Biochimica et Biophysica Acta, 1024 (1990) 323–330
Elsevier

BBAMEM 74862

Studies of zinc transport into brush-border membrane vesicles isolated from pig small intestine

Frédérique Tacnet [1], Don W. Watkins [2] and Pierre Ripoche [1]

[1] *Biomembranes, Service de Biologie Cellulaire, Centre d'Etudes Nucléaires de Saclay, Gif-sur-Yvette (France)* and [2] *Department of Physiology, George Washington University, Washington DC (U.S.A.)*

(Received 14 November 1989)

Key words: Brush-border membrane; Intestinal cell; Zinc transport; Cadmium inhibition; (Pig small intestine)

Zinc transport into brush-border membrane vesicles was investigated by measuring uptake rates at a very short incubation time (2 seconds), during the initial linear uptake. A divalent cation chelator (EGTA) was added to the stop and washout solutions in order to remove the zinc bound to the external surface of the vesicles. Under these conditions, we showed that zinc enters the vesicles by (1) a saturable carrier-mediated process, and (2) an unsaturable pathway. The kinetic parameters we calculated were an affinity of 0.215 ± 0.039 mM, a J_{max} of 17.2 ± 1.7 nmol · min^{-1} · (mg protein)$^{-1}$ and an unsaturable constant of 0.025 ± 0.006 ($n = 6$). The imposition of an outwardly directed K^+ gradient (negative inside) did not affect the J_{max} value of the zinc uptake but increased the K_m value significantly. This suggests that, at least a portion of zinc which crosses the membrane does not do so in a cationic form. Zinc uptake was decreased or increased according to the nature of accompanying anions (Cl^-, SO_4^{2-}, SCN^-) in the absence of any membrane potential. With highly permeant anions such as thiocyanates, zinc uptake was considerably augmented, suggesting a movement of zinc in a complexed form involving the presence of negative species. We also showed that cadmium competitively inhibited the zinc uptake; we measured a K_i value of 0.21 mM, indicating a similar affinity of cadmium for the carrier as zinc itself. By contrast, the presence of calcium had little effect on zinc entry into vesicles. The calcium ionophore A23187 had only a slight stimulating effect on zinc uptake. These results indicate that zinc and calcium transports are probably independent of each other.

Introduction

Zinc is an essential trace element necessary to life. Some of the physiological functions in which zinc is involved are due to the role this metal plays in numerous metalloenzyme systems [1–4]. This cation has also been shown to interact with biomembranes [5,6] and intracellular components [1] and to be influenced by the endocrine system as is the metabolism of the major nutrients [7–10]. At present, the whole metabolic pathway of zinc is well known [1,11,12]. As early as 1964, Cotzias and Papavasiliou [13] introduced the general concept of the homeostatic regulation of intestinal zinc absorption. Nevertheless, the mechanisms of this absorption and the regulatory events which control intestinal zinc movements are still not yet clearly defined.

Extensive studies of zinc transport in isolated perfused intestinal segments [14–16] and on the whole body [14,17] indicated that the transepithelial movement of zinc across the small intestine is a carrier-mediated process. However, they don't explain the precise membranous mechanisms which control zinc entry from the lumen to the cytosol of enterocytes. Ménard and Cousins [18] described a saturable uptake of zinc in brush-border membrane vesicles isolated from the small intestine of rats fed adequate and deficient amounts of the metal.

Zinc, as well as cadmium [19] or calcium [20], is probably associated to intravesicular or extravesicular sites. These binding steps could be limiting steps for zinc transport. In this study, we tried to determine and use conditions which allow to distinguish a transmembranous transport step from an intravesicular or extravesicular binding.

Throughout the small intestine, jejunum was shown to be the main site of zinc reabsorption [15,17]. For the present work, brush-border membrane vesicles isolated from pig jejunal cells have been used to study the transmembrane movements of zinc. A part of the study consisted in dissociating the two events previously de-

Correspondence: P. Ripoche, Département de Biologie, C.E.N. Saclay, 91191 Gif-sur-Yvette, Cédex, France.

scribed by measuring the zinc uptake under conditions far from equilibrium, during the initial uptake rate (2 s of incubation). Zinc transport experiments were performed by a technique of isotopic kinetics using $^{65}ZnCl_2$ as a tracer. The zinc corresponding to an aspecific extravesicular binding was eliminated at the washout step by adding ethylene glycol bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) to the stop solution. Under these conditions, some further characterization of the zinc transport across the apical membrane of pig jejunum epithelial cells has been described.

Experimental procedures

(1) Preparation of brush-border membrane vesicles (BBMV)

An entire pig jejunum obtained at the INRA (National Institute of Agronomic Research, Breeding center at Jouy-en-Josas, France) from animals killed for other purposes was used for each preparation. The brush-border membranes were isolated from scraped enterocytes by the Ca^{2+} precipitation technique described by Kessler et al. [21] with substitution of Ca^{2+} by Mg^{2+}. The final pellet of vesicles was suspended in a buffer solution containing 100 mM mannitol, 10 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (Hepes) adjusted to pH 7.4 with KOH, supplemented with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.01% lithium azide (LiN_3): buffer A.

