J. Membrane Biol. 123, 63–71 (1991)
0022263191001082

The Journal of
Membrane Biology

Intracellular Free Zinc and Zinc Buffering in Human Red Blood Cells

T.J.B. Simons
Biomedical Sciences Division, King's College London, Strand, London WC2R 2LS

Summary. Zn^{2+} has been allowed to equilibrate across the red cell membrane using two agents that increase membrane permeability to this ion: the ionophore A23187 and the specific carrier ethylmaltol. Extracellular free Zn^{2+} was controlled with EGTA (1,2-di(2-aminoethoxy)ethane-NNN'N'tetra-acetic acid)) buffers, except in the case of ethylmaltol, which itself acts as a buffer. Measurement of cellular zinc content at different levels of free Zn^{2+} facilitated the study of intracellular Zn^{2+} binding. It was also possible to estimate intracellular free Zn^{2+} concentration in untreated cells using a "null-point" technique. Intracellular zinc was found to consist of an inexchangeable component of about 129 μmol/10^{13} cells and an exchangeable component of 6.7 ± 1.5 μmol/10^{13} cells, with a free concentration of about 2.4×10^{-11} M. The main component of Zn^{2+} buffering is hemoglobin, with a dissociation constant of about 2×10^{-8} M.

Key Words zinc · erythrocytes

Introduction

Human erythrocytes contain about 150 μmol of zinc per liter cells, over 90% of which is bound to the enzymes carbonic anhydrase and superoxide dismutase (Ohno et al., 1985). Smaller fractions of cellular zinc may be bound to other proteins, such as metallothionein (Grider, Bailey & Cousins, 1990) and hemoglobin (Rifkind & Heim, 1977; Gilman & Brewer, 1978). There is no information concerning the concentration of free zinc ions in the red cell, either in freshly isolated cells, or after their zinc content has been altered. This is needed in order to estimate the electrochemical gradient for zinc across the cell membrane and to be able to interpret zinc transport experiments on a thermodynamic basis. Measurement of erythrocyte free zinc may also have relevance for studies of zinc nutritional status for which there is no convenient assay in humans (Grider et al., 1990). It is known that total erythrocyte zinc is unaffected by dietary zinc manipulation in rats (Bettger & Taylor, 1986).

The approach adopted is similar to that of Flatman & Lew (1980) who studied magnesium buffering in intact human red blood cells. The cell membrane has a very low permeability to zinc. If zinc permeability can be increased sufficiently, free zinc ions will distribute themselves across the membrane passively and come to equilibrium. The intracellular Zn^{2+} concentration can then be calculated if the extracellular concentration and the membrane potential are known. The zinc buffering ability of the cytoplasm can be deduced from the variation of the cell zinc content with the intracellular free Zn^{2+} concentration. Finally, the intracellular free Zn^{2+} concentration of the fresh cell can be calculated from the nullpoint, the external Zn^{2+} concentration which brings about no change in the cellular zinc content when the membrane is made permeable to zinc.

Preliminary accounts of this work have been published (Simons, 1987, 1990).

Materials and Methods

Equilibration of Zn^{2+} across the Red Cell Membrane

Two different chemical agents were used to increase the permeability of the red cell membrane to Zn^{2+}: A23187, the divalent cation ionophore (Pfeiffer & Lardy, 1976), and ethylmaltol, a newly described selective ligand for Zn^{2+} (Hider et al., 1990). Many features of the experiments were common for the two agents, but where the methods differed, these are described separately.

The experiments used either blood bank blood (2–14 days old, stored in citrate phosphate dextrose adenosine solution at 4°C) or freshly drawn blood with heparin as anticoagulant. Red cells were isolated by centrifuging and washing in a 150 mM KCl, 2 mM K-HEPES medium at 4°C, and then suspended at 10% hematocrit in a medium containing (in mM) 100 KCl, 125 sucrose, 5 glucose, 15 K-HEPES (pH 7.6 at room temperature) and $ZnCl_2$, *either* with ethylmaltol *or* with A23187, $MgCl_2$ and EGTA or NTA (nitrilo-triacetic acid). The medium was designed to have an ionic strength of 0.1 and to be slightly hypertonic to minimize hemolysis. A high-K medium also minimizes cell volume and membrane

potential changes which might follow the activation of Ca^{2+}-dependent K^+ channels by traces of Ca^{2+} (Ferreira & Lew, 1976). The cell suspensions were incubated under aerobic conditions at 37°C, usually for 2 hr, and their pH was adjusted to 7.40 ± 0.01 at 37°C by addition of small amounts of HCl or KOH solution. After incubation, extracellular total Zn and Mg were measured by centrifuging portions of suspension in duplicate. The supernatants were removed, diluted as necessary with 150 mM NaCl, acidified with one-tenth their volume of 15% perchloric acid, and the metal concentrations measured by atomic absorption spectrophotometry (Pye Unicam SP 9).

Intracellular cations were measured by layering 0.5-ml portions of suspension on top of 0.5-ml Dow Corning® 550 silicone fluid in a 1.5-ml polypropylene centrifuge tube and centrifuging for 30 sec at 8000 × *g*. The supernatant consisting of extracellular medium and silicone fluid was aspirated, and the tip of the tube containing the cell pellet was cut off. The cells were resuspended in 3 ml 150 mM-NaCl and extracted by addition of 0.3 ml 15% perchloric acid. After mixing and centrifuging, the Zn and Mg content of the supernatant were measured by atomic absorption spectrophotometry (Kalfakakou & Simons, 1990). Measurements were normally made in triplicate, and the results reported as mean ± SEM. The values obtained were corrected for Zn in trapped extracellular fluid by means of subsidiary experiments in which cell Zn content was measured over a range of extracellular Zn^{2+} concentrations in suspensions without A23187 or ethylmaltol. (This amounted to 2–3% of the external zinc concentration.) Intracellular cation measurements were then normalized to a constant number of cells (measured with a Coulter counter model ZF), and expressed as μmol or mmol per 10^{13} cells (approximately equal to a liter of cells). In the null-point experiments, where very small changes in cell Zn content were measured, the normalization to constant numbers of cells was carried out by measuring the hemoglobin concentration in the cell extracts before addition of perchloric acid. This was done by dilution with Drabkin's reagent and spectrophotometry at 541 nm.

