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Zinc binding in intestinal brush-border membrane isolated from pig

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

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

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Zinc binding to brush-border membrane vesicles isolated from pig jejunum was investigated by a rapid filtration method, for long incubation periods (up to 180 min). Zn^{2+} influx revealed a large accumulation of the metal, reaching an apparent intravesicular volume of 160 μl/mg protein at equilibrium, a volume 45-times that of an osmotically reactive sugar, sorbitol (3.6 μl/mg protein). Changes in medium osmolarity had no effect on zinc accumulation. These results suggested a large degree of zinc binding to vesicular components (membrane or core). ^{65}Zn efflux measurements led to the conclusion that two vesicular pools of zinc existed: a small external pool, accessible to different chelators (EGTA) or competitive cations, and a 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 an exchange molecule or a chelator. Scatchard plot analyses revealed, on one hand a first class of high-affinity extravesicular zinc binding sites ($K_a = 8.6 \cdot 10^3$ M^{-1}, $n = 0.455$ nmol Zn^{2+}/mg protein) and a second class of extravesicular sites having a very low affinity ($K_a = 22$ M^{-1}, $n = 25.35$ nmol Zn^{2+}/mg protein) and, on the other hand one type of intravesicular sites ($K_a = 3.3 \cdot 10^4$ M^{-1}, $n = 550$ nmol Zn^{2+}/mg protein). The intravesicular sites have a high affinity for zinc and are specific, since only nonlabelled zinc (or cadmium) but not calcium present in the bathing medium is exchanged with the internally accumulated labelled cation.

Introduction

The intestinal absorption of the essential trace element zinc is an homeostatically regulated process located on the apical membrane of enterocytes [1–3]. However, the details of the membranous mechanisms involved in the transport of the metal from the intestinal lumen to the mucosal cell cytoplasm have not yet been completely characterized.

In the last few years, it has been shown that zinc transport across the brush-border membrane of the small intestine occurs via a regulated and saturable carrier-mediated process [4]. In a previous study [5], we reported some kinetic characteristics of zinc transport into brush-border membrane vesicles (BBMV) isolated from pig jejunum. Uptakes were carried out for short incubation periods during the constant rate of zinc entry, i.e., in the absence of possible rate-limiting steps.

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

Indeed, the interpretation of zinc uptake by intestinal BBMV is complicated as the extent of membrane bound metal can be great relative to the amount of transport into the intravesicular space. Similar binding phenomena were also observed for Fe^{2+} [6] and for Ca^{2+} [7,8] where, at equilibrium, Ca^{2+} was found mostly bound to interior sites of the cell membranes [7].

Interactions of zinc with the apical membrane could be schematically separated as followed: a first step would involve non specific binding at the external surface of the membrane concomitant to the regulated and saturable passage of zinc across the brush-border membrane, and a second step would consist of zinc binding to the internal surface and/or to core components of the brush-border leading to the formation of intravesicular pools of zinc.

The objective of the present paper was to examine more precisely the external and internal zinc binding steps in order to characterize the affinities and capacities of the vesicular zinc binding sites. Actually, it has been previously shown that, in initial velocity conditions, a portion of the metal taken up by the vesicles

was bound to the exterior [4,5]. In this report, we showed that zinc was accumulated in the vesicles as a function of time and reached an equilibrium level after 180 min of incubation. This apparent accumulation corresponds to uptake of the metal into a non osmotically reactive vesicular space, which implies extensive binding of zinc to vesicular components. The analysis of zinc binding at different $^{65}ZnCl_2$ concentrations indicated that the metal was bound to two types of sites on the external surface and to at least one major type of sites inside the BBMV. The internal sites, much more numerous than the external ones, have a high affinity for zinc and are very specific. The nature and the role of these zinc binding sites, as well as their contribution to the intestinal transport process, remain to be defined.

Experimental procedures

1. Vesicle preparation

The BBMV were isolated from pig jejunum epithelial cells according to the $CaCl_2$ precipitation technique described by Kessler [9] with the substitution of $MgCl_2$ for $CaCl_2$. 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)-KOH (pH 7.4) supplemented with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.01% lithium azide (LiN_3): buffer A. The final protein concentration was adjusted to 25 mg/ml. The purity of the preparation and the transport efficiency of the vesicles were also verified as described in a previous work [10]. Freeze-fracture pictures of BBMV suspensions revealed a very homogeneous vesicle population with an average diameter of 200 nm (data not shown). Watkins et al. [10] showed that no aggregation of vesicles, leading to the formation of larger species, occurred in the presence of 0.5 mM $ZnCl_2$. Finally, brush-border membranes are almost exclusively right-side-out oriented (more than 90%) as also shown by Klip et al. [11] in the enterocytes of rabbit small intestine. At the end of the preparation, vesicles were frozen in liquid nitrogen.