Protein concentration was determined in the initial homogenate and in vesicles by the Bio-Rad protein assay according to Bradford [22] with bovine serum albumine as standard. The purity of the preparation was controlled by measuring the activities of the apical membrane marker enzymes leucine aminopeptidase [23] and sucrase [24], and the activity of the basolateral membrane marker K^+-stimulated phosphatase [25]. The enrichment in apical membrane was in the range 10–12-fold over the homogenate; contamination by basolateral membranes represented about 6%. Brush-border membranes are almost exclusively right-side-out oriented (more than 90%) as also shown by Klip et al. [26] in the enterocytes of rabbit small intestine. At the end of the preparation, vesicles were aliquoted and frozen in liquid nitrogen.

The time-course of D-[6-^{3}H]glucose (0.1 mM) uptake was also measured on fresh or thawed vesicles in the presence of an inward gradient of 100 mequiv./l Na^+ and a membrane potential generated by 100 mequiv./l K^+ and 11 μM valinomycin, to verify the integrity of the membrane and the quality of transport properties. The amount of glucose within the vesicles at the maximum of the overshoot (1 min incubation time) was 40- or 20-fold the vesicular content of glucose at the equilibrium respectively for fresh or thawed vesicles. The $t_{1/2}$ of the decay to the equilibrium value was 6 or 7 min.

(2) Mg^{2+} washout from membranes

In the Kessler procedure of preparation, a large amount of divalent cations remains associated with the vesicular material. In order to eliminate the residual Mg^{2+} ions, thawed vesicles were preincubated with 1 mM EDTA during 1 h or 3 h at room temperature. Then, the vesicles were centrifuged 30 min at 50000 × *g* on an ultracentrifuge (Beckman airfuge) and Mg^{2+} content was measured in corresponding supernatants and pellets by atomic absorption spectrometry (BAIRD, Zoeterwoude, The Netherlands). Results were compared to Mg^{2+} concentrations in control experiments.

The pellets of control vesicles and vesicles after 1 h EDTA pretreatment were suspended in a large volume of the buffer A, centrifuged at 28000 × *g*, then zinc uptake was measured at 2 s by vesicles resuspended from the pellets.

(3) Zinc uptake by BBMV

All transport measurements were carried out at room temperature (22° C). Thawed vesicles (around 20 mg/ml) were preequilibrated during one hour in 100 mM KCl in the presence of 11 μM valinomycin to reduce surface charges, allow a better vesicle retention on the filters and maintain a zero transmembranous potential. Each point was carried out in triplicate.

The uptake of zinc was initiated by mixing 5 μl of preequilibrated vesicles (around 0.1 mg protein) with 50 μl of the following incubation medium: buffer A containing 100 mM KCl, 1.5 μM valinomycin and various concentrations of $^{65}ZnCl_2$ ranging from 0.009 to 3.6 mM at an activity of 1.7 μCi/ml of final incubation medium. In some experiments, KCl was replaced either by tetramethylammonium chloride (TMACl) (in this case, the pH of the medium was adjusted to 7.4 with tetramethylammonium hydroxide (TMAOH)), or K_2SO_4, KSCN or NaCl in the conditions detailed in the figure legends.

After suitable incubation times, the mixture was diluted in 3 ml of an ice-cold stop solution (buffer A, 100 mM KCl) containing 5 mM EGTA in order to complex free and bound zinc outside of the vesicles. The mixture was immediatly filtered through nitrocellulose filters (0.45 μm pore size, Sartorius, Göttingen, F.R.G.) under vacuum. Vesicles, trapped in the filter fibres, were washed twice with 5 ml of the cold stop solution in order to eliminate the extravesicular radioactive medium. Radioactivity retained on the filters was measured in a Packard scintillation counter (Zürich, Switzerland).

All transport measurements, carried out at short incubation periods (below 5 s) and at a constant temperature of 22° C, were performed with a semi-automatic

device as described by Kessler et al. [27] and now manufactured by Innovativ Labor A.G. (Adliswil, Switzerland). Zinc influx (J) was expressed in nmol · min^{-1} · (mg protein)$^{-1}$ as a function of $^{65}ZnCl_2$ concentration.

Initial rates of uptake were analyzed by a simplex iterative method, using a PC/XT-like desk computer and a curve fitting program ('VOYONS', Commissariat à l'Energie Atomique, copyright © 1983–1988 [28]). For zinc transport, the best adapted model was a model involving a single carrier with a so-called 'diffusive' component according to the equation:

$$J = \frac{J_{max} \cdot [S]}{[S] + K_m} + K_d \cdot [S]$$

where J_{max} is the maximal rate of uptake expressed in nmol · min^{-1} · (mg protein)$^{-1}$, [S] is the concentration of $^{65}ZnCl_2$, K_m the apparent dissociation constant in mM and K_d a 'diffusional' constant that we recalled more generally K_{ns} for non saturable constant. The K_{ns} value was variable from one vesicle preparation to another. Thus, for a better comprehension and homogeneity of the figures, this unsaturable component (i.e., the product value of $K_{ns} \cdot [S]$ in the equation) was subtracted from all the curves presented in this article except in Fig. 3. All the curves represented the means ± S.E. from at least three experiments, except for Fig. 4 which represents one typical experiment.