Experiments with A23187

A23187 was added to cell suspensions as a 2-mM solution in ethanol to a final concentration of 10 μM (equivalent to about 100 μmol/liter · cells, final ethanol conc. 0.5%). A23187 carries Mg^{2+} as well as Zn^{2+} across the cell membrane, and a preliminary experiment indicated that changes in cell Mg content might have a significant effect on intracellular binding of Zn^{2+} (*not shown*). Such changes were normally avoided by including 0.4–0.5 mM free Mg^{2+} in the external medium. The medium also contained 3 mM EGTA or NTA as Zn^{2+}buffers. The free external Zn^{2+} and Mg^{2+} concentrations were calculated from the external Mg and Zn concentrations, measured by atomic absorption spectrophotometry, and the dissociation constants for EGTA and NTA. These were obtained from the values given for 25°C and ionic strength 0.1 (Martell & Smith, 1974) corrected to 37°C and pH 7.4. The dissociation constants used in the calculations were: Zn-EGTA $10^{-9.19}$ M, Mg-EGTA $10^{-2.15}$ M, Zn-NTA $10^{-8.41}$ M, and Mg-NTA $10^{-3.38}$ M.

Extracellular free Zn^{2+} can be related to intracellular free Zn^{2+} by assuming that Zn^{2+} is distributed across the red cell membrane in accordance with the Nernst equation, so that $[Zn^{2+}_{(in)}]/[Zn^{2+}_{(out)}] = \{[Cl^-_{(in)}]/[Cl^-_{(out)}]\}^{-2}$. The ^{36}Cl ratio (in/out) was measured on three occasions using cells pre-equilibrated in an EGTA buffer containing 1.2×10^{-11} M free Zn^{2+} and A23187. The ratios were 0.84 and 0.78 (blood bank cells) and 0.84 (fresh cells); overall mean 0.82. This value is equivalent to a difference of 0.17 log units between intracellular and extracellular free Zn^{2+} concentrations. Intracellular free Zn^{2+} concentration was estimated by adding 0.17 log · units to the calculated extracellular value.

Experiments with Ethylmaltol

Solid ethylmaltol was dissolved directly in the cell suspensions to a final concentration of 3 mM (calculated on the assumptions that ethylmaltol would equilibrate across the cell membrane and not be accumulated in the cytoplasm). Mg^{2+} was not added to the suspensions because ethylmaltol does not increase membrane permeability to Mg^{2+} (T.J.B. Simons, *unpublished observations*), and the cells' Mg content did not change during incubation. The free Zn^{2+} concentration in the medium was calculated from the medium Zn concentration, measured by atomic absorption spectrophotometry, and subsidiary experiments on the binding of Zn^{2+} by ethylmaltol.

The binding of Zn^{2+} to ethylmaltol was studied using antipyrylazo III as an optical indicator (Scarpa, Brinley & Dubyak, 1978). There were three steps to this: (*i*) measuring the optical density (OD) of antipyrylazo III in Zn^{2+}-NTA buffers of known Zn^{2+} concentration, (*ii*) measuring the OD of antipyrylazo III in solutions containing $ZnCl_2$ alone, and (*iii*) measuring the OD of antipyrylazo III in solutions containing 3 mM ethylmaltol and varying amounts of $ZnCl_2$ (Fig. 1). The OD measured was the difference between the absorbances at 520 and 605 nm at room temperature (25°C) using an Aminco DW-2 spectrophotometer. These wavelengths were determined empirically to give the maximum difference signal for the reaction of Zn^{2+} with antipyrylazo III. All measurements used the same concentration of antipyrylazo III (25 ng/ml). Under these circumstances, any OD value corresponds to a single concentration of free Zn^{2+} (given by measurement (*i*) above) and a concentration of Zn^{2+}-ethylmaltol complex (given by the difference between the $ZnCl_2$ concentrations at the same OD in measurements (*ii*) and (*iii*)). These corresponding values of free Zn^{2+} and Zn^{2+}-ethylmaltol can then be used to calculate the free Zn^{2+} concentration from the total Zn^{2+} concentration in Zn^{2+}-ethylmaltol mixtures in the absence of antipyrylazo III. The method involves empirical fitting of polynomial expressions to the curves corresponding to measurements (*i*), (*ii*) and (*iii*) (*see* Fig. 1) and then solving the resulting cubic equations. Note that this calibration procedure avoids making any assumption about the stoichiometry of the reactions between Zn^{2+} and ethylmaltol or antipyrylazo III.

Nonlinear regressions, both here and elsewhere, were carried out with Multifit 2.01 (Day Computing, Cambridge, UK) on a Macintosh SE computer.

Cell ^{36}Cl Ratio and Water Content

^{36}Cl was measured by adding 1 μCi ^{36}Cl to 2 ml of cell suspension. One min later, 0.5-ml portions were layered onto 0.8 ml silicone fluid and centrifuged (as above). The cell pellets were resuspended in 150 mM NaCl and extracted with 1.5% perchloric acid, and their ^{36}Cl content (and that of the supernatant, similarly treated) were measured by liquid scintillation counting. The water content of packed cells and external medium was measured as loss of weight on drying at 110°C, and the ^{36}Cl ratio calculated as (counts per unit weight of water) (in/out).