2. Time-course of zinc and sorbitol uptakes

Uptake studies of $^{65}ZnCl_2$ and [^{14}C]sorbitol as a function of time were performed at room temperature (21°C) by the rapid-filtration method described by Hopfer [12]. Thawed vesicles were previously equilibrated during 1 h in 100 mM KCl in the presence of 11 μM valinomycin [5]. 5 μl of preequilibrated vesicles (around 120 μg proteins) were incubated with 200 μl of an incubation medium, buffer B, composed of buffer A plus 100 mM KCl and containing 1 mM $^{65}ZnCl_2$ (final activity: 7 μCi/ml) and 0.5 mM [^{14}C]sorbitol (final activity: 2 μCi/ml) or, in some experiments, 1 mM [^{14}C]glucose (final activity: 2 μCi/ml). In this type of experiment, when the effect of the ionophore A23187 on zinc uptake was tested, the ionophore was added to the vesicles 10 min before the end of the KCl equilibration and to the incubation medium at a final concentration of 12 μM. At various times, up to 3 h, the reaction was stopped by addition of 3 ml of an ice-cold stop solution: the incubation buffer B containing nonlabelled zinc and sorbitol. The mixture was rapidly filtered onto 0.65 μm pore size nitrocellulose filters (Sartorius, GmbH, Göttingen, F.R.G.) under vacuum; the vesicles, retained in the filter fibres, were washed twice with 5 ml of the cold stop solution in order to remove all the extravesicular radioactive medium. Triplicate observations were made for each point. Then, the radioactivity associated with the filters was measured by a liquid scintillation counter (Packard, Zürich, Switzerland).

3. Influence of medium osmolarity on the zinc uptake

The effect of altering the osmotic gradient on ^{65}Zn uptake by BBMV was studied by increasing the concentration of mannitol in the incubation medium. 5 μl of thawed vesicles, prepared in buffer A, were incubated for 2 h at room temperature in 200 μl of modified buffer A containing 0.2 mM $^{65}ZnCl_2$ (final activity: 1.9 μCi/ml of buffer A) and increasing mannitol concentrations (100, 200, 300, 400, 500, 600 and 800 mM). At the end of the incubation, the reaction was stopped by addition of 3 ml of ice-cold stop solutions (modified buffer A, 0.2 mM $ZnCl_2$ and 5 mM EGTA). EGTA was added as an impermeant cation chelator that binds only the external untransported zinc [5]. Then, the vesicles were rinsed twice with this solution as described above.

4. Zinc efflux

Thawed vesicles, previously equilibrated in 100 mM KCl in the presence of valinomycin, were loaded at room temperature (21°C) with 0.1 mM $^{65}ZnCl_2$ (final activity: 2.7 μCi/ml of vesicles) for at least 3 h; then, a small amount of the suspension, generally 5 μl (around 120 μg of proteins), was diluted in a large volume (2.5 ml) of different efflux solutions whose composition is detailed below and in the figure legends. The temperature of the efflux medium was adjusted to 15°C. At different times, the mixture was filtered, then the filters were rinsed twice with 5 ml of an ice-cold stop solution and the residual radioactivity was measured as described above.

In a first experiment, we studied the effect of 0.1 mM or 2.1 mM $ZnCl_2$ present in the diluting medium (buffer B). In another set of experiments, the diluting medium, i.e., buffer B, was modified by addition of either 12 μM of the ionophore A23187 or 0.1 mM of EGTA, or both A23187 and EGTA. When A23187 was present in the efflux medium, vesicles were also pretreated with the ionophore by addition 10 min before the end of the $^{65}ZnCl_2$ loading. In a last experiment, the diluting

medium was modified by the addition of either 12 μM A23187 or both A23187 and 2 mM $ZnCl_2$.

The effect of $CaCl_2$, $CdCl_2$ or $ZnCl_2$ present at a final concentration of 1 mM in the diluting medium was also studied. In these experiments, both vesicles and efflux mediums contained 12 μM of A23187; the stop solution was composed of buffer B, 5 mM EGTA.

5. Effect of divalent cations on zinc uptake

In another set of experiments, the effect of $CdCl_2$ or $CaCl_2$ on zinc uptake as a function of time was studied. Preequilibrated vesicles were incubated in buffer B containing 0.2 mM $^{65}ZnCl_2$ (final activity: 1.7 μCi/ml) with or without either 5 mM $CdCl_2$ or 5 mM $CaCl_2$. The reaction was stopped at various times with a cold stop solution composed of buffer B, 5 mM EGTA; then, the mixture was filtered, the filters were rinsed twice with this solution and the radioactivity retained by the filters was determined as described above.

6. Scatchard plot analysis

6a. Intravesicular binding

Zinc uptake was performed on 5 μl of preequilibrated vesicles at room temperature, for an incubation period of 3 h in 200 μl of different mediums, i.e., buffer B containing various increasing $^{65}ZnCl_2$ concentrations (0.01, 0.03, 0.1, 0.3, 1, 2, and 4 mM). These concentrations of zinc were assimilated to the total free zinc (Free). Indeed, the comparison of the extravesicular volume of 200 μl and the intravesicular volume of 0.125 μl (for an initial protein concentration of 25 mg/ml) indicates that the external free zinc concentration does not diminish to more than 10% even for the lower experimental point (0.01 mM $ZnCl_2$) after 180 min. After 3 h incubation, i.e., when zinc entry was at equilibrium, the uptake was stopped by dilution in a cold (nonlabelled) stop solution (buffer B); then, the vesicles were filtered and washed twice with this solution. The radioactivity associated with the vesicles was measured at each $^{65}ZnCl_2$ concentration. This corresponded to the intravesicular bound zinc (Bound).