(4) Chemicals

$^{65}ZnCl_2$ was purchased from Amersham Laboratories (Buckinghamshire, U.K.); D-[6-^{3}H]glucose was obtained from C.E.A., Labelled Compounds (Gif-sur-Yvette, France). Hepes, TMAOH, valinomycin and gramicidin D (as gramicidin) were obtained from Sigma Chemical Company (St Louis, MO, U.S.A.). TMACl was obtained from Flüka Biochemika (Buchs, Switzerland), EGTA from Serva Feinbiochemica (Heidelberg, F.R.G.), PMSF from Boehringer Biochemica and LiN_3 from Eastman Kodack Company (Rochester, U.S.A.). All other chemicals were at least analytical grade.

Results

(1) Mg^{2+} washout

The influence of the Mg^{2+} ions trapped in and outside the vesicles during zinc uptake was determined. EDTA (1 mM) was used to complex and so reduce Mg^{2+} ion content in vesicles. In the absence of any EDTA pretreatment (control vesicles), 72% of total Mg^{2+} ions are present in the vesicle pellet. This corresponds to a final Mg^{2+} concentration in the vesicles of 120 nmol/mg protein. After 1 h and 3 h incubation with 1 mM EDTA, around 85% of Mg^{2+} ions were present in the supernatant. The remaining magnesium in the vesicle pellet was decreased to 30 nmol/mg protein. No difference was observed in the Mg^{2+} content of the vesicle pellets between 1 h and 3 h pretreatment. Zinc uptake into control vesicles and into vesicles after 1 h pretreatment with 1 mM EDTA was then measured. In pretreated vesicles, the maximal rate of the zinc uptake was the same than in control vesicles (J_{max} = 30 nmol · min^{-1} · (mg protein)$^{-1}$ in both cases).

(2) Effect of EGTA on zinc external binding

Zinc uptakes were performed on thawed vesicles as described in Experimental procedures for one fixed $^{65}ZnCl_2$ concentration (0.128 mM). The effect of increased EGTA concentrations in the stop solution was studied to estimate the amount of zinc reversibly bound to the outer surface of the vesicles. In our conditions of low temperature (stop solution at 2° C) and short washing step (6 s), we can assume that EGTA does not enter the vesicles.

In the same way, we also showed that during the washing period, almost no zinc was released from the vesicles: indeed, we performed zinc transport experiments in conditions of maximal uptake rates, i.e., in the presence of thiocyanates both in and outside the vesicles (see below, Fig. 5), always in conditions of initial velocity and we showed that after a 6-s wash only 2.7% of transported zinc was released from the vesicles. If the washing period is prolonged to 60 s, 24% of zinc is lost from the vesicles (data not shown). Results presented in Fig. 1 show that, in the absence of EGTA in the stop solution, the 'apparent' rate of the uptake was equal to 31 nmol · min^{-1} · (mg protein)$^{-1}$. Introduction of EGTA into the stop solution at concentrations from 1 mM to 10 mM led to an approximate diminution of 50% of the uptake rate (around 16 nmol · min^{-1} · (mg protein)$^{-1}$ at all concentrations of EGTA). This decrease corresponded to the displacement of zinc from its binding site on the external vesicle surface, to the chelator. Zinc-EGTA complexes were then eliminated through the filter pores by washing. For all of the following

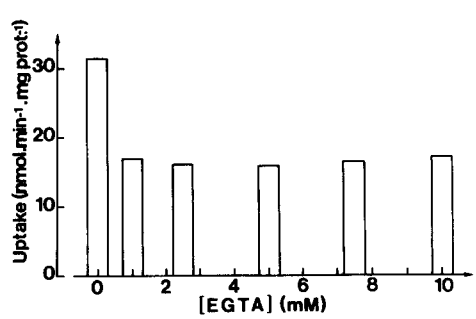

Fig. 1. Effect of increased EGTA concentrations in the stop solution. Zinc uptake was initiated by mixing 5 µl of equilibrated vesicles (100 mM KCl in and outside) with 50 µl of the incubation medium containing 0.128 mM $^{65}ZnCl_2$. At t = 2 s, the reaction was stopped with cold stop solutions containing 0, 1, 2.5, 5, 7.5 or 10 mM EGTA; filters were rinsed with the different stop solutions and residual radioactivity was measured.

