of accumulated isotope by either 2 mM Ca^{2+}, Cd^{2+} or Zn^{2+} present in the diluting medium (Fig. 6). In the presence of 2 mM Cd^{2+} or Zn^{2+}, exchange of the isotope was very rapid. At 5 min, 50% of accumulated $^{65}ZnCl_2$ was released into the medium containing non-labelled zinc and A23187. After 2 h, the efflux accounted for about 84% of accumulated $^{65}ZnCl_2$ in case of cadmium and 90% in case of zinc. However, there was no effect of calcium on $^{65}ZnCl_2$ release.

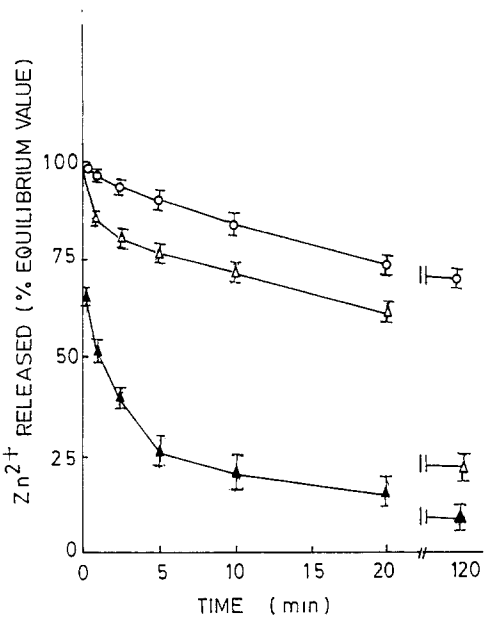

Fig. 5. Effect of ionophore A23187 and EGTA on Zn^{2+} efflux: 10 μl of BBMV (100–140 μg protein) were preloaded to equilibrium by incubation for 2 h at 25°C with buffer containing 150 mM KCl, 15 mM Hepes-KOH (pH 7.0), 0.3 mM $^{65}ZnCl_2$. The suspension (100 μl) was then diluted with 2.5 ml of 50 mM KCl, 15 mM Hepes KOH (pH 7.0) containing 0.1 mM EGTA (○) or 10 μM A23187 (△) or both EGTA and A23187 (▲). The reaction was stopped at various times by filtration and washing with stop solution containing 5 mM EGTA. Data represents the mean ± S.E. of three experiments, each carried out in duplicate with different membrane preparations.

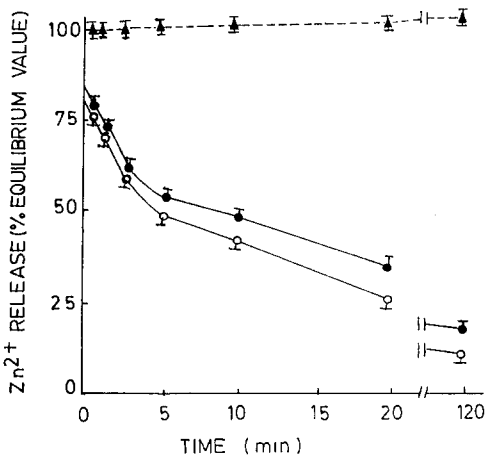

Fig. 6. Effect of divalent cations (calcium, cadmium and zinc) on $^{65}ZnCl_2$ release: 10 μl of BBMV (80–120 μg protein) were loaded for 2 h at 25°C with ^{65}Zn by incubating in buffer containing 150 mM Hepes-KOH (pH 7.0), 0.3 mM $^{65}ZnCl_2$. 10 min before the end of the $^{65}ZnCl_2$ loading, ionophore A23187 (10 μM) was added to the vesicle suspension. The suspension (100 μl) was diluted with 2.5 ml of medium containing 150 mM KCl, 15 mM Hepes KOH (pH 7.0), containing either 2 mM $CdCl_2$ (●), 2 mM $CaCl_2$ (▲) or 2 mM $ZnCl_2$ (○). The reaction was stopped at various times by filtration and washing with ice-cold stop solution containing 5 mM EGTA. Each point represents the mean ± S.E. of three experiments, each carried out in duplicate with different membrane preparations.