6b. Extravesicular binding

In order to determine the number and affinity of the zinc extravesicular binding sites, we measured the zinc uptake under initial velocity conditions (2 s incubation time) as a function of increasing $^{65}ZnCl_2$ concentrations with or without 5 mM EGTA in the stop solution (buffer B). This concentration of EGTA was sufficient to remove the untransported zinc aspecifically fixed on the outer membrane of the vesicles [5]. The difference between the uptake in the absence or presence of the chelator corresponded to the extravesicular bound zinc.

7. Mathematical analyses

The kinetic analysis of zinc time-courses was performed with a curve fitting program using a double-exponential relationship: 'VOYONS', Commissariat à l'Energie Atomique, Copyright C, 1983–1988 [13].

The analysis of the Scatchard plot for the extravesicular binding of zinc was performed with the same program [13] using, in this case, a least-squares iterative method that averaged to the best values of n and their respective association constants, K_a. The numerical values were obtained from the simplified equation of Weder et al. [14].

8. Chemicals

$^{65}ZnCl_2$ and D-[U-^{14}C]Sorbitol were purchased from Amersham Laboratories (Buckinghamshire, U.K.); D-[U-^{14}C]glucose was obtained from the Labelled Compound Laboratories (CEN, Saclay, France). The K^+-ionophore valinomycin was obtained from Sigma Chemical Co. (St Louis, U.S.A.). The ionophore A23187 was obtained from Calbiochem Corporation (San Diego, U.S.A.), EGTA from Serva Feinbiochemica (Heidelberg, F.R.G.), PMSF from Boehringer Biochemica (Mannheim, F.R.G.) and LiN_3 from Eastman Kodak Company (Rochester, U.S.A.). All other chemicals were at least analytical grade.

Buffers. Buffer A is composed of 100 mM mannitol, 10 mM Hepes-KOH (pH 7.4) plus 0.1 mM PMSF and 0.01% LiN_3. Buffer B is composed of buffer A plus 100 mM KCl.

Results

1. Time-course of zinc and sorbitol uptakes

The uptake of ^{65}Zn and [^{14}C]sorbitol is presented in Fig. 1. First, we noticed that zinc entry was a very slow and continuous process, reaching an equilibrium level

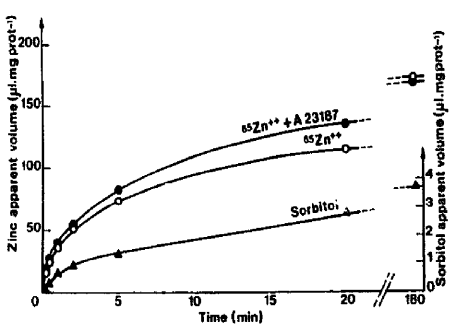

Fig. 1. Time-course of $^{65}ZnCl_2$ and [^{14}C]sorbitol uptake. 5 μl of vesicles, previously equilibrated for one hour in 100 mM KCl in the presence of 11 μM valinomycin, were mixed with 200 μl of the following incubation medium: buffer B, 1 mM $^{65}ZnCl_2$, 0.5 mM [^{14}C]sorbitol with or without 12 μM A23187. The uptake was stopped at various times by a cooled stop solution (buffer B containing 1 mM $ZnCl_2$ and 0.5 mM sorbitol). Vesicles were then filtered and rinsed with this solution.

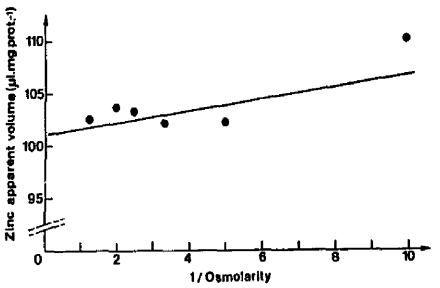

Fig. 2. Influence of medium osmolarity on zinc uptake. Thawed vesicles, prepared in 100 mM mannitol (see Methods), were incubated for 2 h at 21°C in various incubation mediums containing 0.2 mM $^{65}ZnCl_2$ and increasing mannitol concentrations from 100 mM to 800 mM. The uptake was stopped by addition of cold buffer A containing 0.2 mM $ZnCl_2$, the different mannitol concentrations and 5 mM EGTA.

only after 3 h of incubation. The kinetic analysis revealed exponential time constants of $\tau_1 = 0.5$ min, $\tau_2 = 8.8$ min in the absence of ionophore vs. $\tau_1 = 0.2$ min, $\tau_2 = 7.1$ min in the presence of ionophore. The rate of the zinc uptake was slightely enhanced in the presence of 12 µM A23187 whereas the equilibrium uptake of $^{65}ZnCl_2$ was the same as without the ionophore.