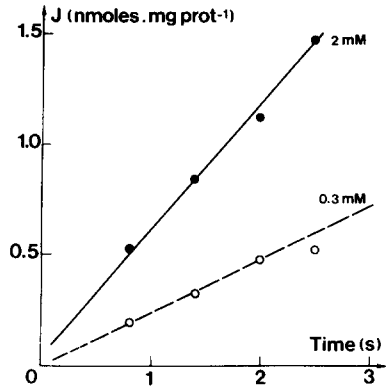

Fig. 2. Initial time course of zinc uptake. 5 μl of vesicles (around 0.1 mg protein) were incubated for various short times in 50 μl of the incubation medium containing 0.3 mM or 2 mM $^{65}ZnCl_2$. At the indicated time, the reaction was stopped, vesicles were filtered and filters were rinsed with the cold stop solution containing 5 mM EGTA.

transport measurements, we decided to use a stop solution containing 5 mM EGTA.

(3) Linearity of the zinc transport for short incubation times

In a set of experiments, the uptake of zinc by BBMV was performed under control conditions (100 mM KCl in and outside the vesicles) for short incubation periods (up to 3 s), with two fixed zinc concentrations, 2 mM and 0.3 mM. Results are shown in Fig. 2. Zinc uptake was found to be time-dependent up to 2 s incubation time for both of the concentrations of $^{65}ZnCl_2$ used. The linear uptake during the first 2 s indicated conditions of initial velocity. The incubation time of 2 s was chosen to examine the effect of various $^{65}ZnCl_2$ concentrations on zinc uptake by BBMV.

(4) Zinc uptake initial velocity

The relationship between BBMV zinc uptake (J) and increasing extravesicular zinc concentrations for 2-s incubations is plotted in Fig. 3. The rate of uptake increased with the zinc concentration, but not in a linear

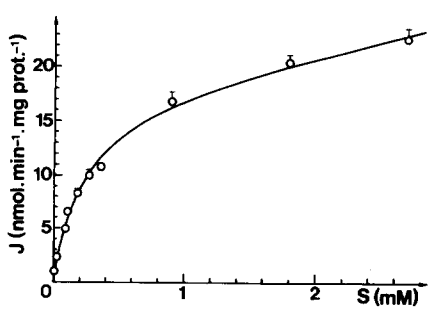

Fig. 3. Zinc uptake in initial velocity conditions. 5 μl of vesicles were added to the incubation medium containing increasing $^{65}ZnCl_2$ substrate concentrations as described in Experimental procedures (control conditions: $KCl_{in} = KCl_{out} = 100$ mM, in the presence of valinomycin). The uptake was stopped at 2-s incubation, vesicles were filtered and rinsed with the stop solution containing 5 mM EGTA. Means ± S.E. on each point ($n = 6$).

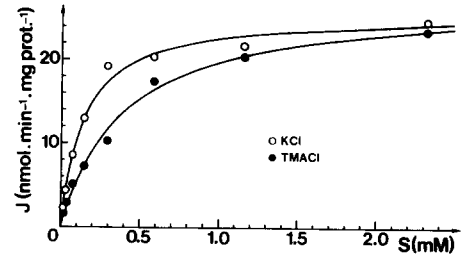

Fig. 4. Effect of a membrane potential on zinc uptake at 2-s incubations. Vesicles, equilibrated in 100 mM KCl, were mixed with the incubation medium as described in Experimental procedures (control conditions, ○) or in a medium where KCl was replaced by 100 mM TMACl to create a transmembrane potential (●). The reaction was stopped, vesicles were filtered and rinsed with the appropriate stop solution: buffer A containing 100 mM KCl and 5 mM EGTA for control conditions or buffer A containing 100 mM TMACl and 5 mM EGTA for treated ones. Typical experiment.

manner and a saturable component became apparent for the high $^{65}ZnCl_2$ concentrations. The kinetic parameters of zinc uptake were: $J_{max} = 17.2 \pm 1.7$ nmol · min^{-1} · (mg protein)$^{-1}$, $K_m = 0.215 \pm 0.039$ mM and $K_{ns} = 0.026 \pm 0.006$ ($n = 6$) at 22° C.

(5) Effect of a transmembranous potential on initial zinc uptake

In the absence of any electrical potential difference across the membrane ($KCl_{in} = KCl_{out}$ in the presence of valinomycin), the uptake of zinc was composed of the two parts described in Experimental procedures (carrier-mediated process and unsaturable pathway) whose characteristics were, after K_{ns} subtraction, a J_{max} of 25.7 ± 0.9 nmol · min^{-1} · (mg protein)$^{-1}$ and an apparent affinity of 0.142 ± 0.018 mM (Fig. 4, which represents one typical experiment). In the presence of 100 mM TMACl instead of KCl in the incubation medium ($KCl_{in} \gg KCl_{out}$ in the presence of valinomycin, establishing an electrochemical potential negative inside), the kinetic parameters were: $J_{max} = 27.5 \pm 1.1$ nmol · min^{-1} · (mg protein)$^{-1}$ and $K_m = 0.4 \pm 0.048$ mM. An identical experiment was carried out using 100 mM choline chloride instead of TMACl, similar results were obtained (data not illustrated). Thus, the maximal rate of the zinc uptake was not increased by the imposition of an outwardly directed K^+ gradient (electrical gradient favourable to a movement of zinc as a cation), whereas the K_m value was significantly increased.