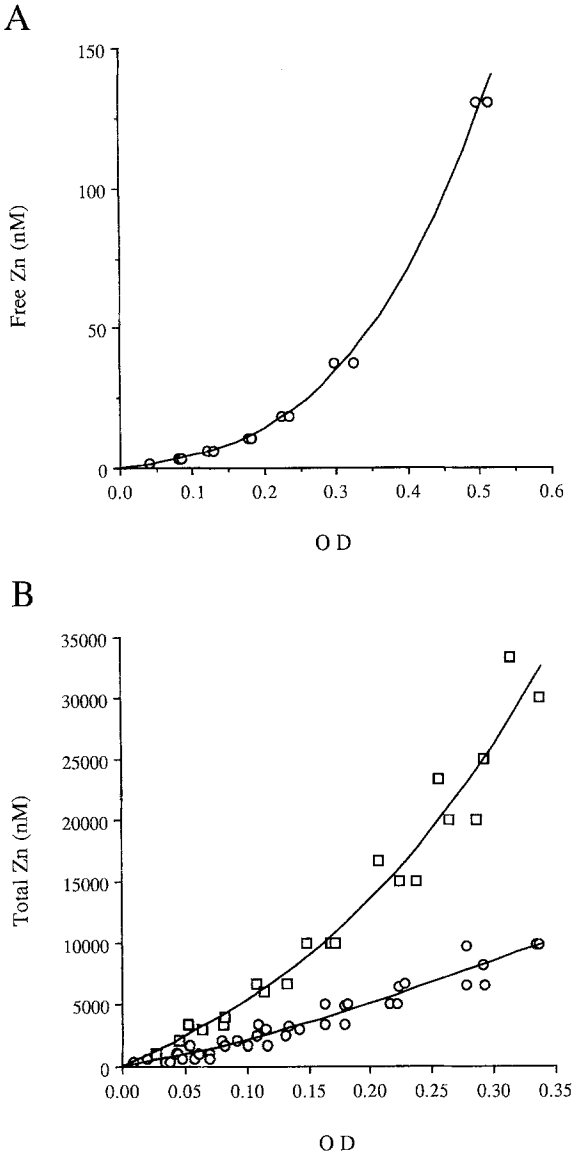

Fig. 1. Calibration of Zn^{2+}-ethylmaltol mixtures with antipyrylazo III. (*A*) Relationship of free Zn^{2+} concentration to Optical Density (OD) with Zn^{2+}-NTA buffers containing 3 mM NTA. (*B*) Relationship of total Zn^{2+} to OD in the presence of varying amounts of $ZnCl_2$, either in the absence (○) or presence (□) of 3 mM ethylmaltol. All measurements were made in media containing 140 mM KCl, 20 mM (K)-HEPES (pH 7.40) and 25 ng/ml antipyrylazo III at room temperature. The graphs are nonlinear regression fits to equations of the form $y = ax + bx^2 + cx^3$, with parameters (*a, b, c*) 30.3, 35.6, and 818 (*A*); 1.52×10^4, 6.27×10^4, and -6.43×10^4 (*B*, lower); and 4.31×10^4, 7.19×10^4, and 2.43×10^5 (*B*, upper)

Sources of Materials

Whole blood, or packed erythrocytes, were obtained from the South London Regional Transfusion Centre, except where fresh heparinized blood was used. ^{36}Cl was from Amersham International plc, ^{65}Zn from New England Nuclear, A23187 from Calbiochem, and antipyrylazo III from ICN Pharmaceuticals, Plainview, NY. All other chemicals were of analytical grade (where possible) either from B.D.H. plc or Sigma.

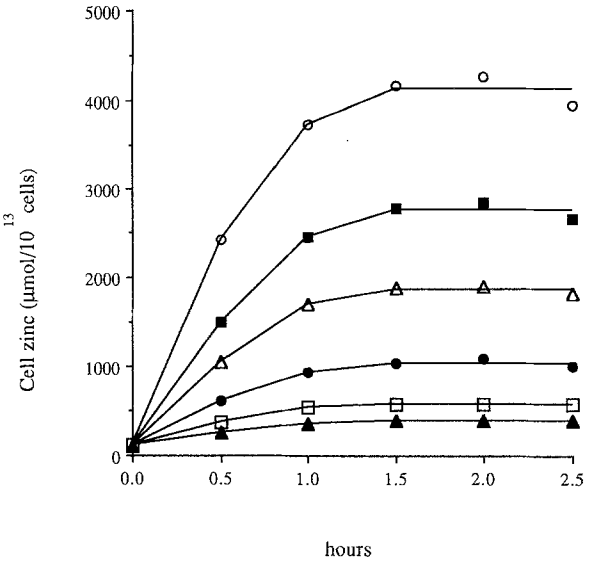

Fig. 2. Equilibration of cell and medium zinc with ethylmaltol. Cells were incubated at 37°C at 4% hematocrit in media containing 145 mM KCl, 5 mM glucose, 15 mM HEPES (pH 7.4), 3 mM ethylmaltol and either 10 μM (▲), 20 μM (□), 40 μM (●), 80 μM (△), 120 μM (■) or 200 μM (○) $ZnCl_2$. The figure shows the cell zinc contents; after 2.5 hr the medium zinc contents (reading from the top downwards) were 8.75, 4.80, 3.24, 1.70, 1.20 and 0.86 μM

Results

Equilibration of Zn^{2+} with Ethylmaltol and A23187

Figure 2 shows the rate at which cells gain zinc when incubated in the presence of 3 mM ethylmaltol and varying concentrations of $ZnCl_2$. The cells reach a steady state in about 1.5 hr. In all the other experiments an incubation time of 2 hr is used to ensure equilibration.