The apparent osmotically reactive space in the vesicles was only 3.5 µl/mg of protein, as determined by the distribution of 0.5 mM sorbitol at 180 min of incubation. Similarly, the apparent volume of [^{14}C]glucose after 3 h of incubation was equal to 3.6 µl/mg of protein (data not shown). At the same time, the apparent zinc intravesicular volume was 45 times larger than the apparent sorbitol or glucose volume and reached a value of 160 µl/mg of protein. This accumulation corresponded to a substantial degree of zinc binding (more than 90% of the total, in this case) to vesicular components.

2. Influence of medium osmolarity on the zinc uptake

The effect of increasing the medium osmolarity on 0.2 mM $ZnCl_2$ uptake after 2 h of incubation is shown in Fig. 2. We noticed that vesicle shrinkage had little effect on the amount of zinc taken up by the vesicles. The extrapolation to infinite medium osmolarity (zero intravesicular space) corresponded to an apparent zinc intravesicular volume of 101 µl/mg of protein. This indicated that zinc uptake is independent of medium osmolarity and confirmed our previous observation that most of the cation associated with vesicles is at equilibrium bound to vesicular material.

3. Zinc efflux measurements

We attempted to determine the proportion of bound zinc in and outside the vesicles by the three following ^{65}Zn efflux experiments: a, in the presence of nonlabelled zinc, b, in the presence of EGTA and ionophore and c, in the presence of ionophore and nonlabelled zinc. The previous double-exponential relationship was also used to measure the time constants of zinc efflux.

3a. Effect of nonlabelled zinc on $^{65}ZnCl_2$ release

In the first experiment, vesicles were loaded with 0.1 mM $^{65}ZnCl_2$ for 3 h at room temperature, then the release of the isotope was produced by dilution of the vesicles in a medium with or without nonlabelled $ZnCl_2$ (Fig. 3). This experiment was carried out in the absence of the ionophore A23187. In control conditions, i.e., in the absence of nonlabelled zinc in the diluting medium, almost no ^{65}Zn was released from the vesicles. In the presence of 0.1 mM $ZnCl_2$ in the external bath, the release of ^{65}Zn occurred in two phases: a first rapid phase ($\tau_1 = 0.47$ min), which could correspond to an exchange between the nonlabelled zinc and the extravesicular bound ^{65}Zn; a second, slower phase ($\tau_2 = 14.5$ min), which could indicate an exchange between the external nonlabelled zinc pool and the intravesicular radioactive pool. The same biphasic phenomenon was observed in the presence of 2.1 mM $ZnCl_2$ in the bathing medium. No further release of ^{65}Zn occurred even up to a 5 mM $ZnCl_2$ concentration (data not shown). These results suggest that the majority of the accumulated zinc was retained inside the vesicles and was very slowly exchanged under these conditions.

3b. Effect of EGTA and A23187 on $^{65}ZnCl_2$ release

In another experiment, we studied the effect of EGTA and the ionophore A23187 on ^{65}Zn efflux from preloaded vesicles (Fig. 4). In control conditions, without EGTA and the ionophore, almost no ^{65}Zn was released from the vesicles. In the presence of 12 µM A23187, added to both the vesicles and the diluting medium,

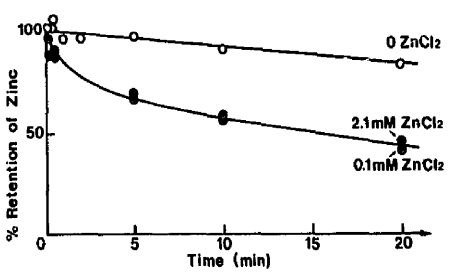

Fig. 3. Effect of $ZnCl_2$ on the release of ^{65}Zn from BBMV. KCl preequilibrated vesicles were loaded for 3 h with 0.1 mM $^{65}ZnCl_2$ at 21°C. At $t = 0$, 5 µl of the vesicle suspension were diluted in 2.5 ml of an efflux medium maintained at 15°C, composed of buffer B and either 0.1 mM (⊙) or 2.1 mM (●) $ZnCl_2$. At different times, the mixture was filtered and rinsed with cold buffer B containing, respectively, 0.1 mM or 2.1 mM $ZnCl_2$. Results are expressed in % of isotopic retention ($t_0 = 100\%$).

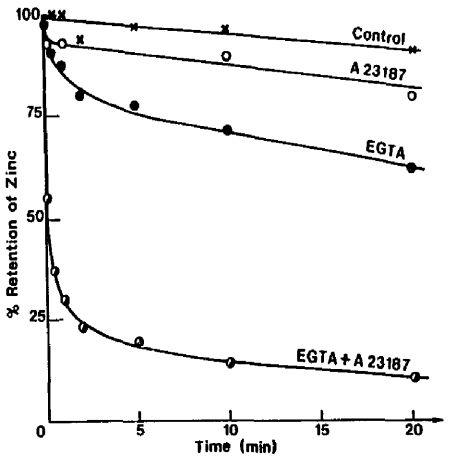

Fig. 4. The effect of EGTA and the ionophore A23187 on ^{65}Zn release. Vesicles were loaded with 0.1 mM $^{65}ZnCl_2$ as described in the legend of Fig. 3; then, they were diluted at various times in 2.5 ml of buffer B (×, control conditions) and in the same buffer containing either 12 μM A23187 (○), or 0.1 mM EGTA (●) or both A23187 and EGTA (◑). The reaction was stopped and vesicles were washed with the stop solution (buffer B).

