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Calcium-Dependent Zinc Efflux in Human Red Blood Cells

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Summary. Zinc efflux from human red blood cells is largely brought about by a saturable mechanism that depends upon extracellular Ca^{2+} ions. It has a V_{max} of about 35 μmol/10^{13} cells hr, a K_m for external Ca^{2+} of 1×10^{-4} M, and a K_m for internal Zn^{2+} of 1×10^{-9} M. External Zn^{2+} inhibits with a $K_{0.5}$ of 3×10^{-6} M. Sr^{2+} is a substitute for external Ca^{2+}, but changes in monovalent anions or cations have little effect on the Zn^{2+} efflux mechanism. It is unaffected by most inhibitors of red cell transport systems, although amiloride and D-600 (methoxyverapamil, a Ca^{2+} channel blocker) are weakly inhibitory. The transport is capable of bringing about the net efflux of Zn^{2+}, against an electrochemical gradient, provided Ca^{2+} is present externally. This suggests it may be a Zn^{2+} : Ca^{2+} exchange, which would be able to catalyze the uphill movement of Zn^{2+} at the expense of an inward Ca^{2+} gradient, which is itself maintained by the Ca^{2+} pump.

Key Words calcium · zinc · erythrocytes

Introduction

Human erythrocytes contain about 150 μmol zinc per liter cells, while the intracellular free Zn^{2+} concentration is estimated to be 2.4×10^{-11} M (Simons, 1991). Under physiological conditions they are suspended in plasma, which contains 15 μM total zinc. The concentration of free Zn^{2+} in human serum is likely to be at least as high as 2.1×10^{-10} M, measured in horse serum (Magneson, Puvathingal & Ray, 1987), for the reasons discussed in the previous paper (Simons, 1991). The resting potential of the red cell membrane is about -8 mV (Jay & Burton, 1969). It therefore seems unlikely that the distribution of Zn^{2+} ions across the membrane is at equilibrium, but more likely that there is an inward electrochemical gradient for Zn^{2+}. No mechanism for maintaining a nonequilibrium distribution of Zn^{2+} is known.

When human red cells are suspended in media containing μM levels of free Zn^{2+}, they gain zinc via the anion transport mechanism (Alda Torrubia & Garay, 1989; Kalfakakou & Simons, 1990). However, zinc-loaded cells show no detectable efflux of ^{65}Zn by this route (Kalfakakou & Simons, 1990). In any case, the anion transport system by its very nature would not be able to produce a Zn^{2+} distribution out of equilibrium with the Cl^- distribution or the membrane potential. This paper deals with the characterization of a novel zinc efflux mechanism in human red blood cells that is wholly dependent upon external Ca^{2+} ions (or Sr^{2+} ions) and is capable of bringing about the extrusion of Zn^{2+} ions against an electrochemical gradient.

A preliminary account of part of this work has been published (Simons, 1987).

Materials and Methods

^{65}Zn Efflux

Washed blood bank cells (not more than 14 days old) were loaded with ^{65}Zn by incubation for 2 hr at 37°C and 20–30% hematocrit in a medium containing (in mM): 145 KCl, 10 (K) HEPES (pH 7.5–7.6 at room temperature), 5 glucose, ^{65}Zn, a Zn^{2+}-citrate buffer (0.5–2 $ZnCl_2$ with 2–3 citrate) and 0.5–10 $KHCO_3$. The ratio of Zn^{2+} to citrate and the concentration of HCO_3^- were varied in order to vary the degree of zinc loading. For example, use of 1 mM Zn^{2+} with 2 mM citrate and 3 mM HCO_3^- generally caused zinc content to increase by 300 μmol/10^{13} cells.

After loading with ^{65}Zn, cells were centrifuged, the supernatant was removed, and the cells were washed 2× with ice-cold medium containing 145 mM KCl, 5 mM (K) HEPES and 1 mM EDTA (ethylene diamine tetra-acetic acid) and then once with ice-cold efflux medium by resuspension and centrifuging. They were resuspended at 5% hematocrit in ice-cold medium containing (in mM): 100 KCl, 125 sucrose, 10 or 15 (K) HEPES and normally either 1 $CaCl_2$ or 0.5 EGTA (1,2-di(2-aminoethoxy)ethane-NNN'N'-tetra-acetic acid). Other substances were added to this medium as required. Changes in monovalent anions or cations were achieved by replacing 100 mM KCl with another salt. The pH was nominally 7.4, but the actual value was usually measured, and is reported in the results (but note the insensitivity of ^{65}Zn efflux to pH in the range 7.0 to 7.5 (*see* Fig. 8)). The cell suspensions were incubated at 37°C, 1.25-ml portions were centrifuged, and 1 ml of supernatant was counted in a Nuclear

Enterprises 8312 gamma counter. Sampling was done either in duplicate at a series of times up to 2 hr (in which case the results are given as rate constants calculated from the slopes of graphs of log (1—counts in supernatant/counts in suspension) against time), or in triplicate at a fixed time (usually 1 hr), in which case the results are given as a fraction of counts lost by the cells (corrected for loss at 0 time). Effluxes in μmol/(10^{13} cells hr) were calculated by multiplying the efflux rate constant by the increase in cell zinc content brought about by ^{65}Zn loading, because ^{65}Zn does not exchange with the zinc initially present in the cells (*see* Fig. 7 of Simons, 1991). The amount of zinc loaded into the cells with ^{65}Zn is referred to as the "^{65}Zn content" of the cells.

The rate of ^{65}Zn efflux is very small in some cases (e.g., in the absence of external Ca^{2+}) so that 1 or 2% hemolysis would be responsible for the majority of the ^{65}Zn appearing in the supernatant. In most experiments hemolysis was monitored by measuring the optical density of the supernatant at 541 nm after addition of Drabkin's reagent. Figure 1 shows the time course of efflux of ^{65}Zn in media containing EGTA or Ca^{2+} over 6 hr, both uncorrected and corrected for hemolysis. After 6 hr in EGTA, there was 1.3% hemolysis, and this had a significant effect on the value of the ^{65}Zn efflux rate constant, which was 0.0055 ± 0.0003 hr^{-1} (uncorrected) or 0.0037 ± 0.0003 hr^{-1} (corrected). In the presence of Ca^{2+}, hemolysis was slightly greater at 1.7% after 6 hr. The graph is linear up to 2 hr, after which the efflux rate decreases, possibly due to cellular heterogeneity. This was not investigated. The calculated efflux rate constant for the period up to 2 hr (0.0866 ± 0.0011 hr^{-1}) is not significantly affected by correction for hemolysis. Results given below are corrected for hemolysis except where stated. (In some experiments chemical reactions of metals with Drabkin's reagent or hemoglobin caused interference with hemolysis measurements.)