The rate at which A23187 brings about Zn^{2+} equilibration depends upon the ratio of A23187 to cells and the free Zn^{2+} concentration. At the ratio used (100 μmol A23187/liter cells), Zn^{2+} equilibration takes place within 15 min with μM free Zn^{2+} (*not shown*), but much more slowly at pM free Zn^{2+} concentrations. Figure 3 shows the change in zinc content when cells are incubated with A23187 in 3 mM EGTA, either in the absence of Zn^{2+}, which causes loss of cell Zn, or in the presence of Zn^{2+}, which brings about a small gain. There is a progressive change in cell zinc content

during the first 2 hr of incubation, after which the differences between the conditions seem to become no greater. The final zinc content was routinely measured 2 hr after addition of A23187, but it is possible that this may not be an equilibrium value.

Buffering of Intracellular Zn^{2+}

The buffering of intracellular Zn^{2+} was measured by equilibrating intra- and extracellular Zn^{2+} using either ethylmaltol or A23187. These measurements give corresponding values of cell zinc content and intracellular free Zn^{2+} concentration, which was calculated by the procedure described in the Materials and Methods.

Figure 4 shows there is little change in the cell zinc content as intracellular free Zn^{2+} concentration increases from 10^{-12} to 10^{-10} M, above which there is a large increase in Zn^{2+} buffering. There is reasonably good agreement between the measurements made with ethylmaltol and with A23187 in the overlapping region. The data can be fitted by a model in which Zn^{2+} binds to a single site, of capacity 10.4 ± 0.1 mmol/10^{13} cells, and affinity 1.8 ± 0.2 × 10^{-8} M Zn^{2+}. An expanded view of the buffering of Zn^{2+} at intracellular concentrations below 10^{-9} M is presented in Fig. 6.

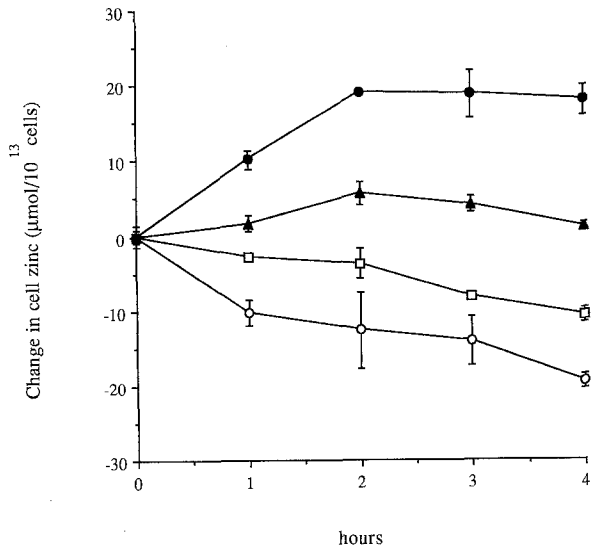

Fig. 3. Change in cell zinc content in EGTA buffers with A23187. Cells were incubated at 37°C at 10% hematocrit in media containing 100 mM KCl, 125 mM sucrose, 5 mM glucose, 0.4 mM $MgCl_2$, 15 mM HEPES (pH 7.4), 3 mM EGTA and either 0 (○, □), 50 μM (▲) or 200 μM (●)-$ZnCl_2$. (Calculated free Zn^{2+} was 1.2 (▲) or 4.8 (●) × 10^{-11} M.) A23187 (10 μM) was added at 0 hr, and the points give the change in cell zinc content during incubation (mean ± SEM (n = 3)). Results from two separate experiments with initial zinc contents of 140 (□, ▲) and 151 (○, ●) μmol/10^{13} cells

Estimation of Intracellular Free Zn^{2+} Concentration (Null-Point)

An example of a typical experiment is shown in Fig. 5. Here, the cells contained 150.8 ± 1.4 μmol zinc/10^{13} cells after washing. This is represented by the horizontal solid line and dashed lines. The cells were incubated with A23187 and a variety of Zn^{2+}/EGTA buffers for 2 hr, after which their zinc contents were analyzed and plotted against the calculated intracellular free Zn^{2+} concentration. The intersection of the interpolated line joining the points with the horizontal line gives the null point, in this case $10^{-10.51\pm0.06}$ M Zn^{2+}.

In all, null points were measured in six separate experiments, two using fresh cells and four with blood bank cells. The values are given in Table 1. The final average is $10^{-10.85\pm0.17}$ M Zn^{2+}, if the individual values are weighted equally, or $10^{-10.62\pm0.02}$ M Zn^{2+}, if the individual values are weighted by $(SE)^{-2}$.