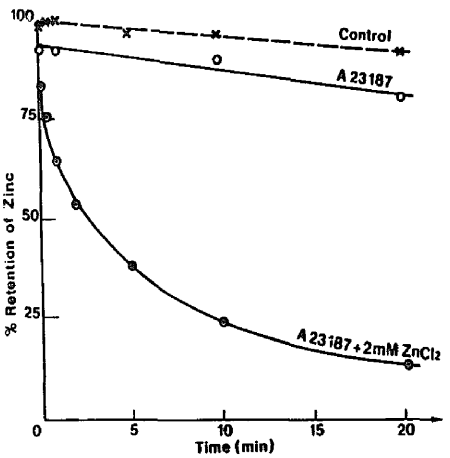

Fig. 5. The effect of $ZnCl_2$ and the ionophore A23187 on ^{65}Zn release. Vesicle load was as stated in the legends of Fig. 3 and Fig. 4; then, they were diluted at various times in the buffer B (×, control conditions) and in the same buffer containing either 12 μM A23187 (○) or both A23187 and 2 mM $ZnCl_2$ (⊙).

there was a slight release of the isotope. As we observed when nonlabelled zinc was added in the efflux medium, the release of ^{65}Zn in the presence of 0.1 mM EGTA in the external bath occurred in two phases. In a first step, EGTA chelated and removed a certain amount of presumably extravesicular zinc ($\tau_1 = 1$ min). Then, in a second step, further isotope was slowly complexed probably because of the delay in moving to the outside of the vesicle ($\tau_2 = 52$ min). When both A23187 and EGTA were present in the diluting medium, the vesicles emptied 80% of their isotopic content within 5 min ($\tau_1 = 0.17$ min, $\tau_2 = 5$ min).

3c. Effect of A23187 and nonlabelled zinc on $^{65}ZnCl_2$ release

Finally, the ^{65}Zn efflux from preloaded vesicles in the presence of both A23187 and 2 mM $ZnCl_2$ in the diluting medium (Fig. 5) was identical to the efflux in the presence of the ionophore and the chelator EGTA, i.e., ^{65}Zn was removed very quickly ($\tau_1 = 0.18$ min, $\tau_2 = 4$ min). This experiment confirmed that most of the bound zinc was inside the vesicles.

These three experiments led us to the conclusion that two pools of zinc existed. First, a small extravesicular pool, which corresponded to zinc bound to the outer membrane of the vesicles and immediatly accessible to the different chelators. Second, a large intravesicular pool, which corresponded to zinc bound on internal sites, from which Zn^{2+} can be displaced provided the membrane has been permeabilized and the external medium contains an exchange cation or a chelator.

4. Extravesicular (EV) and intravesicular (IV) zinc binding sites

4a. Extravesicular sites

A typical Scatchard plot of Zn^{2+} extravesicular binding (see Methods) is represented in Fig. 6. Zinc EV

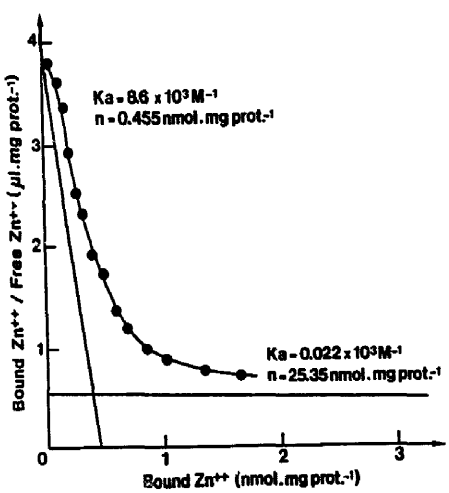

Fig. 6. Scatchard plot of Zn^{2+} extravesicular binding. 5 μl of vesicles were added to 50 μl of the incubation medium (buffer B, 1.5 μM valinomycin) containing increasing $^{65}ZnCl_2$ concentrations from 0.018 to 2.3 mM with a final activity of 1.7 μCi/ml. The uptake was stopped at 2 s incubation, vesicles were filtered and rinsed with the cold stop solution with or without 5 mM EGTA. The difference of uptake in the absence or presence of the chelator represented the zinc bound to the external side of vesicles.

binding was analysed from 2 s uptake data since the amount of extravesicular bound zinc removed by EGTA at 2 s [5] and at 3 h of incubation is the same (around 10 nmol of zinc/mg protein). This indicated that zinc EV binding reached an equilibrium state as soon as after 2 s incubation. The biphasic nature of the Scatchard curve indicated the probable existence of two types of extravesicular binding site: the high-affinity sites ($K_a = 8.6 \cdot 10^3$ M^{-1}) bound 0.455 nmol of Zn^{2+}/mg of protein, and the presence of very low affinity sites for the high $ZnCl_2$ concentrations ($K_a = 22$ M^{-1}, $n = 25.35$ nmol of Zn^{2+}/mg of protein) was suggested by the mathematical treatment [14].