(6) Influence of anions on initial zinc uptake

To study the effect of anion replacement on zinc uptake, experiments were performed under control conditions (100 mM KCl intra- and extravesicular plus valinomycin) and after substitution of chloride anions by either sulfate or thiocyanate (Fig. 5) in the absence of any membrane potential. 50 mM K_2SO_4 or 100 mM KSCN were used to equilibrate the vesicles in the presence of valinomycin and also used in the incubation

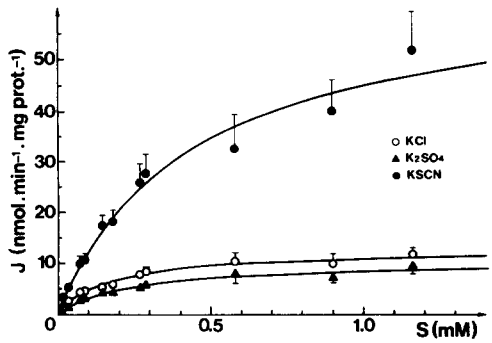

Fig. 5. Effect of sulfates and thiocyanates on zinc uptake. We compared zinc uptakes under control conditions (100 mM KCl intra- and extravesicular in the presence of valinomycin, ○) and after substitution of Cl^- anions by either SO_4^{2-} (▲) or SCN^- (●) in all the media used. In the presence of SO_4^{2-}, the substrate was $ZnSO_4$ instead of $ZnCl_2$; the tracer remained $^{65}ZnCl_2$, corresponding to a range of $4.2 \cdot 10^{-8}$ to $8.8 \cdot 10^{-8}$ equiv./l of Cl^- in the incubation medium. Incubations were performed during 2 s and the reaction was stopped with the appropriate stop solution containing either 100 mM KCl (Cl^-) or 50 mM K_2SO_4 (SO_4^{2-}) or 100 mM KSCN (SCN^-) ($n = 3$).

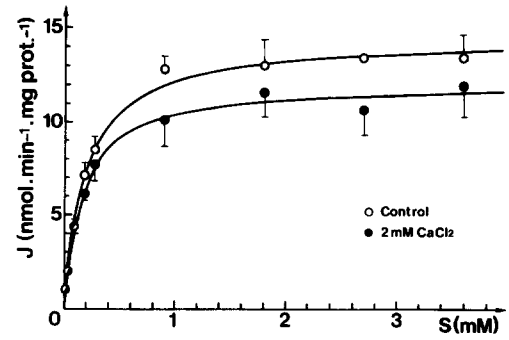

Fig. 7. Effect of calcium on zinc uptake. 5 µl of vesicles equilibrated in 100 mM KCl were incubated in 50 µl of the incubation medium in the absence (○) or presence (●) of 2 mM $CaCl_2$ as described in Experimental procedures ($n = 3$).

medium and stop solution instead of KCl.

When 100 mM KCl was replaced by 50 mM K_2SO_4, the maximal rate of zinc transport was not affected. In these experiments, the J_{max} value was 12.53 ± 0.56 nmol $\cdot \min^{-1} \cdot (\text{mg protein})^{-1}$ under control conditions and 10.14 ± 0.66 nmol $\cdot \min^{-1} \cdot (\text{mg protein})^{-1}$ in the presence of sulfate ions. On the contrary, K_m was increased with K_2SO_4 (0.22 ± 0.038 mM vs. 0.159 ± 0.021 mM) ($n = 3$).

When 100 mM KCl was replaced by 100 mM KSCN, the maximal rate was drastically increased to 64.3 ± 5.3 nmol $\cdot \min^{-1} \cdot (\text{mg protein})^{-1}$ in the presence of thiocyanates (12.53 ± 0.56 nmol $\cdot \min^{-1} \cdot (\text{mg protein})^{-1}$ under control conditions) and K_m was also augmented substantially (0.43 ± 0.078 mM vs. 0.159 ± 0.021 mM). Thus, zinc transport appeared dependent upon the presence and the nature of different anions. Replacement of Cl^- by the more permeant SCN^- increased drastically zinc uptake, whereas a slight decrease was observed in the presence of the less permeant SO_4^{2-}.

Another kind of experiment was performed in order to study the possible effect of bicarbonate anions on zinc uptake. Vesicles were preequilibrated in 100 mM KCl and 10 mM $KHCO_3$ in the presence of valinomycin; incubation was performed in the medium containing 10 mM $KHCO_3$. No difference due to the presence of bicarbonate was observed in the zinc uptake (data not shown).

(7) Effects of cadmium and calcium on initial zinc uptake

The effect of cadmium (Fig. 6) or calcium (Fig. 7) on the uptake of zinc at 2 s incubation time was studied by adding 5 mM $CdCl_2$ or 2 mM $CaCl_2$ in the incubation medium of vesicles.