4.6. Effect of pH on initial zinc uptake

The effect of H^+ gradients on the uptake of zinc is illustrated in Fig. 7. The vesicles were preloaded with H^+ by preincubation in buffers of different pH and the uptake of 1 mM $^{65}ZnCl_2$ performed at pH 7.0 ($[H^+]_i > [H^+]_o$). A marked stimulation of zinc uptake was observed with increasing H^+ gradient. In contrast, when membrane vesicles preincubated at pH 7.0 were incubated with 1 mM Zn^{2+} in uptake buffers of different pH ($[H^+]_i < [H^+]_o$), a marked inhibition of the rate of uptake of Zn^{2+} was observed. It is noteworthy here that we did not use the buffers of pH > 7.0, since zinc salt tends to precipitate.

4.7. Influence of temperature on extravesicular zinc binding

An increase in zinc binding was observed with increased temperature from 10°C to 50°C as depicted in Fig. 8. The temperature dependence of extravesicular zinc bind-

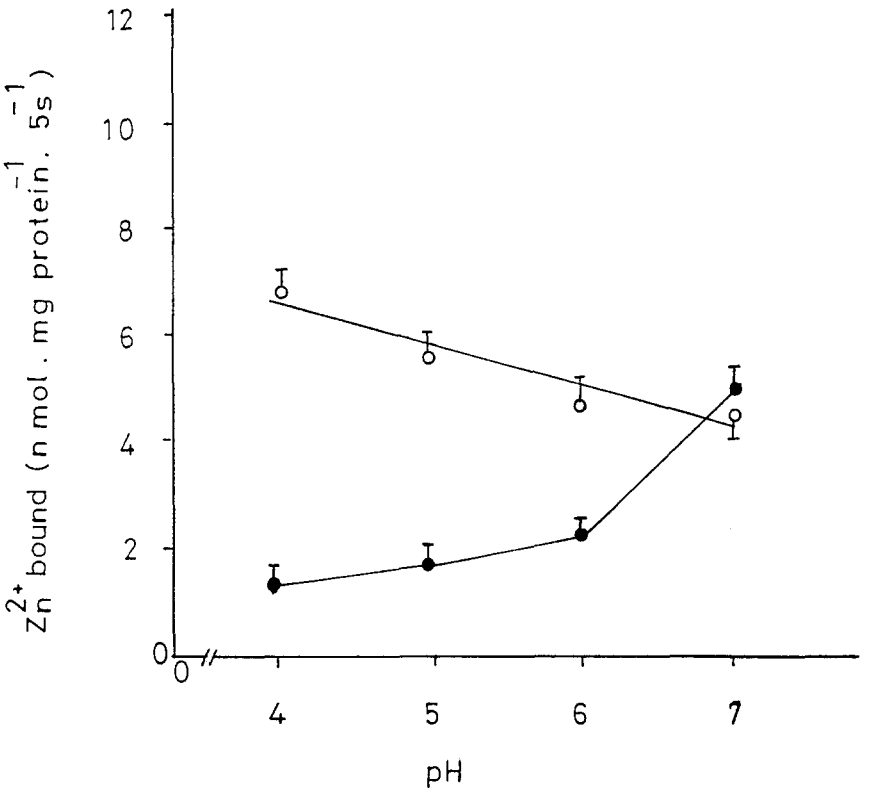

Fig. 7. The effect of pH on 5s zinc uptake: the effect of extravesicular pH (●): 10 μl of BBMV (100–150 μg protein) prepared in 300 mM mannitol, 15 mM Hepes KOH (pH 7.0) were incubated with 90 μl of uptake buffers (pH 4, 5, 6, 7) containing 1 mM $^{65}ZnCl_2$ at 25°C. The effect of intravesicular pH (○): 10 μl of BBMV (100–150 μg protein) prepared in buffers of different pH (4, 5, 6, 7) were incubated with 90 μl of uptake buffer containing 150 mM KCl, 15 mM Hepes KOH (pH 7.0), 1 mM $^{65}ZnCl_2$ at 25°C. At 5 s, the reaction was terminated by stop solution with EGTA. The membrane vesicles were filtered and rinsed with the same buffer. Data represents mean ± S.E. of 3 experiments each carried out in duplicate with separate preparations.

ing was expressed as Arrhenius plot (log V vs 1/T). The linear plots with two slopes were observed. The break point was observed at 30°C. The apparent energy of activation was 13.36 KCal/mol below the break point and 3.69 KCal/mol above the break point. This study clearly shows that extravesicular Zn^{2+} binding is highly sensitive to temperature.