4b. Intravesicular sites

The Scatchard plot of Zn^{2+} intravesicular binding is shown in Fig. 7. The data fitted with a single type of intravesicular zinc binding site whose affinity constant was $K_a = 3.3 \cdot 10^4$ M^{-1} and which bound 550 nmol of Zn^{2+}/mg of protein. The comparison of the data for the extravesicular and intravesicular sites revealed that intravesicular sites were much more numerous than extravesicular sites and that their zinc binding affinity was greater. These results confirmed our previous observation that almost all of the accumulated zinc was bound inside the vesicles.

5. Effect of calcium and cadmium on $^{65}ZnCl_2$ influx

The time-course of $^{65}ZnCl_2$ uptake (final $^{65}ZnCl_2$ concentration: 0.2 mM) was observed under control conditions and in the presence of either 5 mM $CaCl_2$ or 5 mM $CdCl_2$ in the incubation medium (Fig. 8). The zinc fluxes presented in Fig. 8 are expressed in % of the

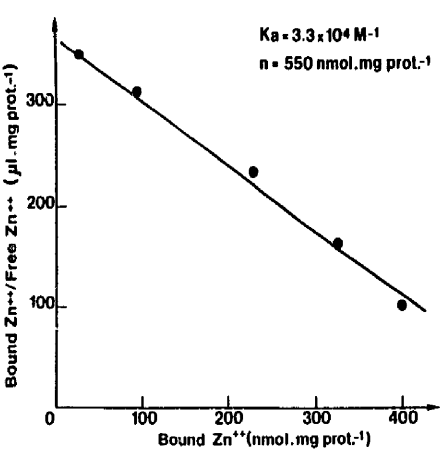

Fig. 7. Scatchard plot of Zn^{2+} intravesicular binding. Vesicles were loaded with various $^{65}ZnCl_2$ concentrations for 3 h at 21°C, until the plateau was reached. Then, the vesicles were diluted with the ice-cold stop solution (buffer B), filtered and rinsed with the same solution and the radioactivity associated with the vesicles was measured.

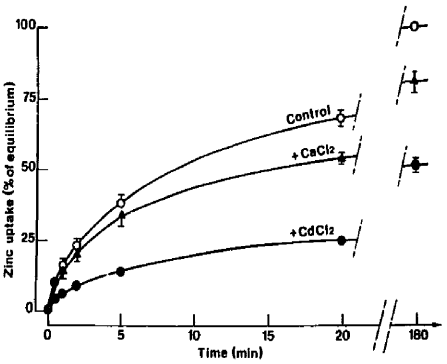

Fig. 8. Effect of calcium and cadmium on zinc accumulation. 5 µl of vesicles were incubated for various times in 200 µl of the buffer B medium containing 0.2 mM $^{65}ZnCl_2$ (control, ○) and either 5 mM $CaCl_2$ (▲) or 5 mM $CdCl_2$ (●). The reaction was stopped by the ice-cold stop solution (buffer B, 5 mM EGTA). Results are expressed in % of the control equilibrium uptake (136 ± 3 µl/mg of protein); each point represents the mean ± S.E. ($n = 3$).

equilibrium value of the control. They represent the mean ± S.E. from three experiments. The presence of 5 mM $CaCl_2$ did not affect significantly the $t_{1/2}$ of the zinc uptake (8.93 ± 1.38 min for the control vs. 8.37 ± 1.33 min in the presence of calcium). However, at $t = 180$ min, calcium reduced by 18.7 ± 4% the equilibrium value of the accumulated zinc in the vesicles. By contrast, when 5 mM $CdCl_2$ was present, the $t_{1/2}$ of the zinc uptake was drastically increased from 8.93 ± 1.38 min for the control to 21 ± 1.35 min in the presence of cadmium, and the equilibrium value was reduced by 49 ± 2.13%. These results indicated that calcium weakly interfered with the zinc accumulation and intravesicular binding (no significant inhibition up to 5 min of incubation), whereas cadmium, the chemical homologue of zinc, strongly inhibited this accumulation.