^{65}Zn Influx

Cells were incubated at 37°C, 4% hematocrit, in a medium containing 100 mM KCl, 125 mM sucrose, 15 mM HEPES (pH 7.4) and 5 or 50 μM $ZnCl_2$, plus ^{65}Zn. Duplicate samples were taken after 0, 20, 40 and 60 min and added to 1.5-ml centrifuge tubes containing 0.4 ml silicone oil and 0.5 ml KCl/sucrose medium containing 1 mM EDTA. After centrifuging and removing the supernatant, the tips of the tubes containing the cell pellets were cut off and counted. Influx rates (μmol/10^{13} cells hr) were determined from the slopes of graphs of cellular ^{65}Zn against time.

Net Zinc Movements

Net zinc movements were estimated from observations of cell zinc content by atomic absorption spectrophotometry, normalized with respect to the hemoglobin content of the cells, as described in the previous paper (Simons, 1991). The cells were preloaded with zinc in media containing 0.5 mM $ZnCl_2$, 1 mM citrate and 3 mM HCO_3^-, washed, and resuspended in media containing 3 mM NTA (nitrilotriacetic acid) and *either* 2.7–2.9 mM $ZnCl_2$ *or* 1.3–2.3 mM $ZnCl_2$ plus 2.5–1.5 mM $CaCl_2$ (Ca^{2+} decreasing in parallel with increasing Zn^{2+}), pH 7.4. These media had free Zn^{2+} concentrations in the 0.1–1 μM range (measured with zincon; Kalfakakou & Simons, 1990) and a free Ca^{2+} concentration of *either* 0 *or* 1 ± 0.1 mM, measured with an Orion Ca^{2+} electrode.

Sources of Materials

These were the same as in the previous paper.

Results

^{65}Zn Efflux—Effect of External Ca^{2+} Ions

^{65}Zn efflux from human red cells is stimulated 10–20-fold by 1 mM external Ca^{2+} (Fig. 1). Figure 2 shows that the variation of ^{65}Zn efflux with external Ca^{2+} concentration obeys Michaelis-Menten kinetics. The V_{max} (expressed as fractional loss of ^{65}Zn) varies with different degrees of ^{65}Zn loading, but the K_m for Ca^{2+} is similar. The average K_m (±SEM) was 110 ± 31 μM (n = 6). In the remainder of this paper "Ca^{2+}-dependent Zn^{2+} efflux" is defined as the difference between the ^{65}Zn efflux observed in 1 mM Ca^{2+} and that observed in 0 Ca^{2+} (0.5 mM EGTA). This is normally 90–95% of total ^{65}Zn efflux.

Variation of Internal Zn^{2+} Concentration

The fraction of ^{65}Zn lost from the cells decreases as the amount of ^{65}Zn preloaded increases (Fig. 2). When these measurements are converted to fluxes, Ca^{2+}-dependent Zn^{2+} efflux is seen to saturate as the internal Zn^{2+} concentration increases (Fig. 3). It was also observed that the metabolic state of the cells affects Ca^{2+}-dependent Zn^{2+} efflux. Cells were usually loaded with ^{65}Zn in the presence of 5 mM glucose, and ^{65}Zn efflux was measured either in the presence or absence of glucose. Figure 3*A* gives combined results from 14 experiments of this type, presented as a graph of Ca^{2+}-dependent Zn^{2+} efflux against ^{65}Zn content. The results can be fitted by the Michaelis-Menten equation, with V_{max} 35.3 ± 2.1 μmol/(10^{13} cells hr) and K_m 0.53 ± 0.10 mmol ^{65}Zn/10^{13} cells. When cells were depleted of ATP, Ca^{2+}-dependent Zn^{2+} efflux was inhibited by about 50%. The equivalent graph of Ca^{2+}-dependent Zn^{2+} efflux against ^{65}Zn content now has V_{max} 24.6 ± 3.9 μmol/(10^{13} cells hr) and K_m 2.2 ± 0.6 mmol ^{65}Zn/10^{13} cells (Fig. 3*B*).

Metabolic depletion of the cells used in the experiments illustrated in Fig. 3*B* was accomplished either by incubation without substrates for 24 hr at 37°C or by incubation for 2 hr with 5 mM inosine and 5 mM iodoacetamide (Lew, 1971). The method used seemed to have no effect on the degree of inhibition observed. Ca^{2+}-dependent Zn^{2+} efflux could not be inhibited any further by adopting more stringent conditions, such as combining the two methods of ATP depletion, or by adding 5 mM tetrathionate to inhibit

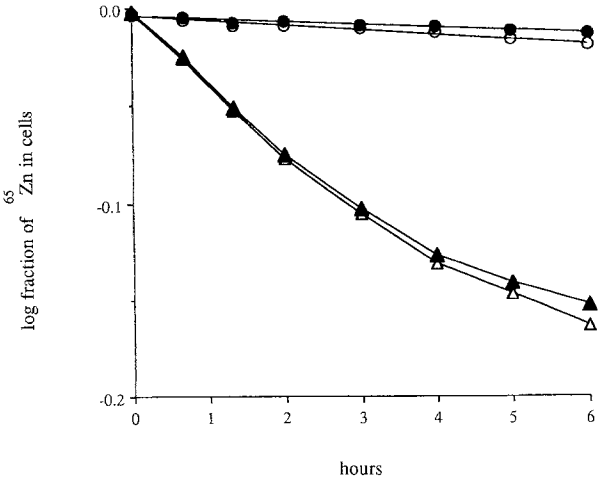

Fig. 1. Efflux of ^{65}Zn from human erythrocytes. Cells were loaded with ^{65}Zn (57 μmol/10^{13} cells) by preincubation with a ^{65}Zn-citrate buffer, as described in Materials and Methods. After washing, ^{65}Zn efflux was measured at 37°C, pH 7.2, in the presence of either 0.5 mM EGTA (●, ○) or 2 mM $CaCl_2$ (▲, △). The abscissa shows the efflux of ^{65}Zn as log (fraction of ^{65}Zn in cells), calculated as log (1—counts in supernatant/counts in whole suspension). Results are shown either uncorrected (open symbols) or corrected for hemolysis (closed symbols). The correction for hemolysis was made on the assumption that lysed cells release ^{65}Zn and hemoglobin in the same proportions as are present in the cells at zero time