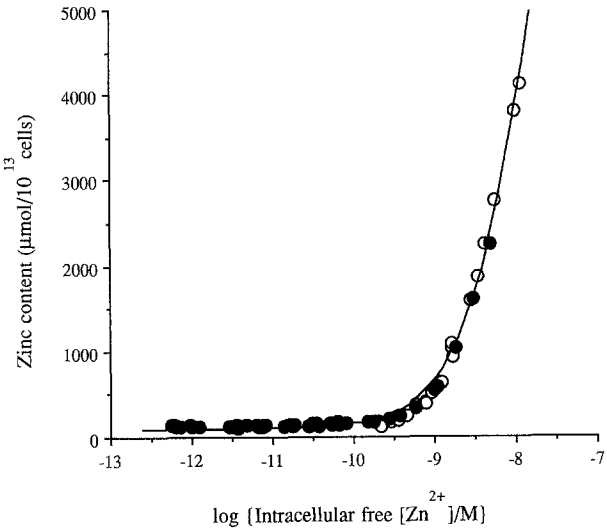

Fig. 4. Variation of zinc content with intracellular Zn^{2+} concentration. Cells were incubated with Zn^{2+}/ethylmaltol mixtures (○) or Zn^{2+}/EGTA buffers (●) for 2 hr at 37°C and then analyzed. Intracellular free Zn^{2+} concentrations were calculated by measuring total Zn in the medium (and Mg when EGTA was used), calculating the extracellular free Zn^{2+} concentration and then adding +0.17 log unit to obtain intracellular free Zn^{2+} (as described in Materials and Methods). The curve drawn is a nonlinear regression fit to the equation $y = a + bx/(c + x)$, with a = 91 ± 11 μmol/10^{13} cells, b = (1.038 ± 0.068) × 10^4 μmol/10^{13} cells and c = (1.78 ± 0.16) × 10^{-8} M. Combined results are from 10 experiments. The hemoglobin content of the cells used in the experiments at the higher free Zn^{2+} levels was either 247 or 269 g/10^{13} cells (mean 3.8 mmol/10^{13} cells)

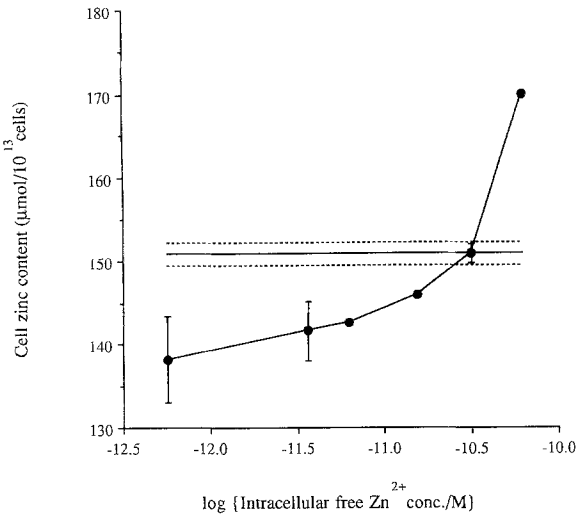

Fig. 5. A null-point experiment. Washed blood bank cells were analyzed for zinc at the start of the experiment. The horizontal line and the two dashed lines represent their initial zinc content, ±SEM (n = 3). The cells were then incubated with A23187 (100 μmol/liter · cells) in Zn^{2+}/EGTA buffers (including 0.4 mM $MgCl_2$) for 2 hr at 37°C. The points represent the final zinc content (±SEM, n = 3) plotted against the intracellular free Zn^{2+} concentration and calculated in the same way as in Fig. 4. The Mg^{2+} content of the cells was 2.00 mmol/10^{13} initially and in the range 1.92–2.05 mmol/10^{13} after the incubation. Experiment no. 1035 from Table 1

Zinc Buffering at Low Intracellular Zn^{2+} Concentrations

Most of the data on Zn^{2+} buffering at low intracellular Zn^{2+} concentrations come from the null-point experiments. The change in cellular zinc measured within a single experiment was comparable to the variability in initial zinc content between experiments, so it was necessary to normalize the results. The initial zinc content was made equal to 136.1 μmol/10^{13} cells in every experiment (the mean value), and all the other values were adjusted upward or downward in parallel. The adjusted data are shown in Fig. 6.

After normalization, all the points lie in a fairly narrow band (Fig. 6). The horizontal line represents the initial zinc content, 136.1 μmol/10^{13} cells. The results suggest that the Zn^{2+} buffering curve is virtually horizontal between $10^{-12.3}$ and $10^{-11.3}$ M intracellular free Zn^{2+}. The cells contain a basal amount of zinc, which cannot be further reduced by incubation with A23187 and EGTA, at least in the short term. The average value for this "inexchangeable" zinc is 129.4 ± 3.8 μmol/10^{13} cells (±SEM, n = 6).The remainder of the zinc initially present in the cells can be regarded as exchangeable. This amounts to 6.7 ± 1.5 μmol/10^{13} cells (±SEM, n = 6) from Fig. 6.

The dotted line on Fig. 6 represents the curve used to fit the data on Fig. 4. It is obviously not a good fit at this expanded scale. Other attempts to fit the data, either by constraining the "inexchangeable zinc" to a different value (*not shown*) or by using the Hill equation for the saturable component (shown as a dashed line), give an improved fit, but were not entirely satisfactory.

Effect of Mg^{2+}

The Mg content of the cells used in the null-point experiments was initially in the range from 1.45 to 2.00 mmol/10^{13} cells. After incubation with A23187 in solutions containing 0.4–0.5 mM free Mg^{2+}, it

Table 1. Null-point measurements of intracellular free $[Zn^{2+}]$

Experiment	Initial Zn^{2+} content (μmol/10^{13} cells)	Null-point log {$[Zn^{2+}_i]$/M}	Minimal Zn^{2+} content (μmol/10^{13} cells)
1016	139.3 ± 1.4	−11.54 ± 0.26	136.8 ± 1.4
1017	140.3 ± 0.8	−10.94 ± 0.04	136.6 ± 2.0
1018 (*F*)	124.3 ± 0.5	−10.44 ± 0.03	115.9 ± 1.4
1033	125.6 ± 1.8	−11.08 ± 0.16	120.9 ± 3.9
1035	150.8 ± 1.4	−10.51 ± 0.06	138.2 ± 5.1
1050 (*F*)	136.3 ± 1.7	−10.58 ± 0.08	128.1 ± 5.6
Mean	136.1 ± 4.1	−10.85 ± 0.17	129.4 ± 3.8

Summary of results from six null-point experiments. The initial zinc content is the value measured with washed red cells, and the minimal zinc content is the value measured after 2-hr incubation in 3 mM EGTA and 0.5 mM $MgCl_2$ plus A23187, without added Zn^{2+}. Both are expressed ±SEM (n = 3). The null point is determined as in Fig. 5, and is expressed in logarithmic units of concentration, with the error given by the intersection of the slanting line with the dotted lines in Fig. 5. Mean values were calculated (±SEM) without weighting. Experiments labelled (*F*) utilized fresh red cells; the remainder utilized blood bank cells.