Cd^{2+} appeared as a potent competitive inhibitor since only the K_m value of the zinc uptake was increased in its presence (from 0.26 ± 0.02 mM (control) to 1.08 ± 0.04 mM) ($n = 3$). J_{max} was essentially the same: 20.7 ± 0.45 nmol $\cdot \min^{-1} \cdot (\text{mg protein})^{-1}$ under control conditions versus 25.5 ± 0.39 nmol $\cdot \min^{-1} \cdot$ (mg

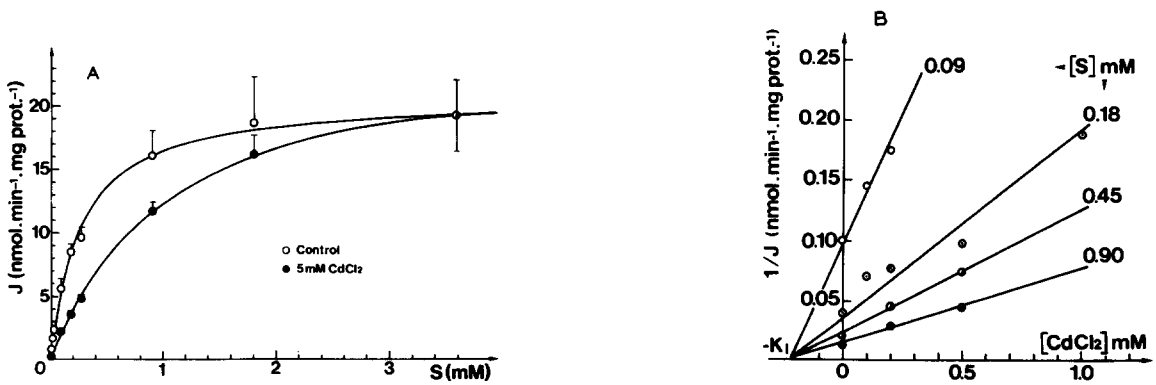

Fig. 6. Effect of cadmium on zinc uptake. 5 µl of vesicles equilibrated in 100 mM KCl were incubated in 50 µl of the incubation medium in the absence (○) or presence (●) of 5 mM $CdCl_2$ (A) as described in Experimental procedures. At 2 s, the reaction was stopped and vesicles were filtered and rinsed with the EGTA containing stop solution ($n = 3$). (B) The Dixon representation for cadmium inhibition of the zinc uptake. 5 µl of vesicles were incubated with 50 µl of incubation media containing increased $CdCl_2$ concentrations for four different $^{65}ZnCl_2$ concentrations. The reaction was stopped at 2 s as described in Experimental procedures.

TABLE I

Some conditions affecting zinc uptake

Values, expressed in percentage of control uptake, represent the means of at least three experiments. The Zn^{2+} uptake at 2 s was ranging between 15 and 25 nmol·min^{-1}·(mg protein)$^{-1}$ in the control. Uptakes of $^{65}ZnCl_2$ were performed as described in Experimental procedures.

Conditions	Zn^{2+} uptake %
Control	100
Lidocaine (10 mM) [a]	497 ± 6
A23187 (10 μM) [a]	120 ±19.8
NaCl (100 mM) [b]	98.7±19

[a] Final $^{65}ZnCl_2$ concentration = 0.1 mM.

[b] Final $^{65}ZnCl_2$ concentration = 0.145 mM; in these experiments, gramicidin D was used instead of valinomycin to equilibrate the vesicles with 100 mM NaCl (11 μM) and was present in the incubation medium (0.75 μM).

protein)$^{-1}$ in the presence of $CdCl_2$ (Fig. 6A). Fig. 6B represents the Dixon plot for the inhibition of zinc uptake by increased $CdCl_2$ concentrations for various $^{65}ZnCl_2$ concentrations. We measured a K_i value of 0.21 mM.

By contrast, Ca^{2+} had no marked inhibitory effect on zinc transport (Fig. 7). The J_{max} value was slightly decreased in the presence of 2 mM $CaCl_2$ in the incubation medium (14.4 ± 0.23 nmol · min^{-1} · (mg protein)$^{-1}$ under control conditions vs. 12.05 ± 0.23 nmol · min^{-1} · (mg protein)$^{-1}$) (n = 3) and no significant modification of the K_m value was noted (0.18 ± 0.012 mM (control) vs. 0.157 ± 0.013 mM). Similar results were obtained with 5 mM $CaCl_2$ in the incubation medium (data not illustrated).

(8) Other experimental conditions

The effects of some reagents on zinc transport are presented in Table I. We noted that sodium ions (100 mM NaCl instead of KCl in all the media) had no marked effects on initial zinc uptake. The calcium ionophore A23187 at a final concentration of 10 μM in the incubation medium slightly increased zinc transport. Lidocaine, a local anesthetic and very lipophilic substance, known for its effects on transport properties of renal brush-border membranes [29], dramatically increased the zinc uptake when used at a final concentration of 10 mM.