4.8. Effect of sulfhydryl and carboxyl blocking agents

In the presence of sulfhydryl blocking agents, N-ethylmaleimide and iodoacetate and carboxyl blocking agent (DCCD), 53%, 57% and 125% zinc binding was observed as compared to the control respectively. Moreover, in the presence of 2-mercaptoethanol, a small molecular weight ligand, 39% zinc binding was observed as compared to the control. Therefore, it could be inferred that sulfhydryl groups are essential for extravesicular zinc binding.

5. Discussion

The zinc uptake process in rat renal BBMV measured under our experimental conditions results from translocation across the membrane and, interestingly, most of the zinc is bound to interior sites of membrane. This conclusion is based on the fact that chelating agents such as EGTA, expected to act mainly on externally bound zinc during the stop and wash steps of the uptake procedure, removed no more than approximately 14% of zinc taken up under equilibrium conditions. Furthermore, the importance of the non-osmotically reactive space in the distribution of divalent cations such as Ca^{2+}, Zn^{2+}, Cd^{2+} have been reported [14,24,25]. The accumulation of Zn^{2+} was 50 times higher than the apparent vesicular volume of glucose at equilibrium which is also an indication of extensive zinc binding to the interior sites of BBMV. A confirmation of extensive zinc binding was given by the classical experiment which consists of increasing the medium osmolarity to modify the osmotically reactive vesicular volume as illustrated in Fig. 2. Both initial (5 s) and equilibrium (2 h) uptakes were not significantly influenced with regard to the osmolarity of incubation medium. Thus, these uptakes correspond to extravesicular and intravesicular zinc bindings respectively. The influx and efflux rates of zinc were markedly enhanced by ionophore A23187. This signifies that zinc crosses the apical membrane, depending on the affinity of zinc for ionophore. These results also suggest that the transmembrane movement of zinc is a rate-limiting step in zinc accumulation.

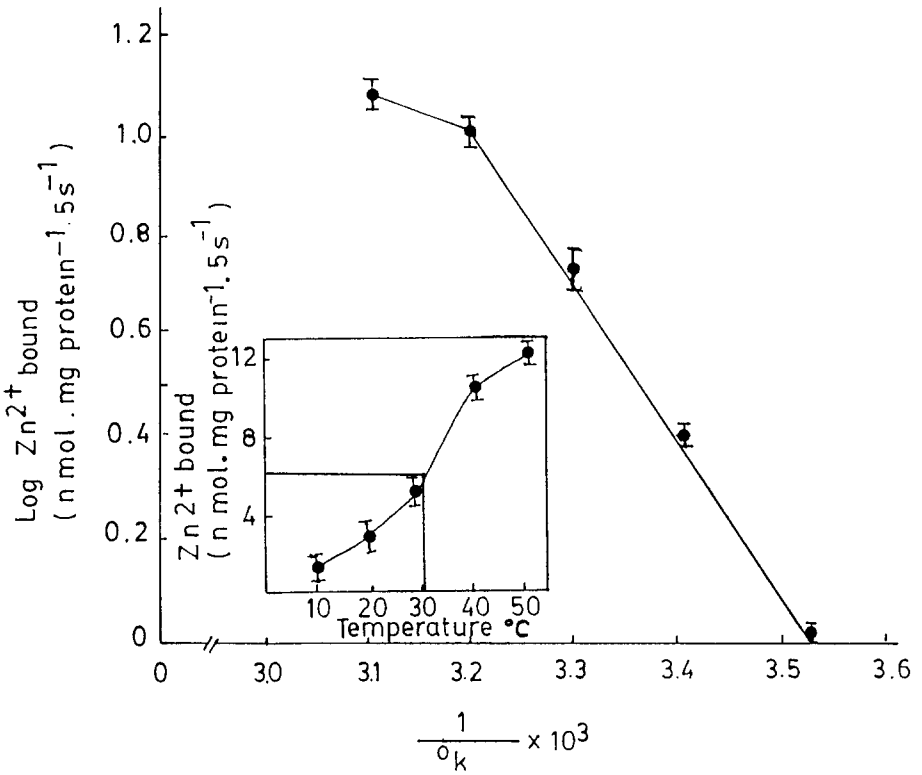

Fig. 8. Arrhenius plot of extravesicular zinc binding: 10 μl of BBMV (80–140 μg protein) were incubated with 90 μl of buffer containing 150 mM KCl, 15 mM Hepes-KOH (pH 7.0), 1 mM $^{65}ZnCl_2$. Incubations were carried out at 10–50°C. Data represents mean ± S.E. of 3 experiments each carried out in triplicate with separate preparations.

The plateau value at 120 min was the same with or without ionophore, indicating that the quantity of bound zinc reached an equilibrium level.