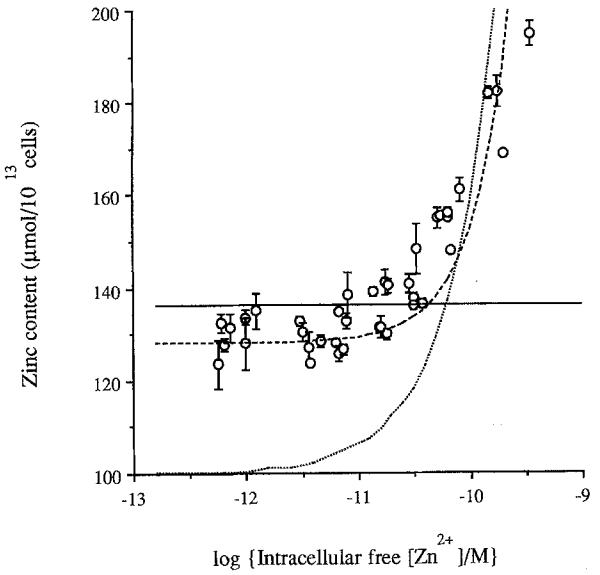

Fig. 6. Zinc buffering at low cell zinc content. The data from Fig. 4 are replotted, with an expanded scale, and normalized to an initial zinc content of 136.1 μmol/10^{13} cells, as described in the text. The points (±SEM, $n = 3$) are plotted against the calculated intracellular free Zn^{2+} concentration, as in Fig. 4. The horizontal line represents the initial zinc content, 136.1 μmol/10^{13} cells. The curves represent nonlinear regression fits: the dotted line to the equation $y = a + bx/(c + x)$, with parameters $a = 100\ U$, $b = 1.01 \times 10^4\ U$, $c = 1.72 \times 10^{-8}$ M, residuals 2.83×10^5; and the dashed line to the equation $y = a + bx^n/(c + x^n)$, with parameters $a = 128\ U$, $b = 6 \times 10^3\ U$, $c = 2.56 \times 10^{-11}$ M, $n = 1.298$ and residuals 1.47×10^5. (U represents μmol/10^{13} cells.) The nonlinear regression analysis includes all the data from Fig. 4, not just the points appearing on Fig. 6

was within 90–123% of the initial value. In two experiments, the $MgCl_2$ concentration in the medium was varied to see what effect this would have on intracellular Zn^{2+} buffering. The results are shown in Table 2. The changes in external $MgCl_2$ concentration have a large effect on cellular Mg content (as seen by Flatman & Lew, 1980), but relatively little effect on the zinc content of the cells. Increasing intracellular Mg decreases intracellular Zn content at higher free Zn^{2+} concentrations, but appears to increase total zinc at $10^{-11.5}$ M free Zn^{2+}.

Exchangeability of Intracellular Zinc

Further evidence that the bulk of intracellular zinc is inexchangeable came from experiments in which cells were loaded with ^{65}Zn and then incubated with A23187 and EDTA (ethylene diamine-N,N,N'N'-tetra-acetic acid) in order to remove zinc from the cells. Figure 7 shows that the zinc content of the cells returns to the same value that it had before Zn loading, while 95% of the tracer ^{65}Zn is lost. This shows that the tracer fails to equilibrate with the zinc initially present in the cells.

Discussion

Intracellular free Zn^{2+}

The best estimate of erythrocyte intracellular free Zn^{2+} is probably given by the weighted mean of the individual values in Table 1, i.e., $10^{-10.62}$ M or 2.4×10^{-11} M. This is comparable with the only other value for intracellular free Zn^{2+} concentration reported in the literature, 10^{-10} M in rabbit skeletal muscle (Peck & Ray, 1971). It is however subject to a number of qualifications. All free Zn^{2+} concentrations in this region depend upon the value chosen for the Zn^{2+}-EGTA dissociation constant, which is believed to be the best available from the chemical literature. The null-point method depends upon the assumption that the ionophore A23187 redistributes Zn^{2+} across the cell membrane in accordance with the Nernst equation, without itself affecting the membrane potential, which is dependent upon the chloride equilibrium. A23187 has a 10^4-fold higher affinity for Mg^{2+} than Zn^{2+} (Pfeiffer & Lardy, 1976), so there should only be insignificant quantities of zinc present as the Zn^{2+}-A 23187 complex. It is possible that Zn^{2+} may not reach complete equilibrium in 2 hr incubation with A23187. However, the results in Fig. 3 suggest that it is quite close to equilibrium by that time. The calculation of intracellular free Zn^{2+} concentration also assumes that the Zn^{2+} distribution is in equilibrium with the Cl^- distribution. This cannot be checked. Mg^{2+} fluctuations may have an effect. Experiments in which Mg^{2+} was varied intentionally are equivocal over whether Mg^{2+} would affect the null-point (Table 2). Mg^{2+} fluctuations in the null-point experiments were much smaller than in Table 2, so it is unlikely that they would have affected the result. Finally, the measurements only relate to washed red cells at the start of the experiment, and the intracellular free Zn^{2+} concentration may have changed either during the washing of the cells, or during previous storage, in the case of blood bank cells. Changes during storage are suggested by the observation that the intracellular free Zn^{2+} concentration in the experiments with fresh cells is higher than in stored cells (Table 1).