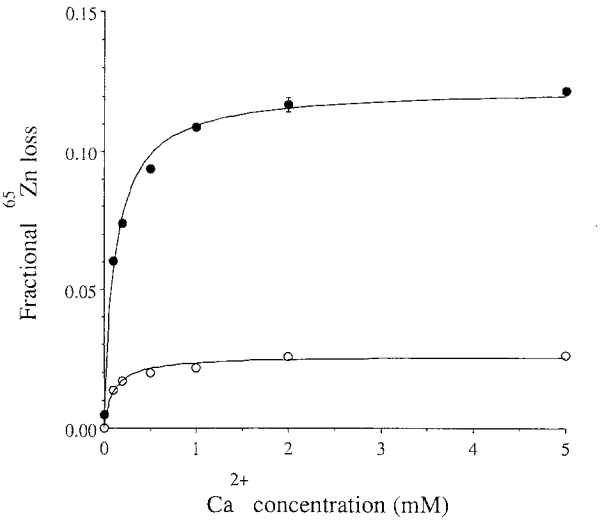

Fig. 2. Dependence of ^{65}Zn efflux on external Ca^{2+} concentration. The graph shows the fractional ^{65}Zn loss over 2 hr when ^{65}Zn-loaded cells were incubated at 37°C, pH 7.22 in media containing the stated concentration of $CaCl_2$ or 0.5 mM EGTA (0 Ca). Results are shown for cells with ^{65}Zn contents of 0.24 (○) and 1.92 (●) mmol/10^{13} cells. They have been fitted by the Michaelis-Menten equation with V_{max} 0.122 ± 0.003 (●), 0.0257 ± 0.0009 (○) and K_m 121 ± 15 μM (●) and 107 ± 19 μM (○)

ATP generation from 2,3-diphosphoglycerate (Duhm, Deuticke & Gerlach, 1968) (*data not shown*).

Figure 3 presents results in terms of ^{65}Zn content of the cells, but it is also of interest to know how Ca^{2+}-dependent Zn^{2+} efflux varies with intracellular free Zn^{2+} concentration. The relationship between cell zinc content and intracellular free Zn^{2+} concentration is given in Figs. 4 and 6 of the previous paper (Simons, 1991). A graph of Ca^{2+}-dependent Zn^{2+} efflux against free Zn^{2+} concentration in metabolically competent cells, analogous to Fig. 3*A*, is also well fitted by a rectangular hyperbola (*not shown*). The parameters calculated for this graph are V_{max} 34.7 ± 1.9 μmol/(10^{13} cells hr) and K_m 1.15 ± 0.21 × 10^{-9} M free Zn^{2+}. (No correction has been made for the fact that the experiments in Figs. 4 and 6 of Simons (1991) were made at pH 7.4, while those in Fig. 3*A* were at pH 7.2.)

Effects of External Cations on Ca^{2+}-Dependent Zn^{2+} Efflux

Na^+, K^+, Li^+

Replacing external K^+ by Na^+ had no effect on ^{65}Zn efflux in either the presence or absence of Ca^{2+} ions. ^{65}Zn efflux rate constants differed by less than 4% in Na^+- and K^+-containing media, which is within experimental error. Replacing K^+ by Li^+ reduced Ca^{2+}-dependent Zn^{2+} efflux by 15% in a single experiment.

Zn^{2+}

Extracellular zinc has several effects on ^{65}Zn efflux. When Ca^{2+} is present, low concentrations of Zn^{2+} cause a profound inhibition of ^{65}Zn efflux (Fig. 4, filled symbols). This inhibition is so pronounced that it was suspected that small quantities of zinc leaving the cells might be affecting ^{65}Zn efflux even in the nominal absence of extracellular Zn^{2+}.[1] Extracellular zinc was therefore measured by atomic absorption spectrophotometry at the start and end of the efflux period, and ^{65}Zn efflux was related to the average extracellular Zn concentration (Fig. 4). The results, in the presence of 1 mM Ca^{2+}, are fitted by an equation characteristic of single-site inhibition ($y = VK_{0.5}/(K_{0.5} + [Zn^{2+}])$). Inspection of the values of

[1] This could not be tested rigorously because the use of chelators to bind traces of Zn^{2+} would also lower the external Ca^{2+} concentration. Reducing the hematocrit from the normal 4–5 to 1% would be expected to reduce the concentration of zinc leaked from the cells, but in two experiments the extracellular zinc concentration was hardly affected. The ^{65}Zn efflux from cells suspended in 1 mM Ca^{2+} and nominally 0 Zn media was insensitive to variation of hematocrit (*data not shown*).

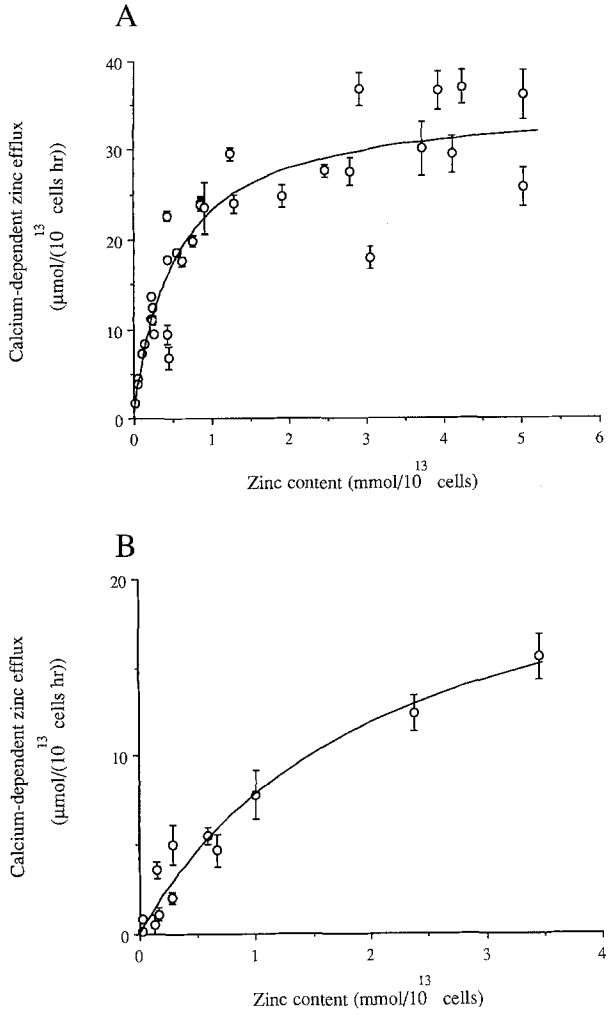

Fig. 3. Dependence of Ca^{2+}-dependent Zn^{2+} efflux on cell ^{65}Zn content in (*A*) fed or (*B*) ATP-depleted cells. Ca^{2+}-dependent Zn^{2+} efflux was calculated by multiplying the difference between the ^{65}Zn efflux rate constants in 1 mM Ca^{2+} and in 0.5 mM EGTA by the cell ^{65}Zn content (i.e., the increase in cell Zn^{2+} content brought about by ^{65}Zn loading) and is shown ± SD. (*A*) Combined results of 14 experiments in which the ^{65}Zn preloading was done in the presence of 5 mM glucose. (*B*) Combined results of four experiments, in two of which the cells were metabolically depleted by 24-hr preincubation at 37°C in the absence of substrates before loading with ^{65}Zn. In the other two, ^{65}Zn loading was carried out in the presence of 5 mM inosine and 5 mM iodoacetamide. In both panels, the results have been fitted by the Michaelis-Menten equation (parameters given in text). The external pH during ^{65}Zn efflux was in the range 7.12–7.27, mean 7.22