6. Effect of calcium, cadmium and zinc on $^{65}ZnCl_2$ efflux

In order to examine both the specificity and affinity of the zinc binding sites, we followed the displacement of the accumulated isotope by either 1 mM $CaCl_2$, $CdCl_2$ or $ZnCl_2$ present in the diluting medium (Fig. 9). The release of ^{65}Zn from vesicles preloaded with 0.1 mM $^{65}ZnCl_2$ was measured in the presence of 12 µM A23187 added to the vesicle suspension and to the external bath. Indeed, if the ionophore was only present in the efflux medium but not preincubated with the vesicles, almost no ^{65}Zn was released (data not shown). in the absence of divalent cations and in the presence of 1 mM $CaCl_2$, almost no ^{65}Zn was removed or exchanged from the intravesicular compartment. By contrast, in the presence of 1 mM $CdCl_2$ or $ZnCl_2$, the exchange of the isotope was very rapid: indeed, at 1 min, 50% of accumulated ^{65}Zn was released into the

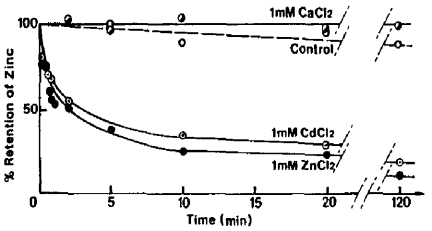

Fig. 9. Effect of calcium, cadmium and zinc on ^{65}Zn release. Vesicles were loaded for 3 h at 21°C with 0.1 mM $^{65}ZnCl_2$; 10 min before the end of the ^{65}Zn loading, 12 μM of the ionophore A23187 was added to the vesicle suspension. Then, 5 μl of vesicles were diluted in 2.5 ml of the following mediums: buffer B, 12 μM A23187 (control, ○) containing either 1 mM $CaCl_2$ (◑), 1 mM $CdCl_2$ (⊙) or 1 mM $ZnCl_2$ (●). The reaction was stopped at various times by filtration and washing with the cold stop solution (buffer B, 5 mM EGTA).

medium containing nonlabelled zinc and 12 μM A23187 (Fig. 9), in comparison with the 17% release after 1 min in the absence of the ionophore (see Fig. 3). After 2 h, the efflux accounted for more than 80% of accumulated ^{65}Zn in the case of cadmium and 90% in the case of zinc. These results confirmed the specificity of the Zn^{2+} binding sites we observed above and also indicated that the internal sites have a strong affinity for zinc.

Discussion

In this study, we focused on the localization and characterization of the vesicular zinc binding sites, after the metal had been incubated for very long periods with intestinal BBMV.

Several authors have already described the importance of the non osmotically reactive space in the distribution of divalent cations such as Ca^{2+} [7,8,15] or the toxic homologue of zinc, Cd^{2+} [16], and the same observation is also valid for zinc [3,10,17]. Indeed, we showed in this report that for long incubation periods, up to 3 h, zinc was extensively accumulated in the vesicles, indicating that the metal bound itself to vesicular components. The accumulation of zinc we observed reached more than 45-times the apparent vesicular volume of sorbitol (Fig. 1). This corresponded to zinc binding, if we assume that sorbitol is not adsorbed or accumulated itself in the vesicles. However, we were surprised to find a sorbitol or glucose apparent volume of 3.5 or 3.6 μl/mg protein, higher than a 'normal' sugar intravesicular volume which is approx. 1–2 μl/mg protein, when determined in the absence of zinc [18,19]. This result could be explained by a slight adsorption of the sugars in the presence of high $ZnCl_2$ concentrations and justifies their augmented intravesicular volumes. Indeed, the extrapolation of the sorbitol apparent volume to infinite osmolarity revealed a significant amount of bound sorbitol: around 2 μl/mg protein (data not illustrated). Taking into account this quantity of bound sorbitol, the amplitude of the zinc accumulation is largely underestimated (i.e., is superior to 45).

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. We showed that the zinc apparent volume was almost the same (around 103 μl/mg protein, after 2 h of incubation with 0.2 mM $^{65}ZnCl_2$, Fig. 2) regardless of the osmolarity of the incubation medium. The less important pool of bound zinc at zero intravesicular space found by Ménard and Cousins [4] could be explained by their measurement of the equilibrium zinc uptake after only 1 h, their use of BBMV isolated from the whole small intestine of rats or their experimental pH value of 6.7.

When the cation-ionophore A23187 (known to accelerate zinc movements in different cells [20]) was added at a final concentration of 12 μM, we observed an increase in the rate of zinc uptake. This increase signifies that zinc crosses the apical membrane via an aspecific pathway, the rate of which depending on the affinity of zinc for the ionophore. This result also indicated that the transmembrane movement of zinc is a rate-limiting step of the zinc accumulation. However, the plateau value at 180 min was the same with or without the ionophore, indicating that the quantity of bound zinc reached an equilibrium level.

The fact that neither a large amount of nonlabelled zinc (Fig. 3) nor the chelator EGTA (Fig. 4) present in the extravesicular medium displaced large quantities of the bound accumulated ^{65}Zn in the first minutes of the efflux measurement strongly suggests that the external zinc binding is negligible compared to the internal binding. When the ionophore A23187 was used in association with the chelator (Fig. 4) or nonlabelled zinc (Fig. 5) in the efflux medium, the accumulated ^{65}Zn rapidly came out of the vesicles emptying 80% of their isotopic content within 5 min. The necessity of ionophore plus chelator or cation for experimental conditions to extrude almost the totality of the accumulated ^{65}Zn argues in favor of an extensive and tight intravesicular binding. The weak percentages (around 10%) of Zn^{2+} that remained associated with the vesicles after treatment by the ionophore was considered to be tightly bound to the inner surface of the membrane. The kinetic analysis of zinc efflux measurements confirmed our previous remark that the membrane barrier was the limiting step in our vesicular system: indeed, in the presence of ionophore, overcoming the carrier capacity, the rates of zinc efflux were greatly enhanced.