The estimate that intracellular free Zn^{2+} concentration is 2.4×10^{-11} M suggests that Zn^{2+} may not be at equilibrium across the red cell membrane in vivo. There are no direct measure-

Table 2. Effect of Mg variation on cellular zinc content

Experiment	Concentration in EGTA buffer		Cellular content after equilibration		Calculated free intracellular $[Zn^{2+}]$ (M)
	Mg^{2+} (μM)	Zn^{2+} (μM)	Mg^{2+} (mmol/10^{13} cells)	Zn^{2+} (μmol/10^{13} cells)	
1033	0	10	0.41 ± 0.01	118.7 ± 1.0	1.5×10^{-12}
	400	10	1.33 ± 0.01	122.4 ± 1.0	1.3×10^{-12}
	800	10	1.92 ± 0.01	127.6 ± 2.2	1.6×10^{-12}
	0	50	0.41 ± 0.02	128.9 ± 0.6	6.0×10^{-12}
	400	50	1.35 ± 0.00	128.7 ± 1.0	6.3×10^{-12}
	800	50	1.94 ± 0.01	127.2 ± 0.9	6.5×10^{-12}
	0	200	0.42 ± 0.02	148.2 ± 0.9	2.4×10^{-11}
	400	200	1.41 ± 0.00	145.0 ± 1.9	2.5×10^{-11}
	800	200	1.95 ± 0.01	141.5 ± 2.5	2.6×10^{-11}
(initial)			1.46 ± 0.01	125.6 ± 1.8	
1035	0	200	0.83 ± 0.01	182.6 ± 2.4	3.0×10^{-11}
	400	200	2.01 ± 0.02	169.9 ± 0.0	2.9×10^{-11}
	1000	200	3.12 ± 0.01	162.5 ± 1.8	3.2×10^{-11}
(initial)			2.00 ± 0.02	150.8 ± 1.4	

Cells were incubated in media containing 3 mM EGTA, the indicated concentrations of $ZnCl_2$ and $MgCl_2$, and A23187 (100 μmol/liter · cells) for 2 hr at 37°C, and then analyzed for their Zn and Mg contents, which are given ±SEM (n = 3). The initial values of the cellular Mg and Zn contents are also given, together with the intracellular free Zn^{2+} concentration at 2 hr, and calculated as described in Materials and Methods.

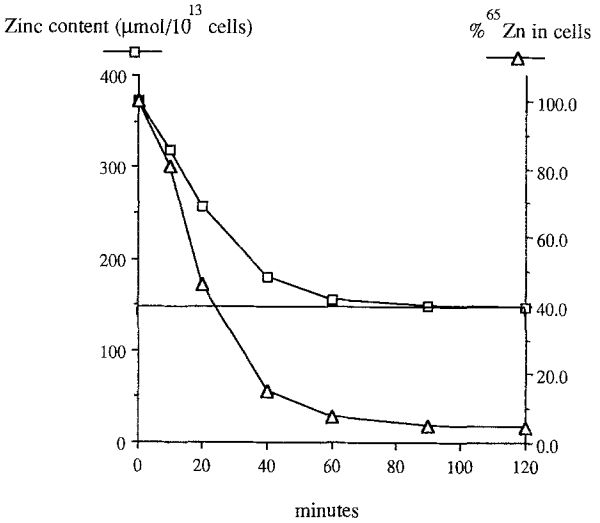

Fig. 7. Selective removal of added ^{65}Zn from cells. Red cells were preincubated for 2 hr at 37°C in standard medium supplemented with 2 mM $ZnCl_2$, 3 mM citrate, 1 mM $KHCO_3$ and ^{65}Zn. They were then washed and incubated at 37°C, 5% hematocrit, in standard medium supplemented with 1 mM EDTA and A23187 (100 μmol/liter · cells). The graph shows the cells' subsequent zinc content (measured by atomic absorption) and ^{65}Zn content (expressed as % of initial value). The horizontal line at 148 μmol/10^{13} cells gives the zinc content before the preincubation. One of three similar experiments is shown

ments of free Zn^{2+}in human serum, but the value of 2.1×10^{-10} M has been measured in horse serum (Magneson, Puvathingal & Ray, 1987). Comparison of horse and human serum suggests that free Zn^{2+} will be at least as high in human as in horses. The zinc content of human serum is 15 μM, compared with 8 μM in horse serum, while the inexchangeable serum zinc bound to α_2-macroglobulin is about the same, at 2.4 μM (Foote & Delves, 1984; Magneson et al., 1987). The vast majority of exchangeable serum zinc is bound to albumin. The higher concentration of exchangeable serum zinc in humans is not accompanied by a higher serum albumin concentration, i.e., the ratio of exchangeable zinc/albumin is higher in human than in horse serum (Foote & Delves, 1984; Magneson et al., 1987; Harris & Keen, 1989). Finally, the dissociation constant reported for Zn^{2+}/human albumin (10^{-7} M) (Giroux & Henkin, 1972) is larger than for Zn^{2+}/horse albumin (3×10^{-8} M) (Magneson et al., 1987). The weight of evidence suggests that the free Zn^{2+} concentration in human serum is greater than 2.1×10^{-10} M.