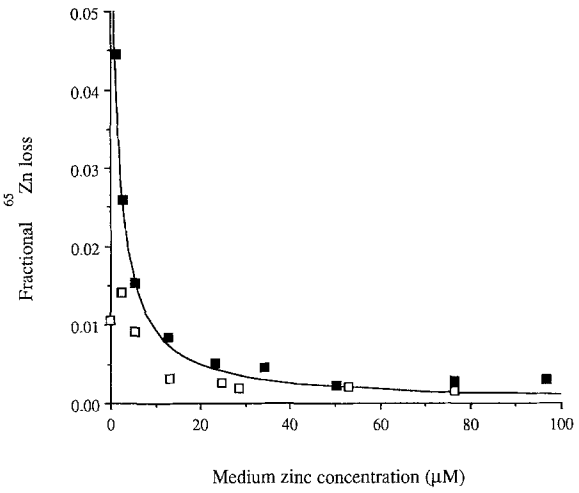

Fig. 4. Dependence of ^{65}Zn efflux on external Zn^{2+} concentration. ^{65}Zn efflux was measured over 1 hr from cells preloaded with 308 μmol $^{65}Zn/10^{13}$ cells, either in the absence (□) or the presence (■) of 1 mM Ca^{2+}, at pH 7.33 and 37°C. The medium zinc concentration was measured by atomic absorption spectrophotometry at 0 and 60 min, and the average value reported, *except* for the (0 Zn, 0 Ca) point, when 0.5 mM EGTA was present, which would have chelated any Zn^{2+} present. The curve is a nonlinear regression fit to the equation $y = VK_{0.5}/(K_{0.5} + [Zn^{2+}])$ with parameters V = 0.081 and $K_{0.5}$ = 1.3 μM. The results have not been corrected for hemolysis because of progressive interference in hemoglobin measurements by Zn^{2+} at the higher Zn^{2+} concentrations, but the hemolysis at 0 Zn^{2+} was about 0.5% (corresponding to a fractional ^{65}Zn loss of 0.005). The SE of the results are smaller than the size of the points used. This is experiment 1010 of Table 1

$K_{0.5}$ measured at different external Ca^{2+} and internal Zn^{2+} concentrations suggest that there are no significant interactions between the relevant ion-binding sites (Table 1). The average value for $K_{0.5}$ is 2.5 ± 0.3 μM Zn_o^{2+} (±SEM, n = 8).

Figure 4 also shows an apparent stimulation of ^{65}Zn efflux by low concentrations of Zn^{2+} in the nominal absence of Ca^{2+} (open symbols). This is probably due to traces of Ca^{2+} ions in the medium. In two other similar experiments, external Ca concentrations of 7 and 9 μM were measured by atomic absorption spectrophotometry.

One possible explanation for the inhibition of ^{65}Zn efflux by external Zn^{2+} might be the dilution of intracellular radioactivity by unlabeled zinc entering the cells. This was tested by measuring ^{65}Zn influx and efflux in parallel incubations with the same batch of cells (Table 2). The results show that the Zn^{2+} influx with 5 μM external Zn^{2+} would have caused negligible dilution of intracellular radioactivity, while with 50 μM external Zn^{2+} there would have been 27% dilution, insufficient to explain the observed 86% inhibition of ^{65}Zn efflux.

Higher concentrations of external Zn^{2+}, above 100 μM, were seen to accelerate ^{65}Zn efflux (Fig. 5). This is further evidence against dilution of intracellular specific activity being responsible for inhibition of ^{65}Zn efflux. The stimulation given by 1 mM Zn^{2+}

Table 1. Inhibition of calcium-dependent ^{65}Zn efflux by external zinc

Experiment	External $[Ca^{2+}]$ (mM)	Internal $[^{65}Zn]$ (mmol/10^{13} cells)	V	$K_{0.5}$ (μM)
1010	1.0	0.31	0.081 ± 0.008	1.3 ± 0.2
1013	1.0	0.39	0.034 ± 0.001	2.8 ± 0.2
	1.0	2.86	0.015 ± 0.004	3.3 ± 2.3
1016	1.0	0.33	0.048 ± 0.007	2.9 ± 0.9
	5.0	0.33	0.048 ± 0.007	4.3 ± 1.7
1034	0.1	0.23	0.081 ± 0.006	1.8 ± 0.3
	1.0	0.23	0.105 ± 0.009	1.8 ± 0.4

This table gives values of V (fractional loss of ^{65}Zn at 0 external Zn^{2+}) and $K_{0.5}$ for the inhibition of Ca^{2+}-dependent ^{65}Zn efflux by external Zn^{2+} (both expressed ±SD) at different levels of external $[Ca^{2+}]$ and internal $[^{65}Zn]$. The experiments were carried out in the same way as in Fig. 4. External pH was 7.33 (1010), 7.37 (1013), 7.42 (1016) or 7.44 (1034).