In the isolated membrane the process is a composite of two interlinked phenomena, i.e., binding of zinc to the extravesicular surface of the membrane and the concomitant translocation of zinc and its subsequent apparent binding to the interior surface of BBMV. The Scatchard plot analysis of the external binding data (Fig. 3) revealed the presence of two types of zinc binding sites having high and low affinity which could correspond to either protein or phospholipids [26,27]. The transition point in the Arrhenius plot (Fig. 8) for extravesicular zinc binding in renal BBMV is compatible with the hypothesis that carrier proteins of the transport system are associated with the lipid components of the plasma membrane. This system undergoes a phase transition at a temperature that is a function of the fatty acid composition of the lipid components. There is considerable evidence that membrane transport across the human erythrocyte membrane [28], sarcoplasmic reticulum [29] and intestinal BBM [30] is mediated by proteins that are intimately associated with the membrane lipids spanning the hydrophobic region of the membrane. The influence of the physical state of the membrane on the rate-limiting step of the transport process can be deduced from the appearance of break points in the Arrhenius plot. Temperature-sensitive transmembrane movement of cadmium and zinc has been demonstrated in isolated renal brush border membrane vesicles [16,25]. These observed components for zinc binding to renal BBM are comparable to the biphasic transport system of zinc in intestinal BBMV [13–15]. It is tempting to speculate the presence of two distinct zinc binding carriers in the brush border membrane. Similar types of receptor sites have been previously reported for other cations like Zn^{2+} and Ca^{2+} in intestinal BBM [14,24].

The extravesicular zinc binding was inhibited in the presence of cadmium. Kinetic analysis revealed that there was no change in maximal binding but an increase in K_d. Inhibition of zinc uptake by cadmium has also been observed in other mammalian systems where a carrier-mediated uptake of zinc has been demonstrated in liver parenchymal cells [31], intestine, kidney [14,16] and human lymphocytes and fibroblasts [32,33]. The competition among Cd^{2+} and Zn^{2+} at the intestinal brush border membrane levels explains the appearance of zinc deficiency in young turkeys, calves and growing rats fed cadmium as well as the ability of dietary supplement of zinc to offset some toxic effects of feeding cadmium [34–36].

The analysis of internal binding data showed a single type of intravesicular binding site (Fig. 4). The K_d value for the intravesicular binding site was comparable to that of the extravesicular binding site and confirmed that the membrane crossing was the rate-limiting step of intravesicular zinc uptake across the membrane. Surprisingly, the

intravesicular zinc binding component has a large number of binding sites which can accommodate 400 nmol Zn^{2+}/mg protein. The phosphate groups of phospholipids and carboxyl and thiol groups of proteins could be the most prominent determinants in binding to zinc. Recently we have isolated a protein with a large number of zinc binding sites from rat renal brush border membranes (unpublished observation). Extravesicular zinc binding was inhibited in the presence of exofacial membrane sulfhydryl group modifying agents such as N-ethylmaleimide and iodoacetate as well as -SH containing compounds such as 2-mercaptoethanol. These studies, therefore, suggest that the sulfhydryl groups of the carrier proteins which are exposed to the outer surface of the membrane are essential in the zinc transport event. Moreover, sulfhydryl groups have been shown to affect the activity of membrane transporters [37,38], properties of membrane [39] and the activity of enzymes [40]. Sulfhydryl groups in the intestinal brush border membrane have been implicated in the calcium transport process [41].

Increase in outwardly directed proton gradient enhanced the zinc uptake, in contrast to inwardly directed proton gradient which reduced the zinc uptake. Therefore, these results suggest the existence of a coupled influx of zinc with concomitant efflux of proton across renal brush border membrane. To demonstrate the presence of Zn^{2+}-H^+ exchanger in renal BBM, further studies involving the measurement of counter flow of protons in exchange for Zinc are required. Zinc efflux experiments in the presence of ionophore A23187 showed that non-labelled cadmium and zinc efficiently displaced all the intravesicular bound zinc, whereas calcium was unable to do so. Therefore, these studies provide evidence that intravesicular binding sites also are specific for zinc. A possible relationship between the mode of inheritance of the disease acrodermatitis enteropathica and general characteristics of Zn transport in tissues other than intestine and kidney remain entirely unclear. Studies involved with the isolation of the binding and translocation elements from brush border membranes and their reconstitution into liposomes would no doubt be very useful for the further characterization of zinc transport process in tubular cells.

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