Discussion

The aim of this study was to characterize some processes of zinc uptake across the apical plasma membrane of the intestinal epithelial cells of pig jejunum. Our results indicate that zinc is transported into pig brush-border membrane vesicles (BBMV) by a saturable carrier-mediated process and an unsaturable pathway.

Zinc uptake experiments were performed on apical membranes of the enterocytes isolated by the Mg^{2+} precipitation technique. The low percentage of basolateral contaminants compared to the high purification factor (10–12-fold) in apical membrane and the amplitude of the glucose overshoot indicated the quality and integrity of transport properties of the vesicles, even after being frozen and thawed. In this case, the overshoot value was decreased indicating that thawed vesicles are more leaky than the fresh vesicles. This could explain the variability we observed in the unsaturable component of zinc uptake, when performed on thawed material.

As expected with this technique, vesicles contained rather large amount of bound cations (Mg^{2+}), which were partially removed by the washing step of the preparation. Using the calcium precipitation procedure to prepare BBMV from rat small intestine, Kessler et al. [21] showed that 120–130 nmol Ca^{2+}/mg protein remained bound to the vesicles in the absence of chelating agents. We used the equivalent magnesium precipitation method and we tested whether residual Mg^{2+} cations could interfere with the Zn^{2+} uptake by occupying eventual sites utilized for zinc uptake. Pretreatment of the vesicles with 1 mM EDTA for one hour decreased the Mg^{2+} content of the vesicles from 120 nmol/mg protein to 30 nmol/mg protein. Zinc uptakes performed at 2-s incubation times on control and treated vesicles were not different from each other, indicating that residual Mg^{2+} ions probably don't interfere with Zn^{2+} transport.

Murer et al. [30] studied the calcium uptake into rat small intestinal BBMV for very short incubation periods (1 s) and showed that a great part of the 'uptake' they measured was actually an extravesicular binding of calcium. In our case, the zinc corresponding to extravesicular pools was eliminated by adding 5 mM EGTA to the stop solution. These are the only pools affected by this molecule since the membrane of vesicles was shown to be impermeable to EGTA [20,31]; thus, the intravesicular space is inaccessible to the chelator. An other possibility was a release of zinc from this intravesicular space during the washout period; we also verified this point: almost no zinc was lost from the vesicles during the 6 s of the washing period (less than 3%). We showed that EGTA complexed and eliminated a certain amount of external untransported zinc, reversibly fixed on the outer membrane of the vesicles. This fixed zinc must have an affinity for the chelator stronger than its affinity for the exterior membranous binding sites. Increasing the EGTA concentration in this solution from 1 mM to 10 mM did not augment the proportion of bound zinc removed by washing (Fig. 1). This absence of further effects at higher concentrations indicated that EGTA is acting only as an impermeant chelating agent and suggested that if another pool of

extravesicular zinc exists, its binding affinity is very high or corresponds to binding sites deeply embedded in the membrane and not accessible to the chelator.

After being transported across the apical membrane, zinc is bound to intravesicular sites since the metal was shown to be accumulated in a non osmotically active space [32] inaccessible to an external chelator. Such a binding could be a rate-limiting step for the zinc transport process and the incubation period must be sufficiently brief to limit internal binding so that it cannot modify the zinc influx. For the two $^{65}ZnCl_2$ concentrations used (0.3 and 2 mM), we observed a linearity of zinc uptake during at least 2 s (Fig. 2) indicating initial linear uptake rate conditions.

We determined the kinetic parameters of the initial zinc uptake in our control conditions, i.e., $KCl_{in} = KCl_{out}$ in the presence of valinomycin, from the curve presented in Fig. 3. Our values appeared relatively near the values obtained by Ménard and Cousins [18] on rat BBMV at 1-min incubation time. These authors measured a K_m of 0.38 mM (versus 0.22 mM in our experiments); their J_{max} values were much lower than ours (5.4 $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$ versus 17.2 $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$). At one minute of incubation, they were no longer in conditions of initial linear uptake rate and the zinc intravesicular content is already superior to the equilibrium free zinc concentration.

All our curves presented a biphasic profile: the first component (from 0 to 0.5 mM $^{65}ZnCl_2$) corresponded to the saturable process and the second one represented an unsaturable pathway of zinc. The same phenomenon was observed for calcium, a divalent cation also transported via a carrier-mediated process [20,30,33].

An important question arose concerning the electrogenicity of the zinc uptake, that is whether the metal was transported as a cation (Zn^{2+}) or a neutral or negative complex. We showed that the imposition of an outwardly directed potassium gradient in the presence of valinomycin (negative inside) did not enhance zinc transport in our initial velocity conditions (Fig. 4). On the contrary, the K_m value was increased whereas J_{max} was not affected. Since the presence of a membrane potential (negative inside) did not stimulate zinc influx, but rather diminished the affinity of the carrier for zinc, in view of an augmented K_m, the metal probably does not cross the membrane as a cation. A possible explanation for inhibitory effects could be that both weakly permeant cations (TMA^+ and $choline^+$), used to replace K^+ in the external incubation medium in order to create this potential, have their own inhibitory effect on the carrier. In any case, zinc entry appeared different from that of another divalent cation, calcium, whose uptake was studied under the same conditions as ours by Murer et al. [30]. They showed that calcium uptake into rat intestinal BBMV was potential dependent and was increased in the presence of an outwardly directed potassium gradient.