Table 2. Parallel measurements of ^{65}Zn influx and efflux

External Zn^{2+} concentration (μM)	0	5	50
^{65}Zn influx (μmol(10^{13} cell hr)$^{-1}$)	0	20.5 ± 0.7	196 ± 6
^{65}Zn efflux (μmol(10^{13} cell hr)$^{-1}$)	13.5 ± 0.2	5.1 ± 0.1	1.9 ± 0.3
Predicted ^{65}Zn efflux (μmol(10^{13} cell hr)$^{-1}$)	(13.5)	13.0	9.9

Red cells were preincubated with a Zn^{2+}-citrate buffer for 2 hr and then incubated at 37°C, pH 7.35, in a medium containing 1 mM $CaCl_2$ and either 0, 5 or 50 μM $ZnCl_2$. ^{65}Zn was present either in the preincubation medium (for efflux) or the final incubation medium (for influx). ^{65}Zn influx was measured as described in Materials and Methods; efflux was measured by means of triplicate samples after 1 hr incubation. The ^{65}Zn content of the cells used for efflux was 267 μmol/10^{13} cells. This value was used to calculate both actual and predicted ^{65}Zn efflux. The predicted efflux was calculated on the assumption that the ^{65}Zn in the cells would have been diluted on average by half the concentration of unlabeled Zn^{2+} entering the cells in 1 hr. As an extra check on the measurements, the zinc content of the cells used for influx was measured by atomic absorption. It was found to be 365 μmol/10^{13} cells at the start and 581 μmol/10^{13} cells after 1 hr in 50 μM Zn^{2+}. The increase (216 μmol/10^{13} cells) is close to the difference between the tracer fluxes (194 μmol/10^{13} cells).

is considerably less than that caused by 1 mM Ca^{2+}. It was not possible to correct for hemolysis in these experiments, so lysis may contribute to the stimulation of ^{65}Zn efflux.

Sr^{2+}, Ba^{2+}, Mg^{2+}

Strontium behaved like Ca^{2+} and activated ^{65}Zn efflux along a section of a rectangular hyperbola, as in Fig. 2. In two experiments the V_{max} was 55 and 66% of the corresponding values seen with Ca^{2+}, and the K_m was 95 and 117 μM Sr^{2+} (*graphs not shown*). Ba^{2+} (100 μM) gave about 30% of the stimulation of ^{65}Zn efflux by 1 mM Ca^{2+}, but increasing the concentration of Ba^{2+} up to 10 mM gave no further activation or inhibition (*not shown*). It was therefore not possible to determine a K_m value. The effect of Ba^{2+} may possibly be due to Ca^{2+} contamination. Mg^{2+} (100 μM) also stimulated ^{65}Zn efflux to about 30% of the effect of 1 mM Ca^{2+}. Raising the Mg^{2+} concentration to 10 mM caused inhibition of ^{65}Zn efflux (Fig. 6). Mg^{2+} had a smaller inhibitory effect in the presence of 1 mM Ca^{2+} (*not shown*).

Co^{2+}, Mn^{2+}, Cd^{2+}

One mM Co^{2+} or Mn^{2+} also stimulated ^{65}Zn efflux, but less well than Ca^{2+}. The mean rates in three separate experiments, normalized to a value of 100 with 1 mM Ca^{2+} were: 30 ± 2 (Co^{2+}), 32 ± 2 (Mn^{2+}) and 16 ± 3 (EGTA). These values are not corrected for hemolysis because of interference from metal hemoglobin interactions. The lack of correction accounts for the relatively high rate of efflux seen in the presence of EGTA. Low concentrations of Cd^{2+} inhibited ^{65}Zn efflux in the presence of 1 mM Ca^{2+} (similar to Zn^{2+}, Fig. 4), and a $K_{0.5}$ value of 12 ± 2 μM was estimated from a few observations (*data*

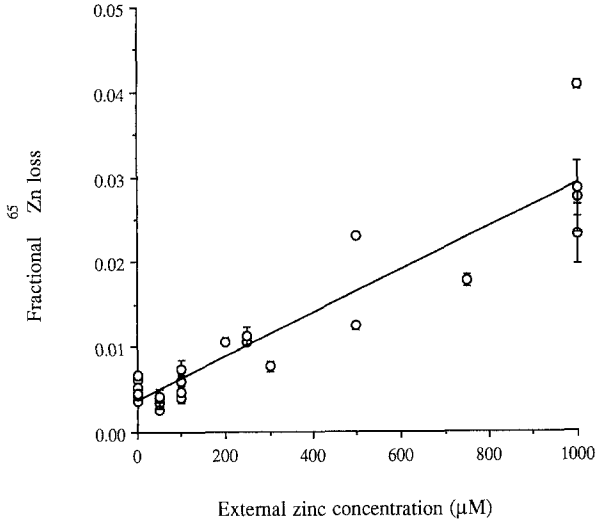

Fig. 5. Variation of ^{65}Zn efflux with external Zn^{2+} concentration. ^{65}Zn efflux (fractional loss in 1 hr) was measured with cells suspended in nominally Ca^{2+}-free media containing either 50 μM to 1 mM $ZnCl_2$, or 0.5 mM EGTA, and is shown ± SEM (n = 3). Points in the 0–50 μM Zn^{2+} range are excluded for clarity—*see* Fig. 4. The graph gives the combined results of five experiments in which cellular ^{65}Zn content was in the range 0.22–1.9 mmol/10^{13} cells and external pH was in the range 7.07–7.30. A regression line is drawn

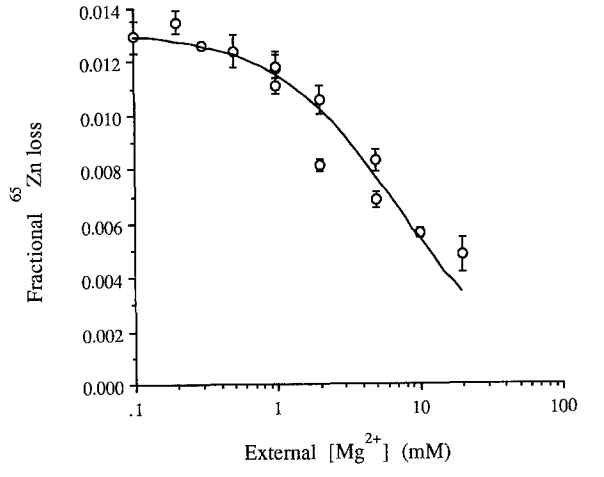

Fig. 6. Variation of ^{65}Zn efflux with external Mg^{2+} concentration. ^{65}Zn efflux (fractional loss in 1 hr) was measured, using cells suspended in nominally Ca^{2+}-free media containing 0.1–20 mM $MgCl_2$, and is shown ± SEM (n = 3). The graph gives the combined results of three experiments in which cellular ^{65}Zn content was in the range 0.22–0.34 mmol/10^{13} cells and external pH was in the range 7.27–7.32; the results were corrected for hemolysis. The line is drawn to fit the equation $y = VK_{0.5}/(K_{0.5} + [Mg^{2+}])$ with V = 0.0131 ± 0.0004 and $K_{0.5}$ = 7.1 ± 1.2 mM. The abscissa has been plotted on a logarithmic scale for clarity