The Scatchard plot analysis of the external binding data (Fig. 6) revealed the presence of two types of extravesicular (EV) zinc binding sites, high and low affinity, which could correspond to either proteic or phospholipidic sites [7,8].

In contrast, the analysis of the internal binding data was fitted with one 'apparent' single type of intravesicular (IV) binding site (Fig. 7). The nature of the anionic groups susceptible to interaction with Zn^{2+} (both at the external and internal levels) has not yet been identified. The phosphate groups of phospholipids or the thiol or carboxyl groups of proteins could be implicated. Since 50% of the total lipids in the intestinal brush-border membrane are phospholipids and nearly 50% of them are phosphatidylethanolamine and phosphatidylserine [21,22], the phospholipids may account for sites with low binding affinity and high capacity. On the other hand, specific cysteine groups could be sites having a high binding affinity for zinc. High affinity Zn^{2+} binding sites could be involved with specific intestinal metall enzyme activities such as phospholipase [23], aminopeptidase or alkaline phosphatase [24] activities.

Other cation binding characteristics have been reported in different intestinal membranes: Miller and Bronner [7] described two types of calcium binding site in rat duodenal BBMV, when the uptake was prolonged until equilibrium (60 min of incubation). Merrill et al. [8] measured two types of intravesicular calcium binding site in rabbit intestinal BBMV under conditions in which the external binding was eliminated. Although Zn^{2+} and Ca^{2+} are different divalent cations, our observation that only one very large pool of intravesicular zinc binding sites exists is surprising because of the high value of the affinity constant ($K_a = 3.3 \cdot 10^4$ M^{-1}) and the number of sites, very elevated, accomodating up to 550 nmol Zn^{2+}/mg of protein. A possible explanation is that internal phospholipids, distributed asymmetrically between the two leaflets of the vesicle membrane [25], have a high affinity for zinc. Vesicles could also contain some zinc-binding proteins [26–28] such as metallothionein [29], and this putative binding of zinc to these proteins could be masked by the enormous binding of the metal to phospholipids. Anyway, the amount of these proteins is probably insufficient to explain such an IV zinc accumulation.

The K_a value of $3.3 \cdot 10^4$ M^{-1} for intravesicular zinc binding sites was compared to that of $4.5 \cdot 10^3$ M^{-1}, corresponding to the affinity of zinc for its membrane carrier [5], and confirmed that the membrane crossing was the rate-limiting step of zinc intravesicular binding.

In order to further study the high-affinity IV zinc binding sites, located on the inner membrane surface or on core components, we compared the effect of calcium and cadmium on ^{65}Zn accumulation and binding. Ca^{2+} did not interfere significantly with the rate of zinc influx or binding inside the vesicles (Fig. 8). This result is not surprising since it is known that calcium and zinc are reabsorbed by separate and distinct mechanisms across rat intestine [26]. Moreover, in our kinetic analysis of initial zinc uptake [5], we did not observe competition between calcium and zinc for transport across the apical membrane. Finally, the result described here did not indicate a strong interaction between these cations at the level of zinc internal binding.

In contrast, cadmium significantly decreased the rate of zinc influx and also strongly reduced the final quantity of the metal bound. Cadmium effect could be explained at two levels: first, it interferes with the membrane zinc pathway (indeed, we previously showed that cadmium was a potent competitive inhibitor of the initial zinc uptake with a K_i value of 0.21 mM, similar to the K_m of zinc uptake, 0.215 mM) [5]. This strongly suggests that cadmium could be transported via the zinc transporter since it has the same affinity for the carrier as zinc itself. Second, direct cadmium interaction with zinc binding sites would explain why we obtained such an inhibition of zinc influx and binding in the presence of this toxic metal.

The last and complementary experiments of ^{65}Zn efflux in the presence of the ionophore A23187 (Fig. 9) showed that nonlabelled zinc and cadmium efficiently displaced almost all the intravesicular bound zinc, whereas calcium was unable to do so. This constituted further evidence that the high-affinity binding sites are also very specific for zinc.

In our vesicular system, the ionophore A23187 produced an increase in the rate of zinc influx (Fig. 1), without change in the maximal binding of zinc at equilibrium. The presence of this ionophore, associated with an external chelator or a competitive divalent cation, was also required to enhance ^{65}Zn efflux. A non-competitive cation such as calcium had no effect on the zinc release, even in the presence of A23187, the ionophore commonly used in Ca^{2+} transport measurements [30].

Studies involving the isolation and characterization of the binding and translocation components from brush-border membranes would be very useful for the further understanding of the Zn^{2+} absorption process.

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