With the anion replacement experiments (Fig. 5), we studied the influence of anionic environment on zinc transport in the absence of any electrical potential (indeed, these experiments were performed in the presence of valinomycin and a high potassium concentration, i.e., the potential generated by the movement of highly permeant accompanying anions such as thiocyanates was continuously clamped to zero by K^+ fluxes). Under these conditions, we showed that the nature of the accompanying anion plays an important role on the zinc uptake process. Two hypothesis can be formulated to explain our results: either zinc is co-transported with an anion or is transported in a complexed form. Several authors showed that zinc could be transported as negatively charged complexes: Torrubia and Garay [34] reported that zinc crossed the red blood cell membrane as a $(Zn(HCO_3)_2Cl)^-$ complex. However, in our vesicles, we did not observe any effect of bicarbonate ions on the initial zinc uptake. Hunt et al. [35] described $Zn(OH)_4^{2-}$ complexes in human pancreatic secretions. The formation of such anionic complexes in the enterocyte apical membrane could explain our results, an aspect of our study which remains to be elucidated.

In any case, the most striking effect on zinc uptake we observed occurred with thiocyanates (Fig. 5). Possibly some transporters, inactive in the presence of SO_4^{2-} or Cl^-, became efficient in the presence of SCN^-. However, considering the augmented K_m value with SCN^-, these hypothetical transporters would have an affinity for zinc much weaker than the affinity of carriers observed in a KCl medium. The formation of a zinc-thiocyanate complex could explain the great effect of thiocyanates on zinc uptake we observed. Eriksson et al. [36] already described such a complex at the substrate binding site of human carbonic anhydrase II.

It is known that the formation of the negatively charged $Zn(SCN)_4^{2-}$ complex is very favourable in aqueous solutions [37]. These observations suggest that, in our experimental conditions, zinc enters vesicles in a complexed form, probably as a salt of zinc, aspecifically coupled to an anion.

Studying sodium dependent transport processes, Wright [38] observed that changes in anion composition altered the potential produced by glucose, phenylalanine, malate and citrate in renal brush borders; nevertheless, when the membrane potential was short-circuited with K^+ plus valinomycin, he found that initial rates of uptake were independent of the anion present. We did not observe the same phenomenon with zinc uptake since we showed a net effect of anion replacement, even after clamping the potential to zero.

Several authors described an exchange Ca^{2+}/Na^+ on isolated baso-lateral membranes from rat kidney cortex [39] or rat small intestinal epithelial cells [40]. In

our case, the possibility of an exchange between zinc and sodium ions was tested by replacing KCl in and outside the vesicles by NaCl in the presence of gramicidin D instead of valinomycin (Table I). The presence of sodium did not enhance the zinc uptake, suggesting that the exchange, if it really occurs, probably resulted in a balancing of electrical charges.

The great enhancement of zinc uptake observed in the presence of lidocaine (Table I) could be explained by the capacity of this substance to modify the membrane permeability of various cations [29].

In a last set of experiments, we showed that cadmium (5 mM) was a potent competitive inhibitor of the zinc entry into vesicles (Fig. 6A). Using $CdCl_2$ concentrations in the same range as $^{65}ZnCl_2$ concentrations, we measured, by a Dixon representation, a K_i of 0.21 mM (Fig. 6B). This indicates that the inhibitor had practically the same affinity for the transporter as the substrate itself. This result differs from that obtained by Foulkes [41], who showed that a greater molar excess of cadmium was necessary to inhibit the zinc absorption in the intact rat jejunum. This author worked on the isolated perfused intestine and his measurements were made at the level of the entire epithelium when different zinc pathways (apical and basolateral plasma membranes, cytoplasm or paracellular pathways) could be implicated. In our system of isolated apical membranes, we found competition between cadmium and zinc for entry into vesicles. By contrast, calcium (2 mM) had no marked inhibitory effect on the zinc uptake (Fig. 7).

Ca^{2+} and Zn^{2+} never behaved in an analogous fashion at any point in this study, although both are divalent cations and they conceivably could share portions of the uptake mechanism. Our results taken together seemed to indicate that zinc follows a very specific pathway to enter into vesicles.

To confirm this hypothesis, results presented in Table I showed that the calcium ionophore A23187 (10 μM) had only a slight stimulating effect on the initial zinc uptake.

Kowarski et al. [15] also arrived at conclusion that Ca^{2+} and Zn^{2+} are transported by separate and distinct mechanisms across rat intestine: they showed that the proximal duodenum was the major site for calcium uptake whereas it was the jejunum in the case of zinc.

Acknowledgements

We thank Mrs. Berthonaud, V. and Ly, S.M. for their technical assistance, Mrs. Lucarain for drawing and Dr. C. Ibarra for critically reading the manuscript.

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