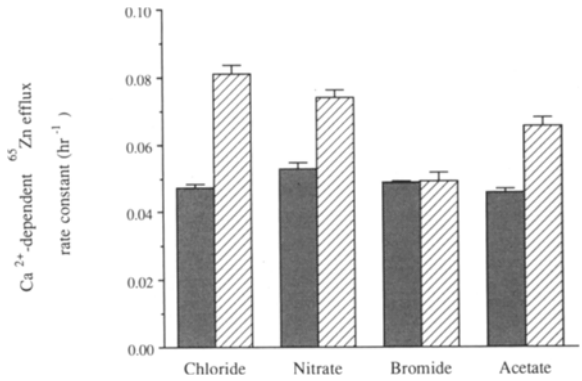

Fig. 7. Effects of anions on Ca^{2+}-dependent ^{65}Zn efflux. The results of two separate experiments are shown by means of different hatching. The cells were loaded with ^{65}Zn in the usual way but washed, and ^{65}Zn efflux was measured using media based upon 100 mM KCl, KNO_3, KBr or K acetate. The efflux rate constants reported above are the differences between those measured in the presence of 1 mM $CaCl_2$ and 0.5 mM EGTA. (Over 90% of ^{65}Zn efflux was Ca^{2+}-dependent.)

not shown). Higher concentrations, up to 1 mM, appeared to stimulate ^{65}Zn efflux, in a similar fashion to Zn^{2+} (*data not shown*).

Effects of Anions on Ca^{2+}-Dependent Zn^{2+} Efflux

Replacement of chloride by other monovalent anions (nitrate, bromide, acetate) had little effect on ^{65}Zn efflux, either in the presence or absence of 1 mM Ca^{2+}. Figure 7 gives the results of two experiments. In one, replacement of chloride by acetate or bromide had an inhibitory effect on Ca^{2+}-dependent Zn^{2+} efflux, but this was not seen in the other experiment.

In a third experiment (*not shown*) partial replacement of chloride by 2, 5 or 10 mM bicarbonate, or by 1 or 2.5 mM phosphate had no significant effect on Ca^{2+}-dependent ^{65}Zn efflux.

Variation of pH

^{65}Zn efflux in the presence of Ca^{2+} is at a maximum, and fairly constant, in the pH range 7.0 to 7.5 (Fig. 8). The flux is inhibited by acid conditions with a pK of roughly 6.5. There are also suggestions of inhibition in alkaline conditions, although insufficient data is available to be certain of this. The residual ^{65}Zn efflux in the absence of Ca^{2+} appears to be insensitive to pH changes (Fig. 8).

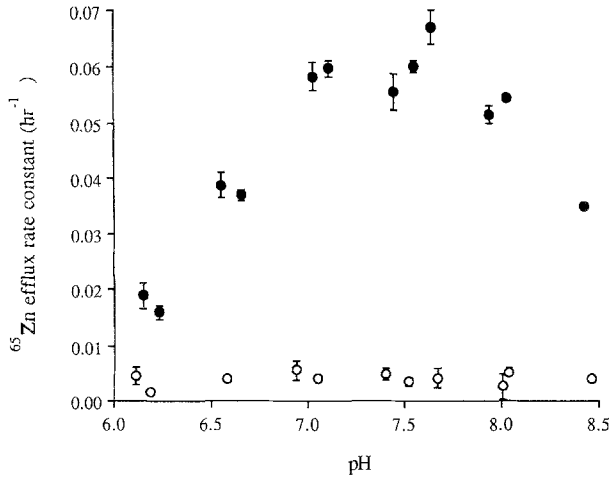

Fig. 8. Variation of ^{65}Zn efflux with pH. ^{65}Zn efflux rate constants were measured in the presence of either 1 mM Ca^{2+} (●) or 0.5 mM EGTA (○) and are shown corrected for hemolysis, ±SD. They are plotted against the mean external pH value and measured at the start and end of the efflux incubation. The media were buffered with 20 mM bisTris (pH < 6.8), HEPES (6.8 < pH < 7.8) or glycylglycine (pH > 7.8). Combined results from two experiments

Table 3. Effects of inhibitors on Ca^{2+}-dependent ^{65}Zn efflux

Inhibitor	% flux
Ouabain, 20 μM	94 ± 2, 96 ± 4
DIDS, 10 μM	93 ± 3, 98 ± 4
Cytochalasin B, 10 μM	99 ± 1
Bumetanide, 100 μM	101 ± 2, 88 ± 2
1 mM	93 ± 3, 60 ± 2
Amiloride, 100 μM	81 ± 2, 84 ± 2
1 mM	48 ± 2, 50 ± 2
D-600, 200 μM	58 ± 2, 51 ± 2

This table gives the Ca^{2+}-dependent ^{65}Zn efflux, expressed as a percentage of the control efflux in the absence of inhibitor, in the same experiment. Cytochalasin B and D-600 were dissolved in ethanol and amiloride and bumetanide in DMSO, before addition to cell suspensions. Addition of the solvents alone (0.5% vol/vol) had no effect on ^{65}Zn efflux.

Effects of Inhibitors

Well-known inhibitors of red cell transport systems, such as ouabain (Na^+ pump), DIDS (anion exchanger), cytochalasin B (sugar transport) and bumetanide (Na^+, K^+, Cl^- cotransport) had no effect on Ca^{2+}-dependent ^{65}Zn efflux at concentrations expected to produce maximal inhibition (Table 3). High concentrations of amiloride (1 mM) and D-600 (methoxyverapamil) (200 μM) caused about 50% inhibition, but the significance of this must be uncertain.

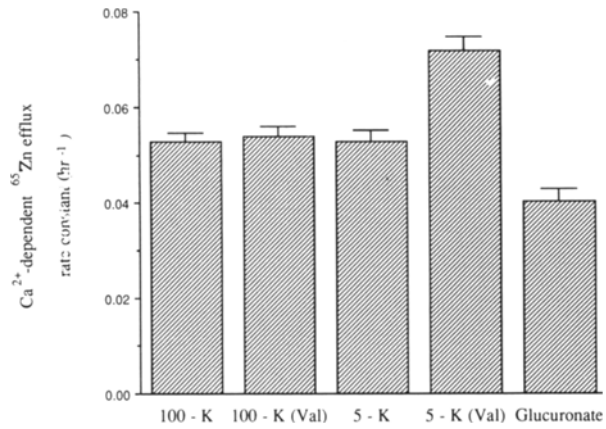

Fig. 9. Effect of changes in membrane potential on Ca^{2+}-dependent ^{65}Zn efflux. Cells were loaded with ^{65}Zn and then incubated in media containing either 100 mM KCl or 5 mM KCl and 95 mM NaCl, or 2.5 mM K_2SO_4 and 95 mM Na glucuronate, in all cases with the addition of 125 mM sucrose, 5 mM glucose and 15 mM (Na) HEPES, final pH 7.33–7.47. Valinomycin was added in ethanol at a final concentration of 2.5 μM (50 μmol/liter · cells) where shown (final ethanol concentration 0.13%). ^{65}Zn efflux rate constants were measured from samples of suspension taken after 5, 15, 25 and 35 min incubation at 37°C. The figure reports the Ca^{2+}-dependent ^{65}Zn efflux rate. The Ca^{2+}-*independent* ^{65}Zn efflux (i.e., the rate measured in EGTA) was less than 8% of the total in all conditions, except for the cells suspended in a glucuronate medium where it was 18%. One of two similar experiments

Changes of Membrane Potential

The red cell membrane may be hyperpolarized by incubation in a low-K^+ medium in the presence of the K^+ ionophore valinomycin or depolarized by incubation in a medium containing an impermeant anion, such as glucuronate (Lassen, 1977). Figure 9 shows that hyperpolarization stimulates Ca^{2+}-dependent ^{65}Zn efflux. Depolarization appears to inhibit it slightly, but about half the inhibition seen in Fig. 9 can be accounted for by an increase in ^{65}Zn efflux in the presence of EGTA. There is also no control against a specific inhibitory effect of external glucuronate. The results are consistent with the hypothesis that Ca^{2+}-dependent Zn^{2+} efflux is accompanied by the net movement of negative charge out of the cell.

Net Zinc Movements

The properties of the ^{65}Zn efflux mechanism described by the previous experiments raise the possibility that Zn^{2+} leaves the red cell in exchange for the entry of Ca^{2+}. It would be quite hard to demonstrate Ca^{2+} entry in exchange for Zn^{2+}, because the Ca^{2+} pump would normally rapidly extrude any Ca^{2+} entering the cell. An alternative approach is to look at

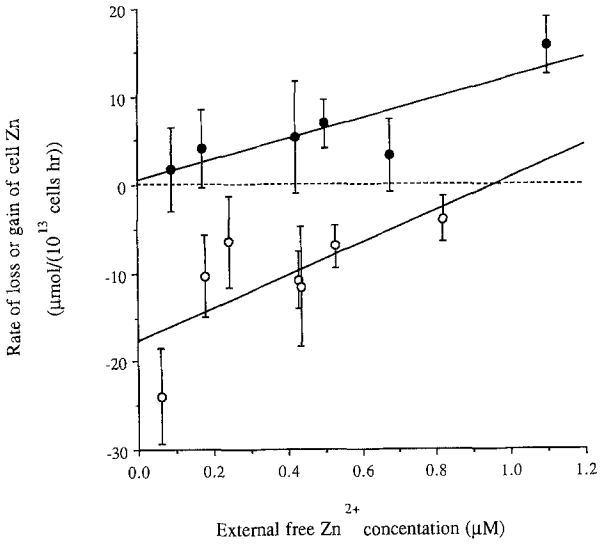

Fig. 10. Net zinc movements in the presence of an inward Zn^{2+} gradient. Cells were preloaded with zinc by 2-hr incubation in a Zn^{2+}/citrate buffer. Their zinc content was 524 or 453 μmol/10^{13} cells after this preincubation. They were resuspended in Zn^{2+}/NTA buffers with 1 mM HCO_3^-, either in the absence (●) or presence (○) of 1 mM free Ca^{2+} and incubated for 2 or 2.5 hr at 37°C. The graph shows the change in Zn^{2+} content, ± SEM (n = 3), expressed as a gain (positive) or loss (negative) per hour, plotted against extracellular free Zn^{2+} concentration. The regression lines have correlation coefficients of 0.72 (without Ca^{2+}) and 0.51 (with Ca^{2+}). Combined results of two experiments

the net movement of zinc in cells in the presence of an inward gradient for free Zn^{2+} ions. Cells were loaded with zinc by preincubation in Zn^{2+}-citrate buffers so that their Zn content was about 500 μmol/10^{13} cells. These cells would have an intracellular free Zn^{2+} concentration of about 10^{-9} M (*see* Fig. 4 of Simons, 1991). They were then incubated in media containing Zn^{2+}/NTA buffers, either with or without Ca^{2+} ions. These buffered media contained 10^{-7} to 10^{-6} M free Zn^{2+} and either 0 or 1 mM free Ca^{2+}. The free ion concentrations in the media were measured with zincon (Kalfakakou & Simons, 1990) and a Ca^{2+} electrode. Figure 10 shows that, in the absence of external Ca^{2+}, the cells gained zinc. This is to be expected because of the inward Zn^{2+} gradient. However, in the presence of external Ca^{2+}, the cells lost zinc in spite of the presence of an inward Zn^{2+} gradient. This demonstrates that the presence of external Ca^{2+} allows cells to transport Zn^{2+} against a concentration (and electrochemical) gradient. (Note that the loss of zinc in the presence of Ca^{2+} could not be accounted for by hemolysis as the zinc content of the cells was normalized to the hemoglobin concentration in the cell extracts.)

Figure 10 also shows a positive correlation between the rate of gain of zinc and the external Zn^{2+} concentration. This is in agreement with the observation that the rate of Zn^{2+} uptake into red cells is linearly related to the external Zn^{2+} concentration (Kalfakakou & Simons, 1990). The rate of Zn^{2+} uptake is also dependent on the bicarbonate concentration. This was set at 1 mM in the experiments of Fig. 10 in order that the Zn^{2+} efflux (in the presence of Ca^{2+}) should not be overwhelmed by Zn^{2+} influx. Similar experiments with 5 mM bicarbonate showed a steeper relationship between net zinc movements and external free Zn^{2+} concentration. In the presence of Ca^{2+}, the loss of zinc changed to a gain at about 0.6 μM external Zn^{2+} (*results not shown*).

Discussion

Nature of the Zn^{2+} Transport System

The experiments in this paper present evidence for a mediated Zn^{2+} efflux mechanism in human erythrocytes with the following properties. It is activated by intracellular Zn^{2+}, with a K_m of 5×10^{-4} M total Zn or 1×10^{-9} M free Zn^{2+}, and by external Ca^{2+}, with a K_m of 1×10^{-4} M. Sr^{2+} is the only good substitute for external Ca^{2+}. These observations suggest the possibility of a $Zn^{2+}:Ca^{2+}$ exchange mechanism in which Zn^{2+} efflux is coupled to Ca^{2+} influx. The attraction of this hypothesis is that Ca^{2+} entering red cells is immediately expelled by the Ca^{2+} pump (Schatzmann, 1982), which would therefore provide a source of energy to bring about the net movement of Zn^{2+} against an electrochemical gradient. The evidence that external Ca^{2+} is needed for the uphill transport of Zn^{2+} (Fig. 10) is hard to explain other than by a $Zn^{2+}:Ca^{2+}$ exchange process. $Zn^{2+}:Ca^{2+}$ exchange would also account for the circumstantial evidence (discussed in the Introduction) that intracellular free Zn^{2+} is below the concentration expected for passive equilibrium across the red cell membrane.

Ca^{2+} influx measurements would obviously be needed in order to demonstrate $Zn^{2+}:Ca^{2+}$ exchange. It is hoped to make these the subject of a later paper. The observation that hyperpolarization accelerates Zn^{2+} efflux, while depolarization appears to inhibit it (Fig. 9), suggests that Zn^{2+} efflux is associated with the net movement of positive charge into the cell. It is premature to speculate whether Zn^{2+} efflux might be associated with the influx of two Ca^{2+} ions or with one Ca^{2+} in combination with another cation entering or anion leaving the cell.

Low concentrations of external Zn^{2+} inhibit Zn^{2+} efflux with a $K_{0.5}$ of 3×10^{-6} M (Fig. 4). The

simplest explanation for this is product inhibition with Zn^{2+} inhibiting at a Zn^{2+} discharge site. The lack of competition with Ca^{2+} (Table 1) suggests that Ca^{2+}- and Zn^{2+}-binding sites are separate.

External acidification inhibits Zn^{2+} efflux with a pK of about 6.5 (Fig. 8). In these experiments there would also have been internal acidification, which, if anything, would have tended to increase the intracellular free Zn^{2+} concentration. Inhibition of Zn^{2+} efflux cannot be explained by a decrease in intracellular Zn^{2+}, but presumably reflects a H^+-sensitive site associated with the Zn^{2+}-transport system.

ATP depletion also inhibits Zn^{2+} efflux, apparently by reducing both the V_{max} and the K_m with respect to total Zn^{2+} (Fig. 3). Interpretation of this is made more difficult by a lack of data on cytoplasmic Zn^{2+} binding in ATP-depleted cells. One possible explanation is that the Zn^{2+} transporter exists in two different states interconverted by phosphorylation. ATP-depletion would be predicted to cause the predominance of a dephosphorylated, less active state. Another possibility is that ATP-depleted cells may gain Ca^{2+} because of the inactivity of the Ca^{2+} pump. Internal Ca^{2+} ions might inhibit Zn^{2+} efflux, although this remains to be tested.

One final question concerns whether the Zn^{2+} transport system might be identical to another, previously characterized mechanism. Most of the evidence comes from the data on inhibition by drugs (Table 3). The lack of inhibition by ouabain, DIDS and cytochalasin B makes any involvement by the Na^+ pump, the anion exchanger or the glucose transporter extremely unlikely. The human red cell probably has both Na^+,K^+,Cl^- and K^+,Cl^- cotransporters, but they require Cl^- or Br^- and do not work with NO_3^- or acetate (Dunham, Stewart & Ellory, 1980; Brugnara, Van Ha & Tosteson, 1989). Additionally, bumetanide inhibits the Na^+,K^+,Cl^- cotransporter with a $K_{0.5}$ below 1 μM in avian red cells (Palfrey, Feit & Greengard, 1980). These facts would seem to rule out a role for either cotransporter in Zn^{2+} efflux. Amiloride is a weak inhibitor of Zn^{2+} efflux, requiring about 1 mM for 50% inhibition (Table 3). This is much higher than the concentration required to inhibit Na^+ channels or $Na^+:H^+$ exchange in the majority of tissues (Benos, 1982). Recently, however, a $Na^+:H^+$ exchanger has been described in human red cells that is inhibited about 60% by 1 mM amiloride (Semplicini, Spalvins & Canessa, 1989). It seems unlikely that Zn^{2+} efflux is related to $Na^+:H^+$ exchange since it has no requirement for external Na^+, but this requires further clarification. The weak inhibitory effect of D-600 on Zn^{2+} efflux probably has little significance. Many Ca^{2+}-related phenomena are weakly inhibited by L-type Ca^{2+} channel blockers (Zernig, 1990).

Physiological Relevance

The intracellular free Zn^{2+} concentration is estimated to be 2.4×10^{-11} M (Simons, 1991), which is much lower than the K_m of Zn^{2+} efflux for internal Zn^{2+}, 1.2×10^{-9} M. Under physiological circumstances external Ca^{2+} would be saturating, so the predicted Zn^{2+} efflux would be about 0.7 μmol/(10^{13} cells hr). This is a tiny flux, but there is no information on the size of the Zn^{2+} influx under physiological circumstances, and it may be sufficient to balance it. The Zn^{2+} efflux mechanism appears to have the function of preventing the erythrocytes from accumulating too much zinc. An hypothesis is that zinc is spared by the red cells so that it can be used elsewhere in the body. The red cells would, however, need sufficient zinc to support their own functions. Ohno et al. (1985) have shown that red cells contain slightly more zinc than can be accounted for by carbonic anhydrase and superoxide dismutase. Reduction of the red cell intracellular free Zn^{2+} concentration to below 10^{-12} M leads to a loss of about 7 μmol $Zn^{2+}/10^{13}$ cells, which is only a small fraction of the total zinc content (Simons, 1991). Carbonic anhydrase has a K_d for Zn^{2+} between 10^{-13} and 10^{-12} M at physiological pH (Lindskog & Nyman, 1964). This could well be sufficiently far below the estimated K_d for the Zn^{2+} efflux mechanism (10^{-9} M) for the latter not to compromise the activity of carbonic anhydrase. It would also be expected that Zn^{2+} influx would prevent the intracellular free Zn^{2+} concentration from falling too low. The other erythrocyte Zn^{2+}-containing enzymes can be expected to have a K_d for Zn^{2+} of 10^{-12} M or less.

A $Zn^{2+}:Ca^{2+}$ exchange mechanism would be capable of transporting Zn^{2+} against a concentration gradient, if it were present in other tissues. There is always a large electrochemical gradient favoring Ca^{2+} entry into cells, so $Zn^{2+}:Ca^{2+}$ exchange would only be suitable for Zn^{2+} exit and not for Zn^{2+} entry into cells. The rate-limiting step for zinc absorption in the gut is thought to be mucosal entry (Lönnerdal, 1989), so involvement of a $Zn^{2+}:Ca^{2+}$ exchange seems unlikely. Speculation about other physiological processes seems premature.

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