Intracellular Zinc Buffering

The buffering of free Zn^{2+} at intracellular concentrations above 10^{-10} M is almost certainly due to hemoglobin. Rifkind and Heim (1977) showed by equilibrium dialysis that one Zn^{2+} binds to every two heme groups, with association constant 1.3×10^7 M^{-1}, at

pH 7.2, 2°C and in 0.1 M NaCl. Under somewhat different conditions Gilman and Brewer (1978) also observed two Zn^{2+} binding sites per tetramer, with an average association constant of 1.9×10^6 M^{-1} over the pH range 6.83–7.11, at 20°C and in 0.25 M NaCl. The lower association constant can probably be ascribed to the more acid conditions and higher ionic strength. The curve fitted to Fig. 4 corresponds to 1.37 Zn^{2+} for every two heme groups and an association constant of 5.6×10^7 M^{-1} at 37°C and an intracellular pH of 7.3. The number of Zn^{2+} binding sites per hemoglobin tetramer could well be in error, because of the lack of observations above a Zn^{2+} content of 4 mmol/10^{13} cells.

There are other red cell constituents which might conceivably bind zinc, such as ATP, 2,3-diphosphoglycerate and glutathione. The effective dissociation constants for Zn^{2+}–ATP and Zn^{2+}–glutathione at pH 7.3 ($10^{-4.76}$ and $10^{-4.67}$ M, respectively) are too large for these molecules to play a significant role in intracellular Zn^{2+} buffering (Martell & Smith, 1982; Smith & Martell, 1989). There is no information available on the Zn^{2+}-2,3-diphosphoglycerate dissociation constant.

Exchangeability of Red Cell Zinc

In 1952, Tupper, Watts and Wormall showed there is no exchange of ^{65}Zn for zinc in carbonic anhydrase during 32 days at 0°C in vitro. They also labeled ox and rabbit red cells with ^{65}Zn and found no tracer in the carbonic anhydrase. About 87% of the zinc in human red cells is in carbonic anhydrase (Ohno et al., 1985), so it is not surprising that the majority of cellular zinc is inexchangeable (Figs. 6 and 7). The amount of exchangeable zinc in the red cell is estimated here as 6.7 ± 1.5 μmol/10^{13} cells (Fig. 6 and Table 1). This value is subject to the qualification that it may not be the same in vivo, because of changes during storage or washing of the cells. It is quite close to the estimate of 5 μmol exchangeable zinc/liter · cells obtained by Van Wouwe et al. (1990) who incubated red cells to equilibrium in media containing 7.6 μM $^{65}Zn^{2+}$ and 580 μM albumin (to mimic serum). It is considerably lower than Ohno et al. (1985) estimate of "other zinc" of 87 μg/100 ml cells (equivalent to about 12 μmol/10^{13} cells), but their estimate only excluded zinc in carbonic anhydrase and superoxide dismutase. Red blood cells also contain zinc in metallothionein (Grider et al., 1990) and probably also in δ-amino levulinic acid dehydratase and AMP deaminase (Galdes & Vallee, 1983). The contribution of these components to total cellular zinc, or to exchangeable zinc, remains unknown.

This work was supported by the Wellcome Trust. I thank Dr. G. Pocock for reading the manuscript.

References

Bettger, W.J., Taylor, C.G. 1986. Effects of copper and zinc status of rats on the concentration of copper and zinc in the erythrocyte membrane. *Nutr. Res.* **6:**451–457

Ferreira, H.G., Lew, V.L. 1976. Use of ionophore A23187 to measure cytoplasmic Ca buffering and activation of the Ca pump by internal Ca. *Nature* **259:**47–49

Flatman, P.W., Lew, V.L. 1980. Magnesium buffering in intact human red blood cells measured using the ionophore A23187. *J. Physiol.* **305:**13–30

Foote, J.W., Delves, H.T. 1984. Albumin bound and α_2-macroglobulin bound zinc concentrations in the sera of healthy adults. *J. Clin. Pathol.* **37:**1050–1054

Galdes, A., Vallee, B.L. 1983. Categories of zinc metalloenzymes. *In:* Metal Ions in Biological Systems. H. Sigel, editor. **15:**1–54, Marcel Dekker, New York

Gilman, J.G., Brewer, G.J. 1978. The oxygen-linked zinc-binding site of human haemoglobin. *Biochem. J.* **169:**625–632

Giroux, E.L., Henkin, R.I. 1972. Competition for zinc among serum albumin and amino acids. *Biochim. Biophys. Acta* **273:**64–72

Grider, A., Bailey, L.B., Cousins, R.J. 1990. Erythrocyte metallothionein as an index of zinc status in humans. *Proc. Natl. Acad. Sci. USA* **87:**1259–1262

Harris, W.R., Keen, C. 1989. Calculations of the distribution of zinc in a computer model of human serum. *J. Nutr.* **119:**1677–1682

Hider, R.C., Ejim, L., Taylor, P.D., Gale, R. Huehms, E., Porter, J. 1990. Facilitated uptake of zinc into human erythrocytes. Relevance to the treatment of sickle-cell anaemia. *Biochem. Pharmacol.* **39:**1005–1012

Kalfakakou, V., Simons, T.J.B. 1990. Anionic mechanisms of zinc uptake across the human red cell membrane. *J. Physiol.* **421:**485–497

Magneson, G.R., Puvathingal, J.M., Ray, W.J. 1987. The concentrations of free Mg^{2+} and free Zn^{2+} in equine blood plasma. *J. Biol. Chem.* **262:**11140–11148

Martell, A.E., Smith, R.M. 1974. Critical Stability Constants: Amino Acids. Vol. 1. Plenum, New York

Martell, A.E., Smith, R.M. 1982. Critical Stability Constants: First Supplement. Vol. 5. Plenum, New York

Ohno, H., Doi, R., Yamamura, K., Yamashita, K., Iizuka, S., Taniguchi, N. 1985. A study of zinc distribution in erythrocytes of normal humans. *Blut* **50:**113–116

Peck, E.J., Ray, W.J. 1971. Metal complexes of phosphoglucomutase in vivo. Alterations induced by insulin. *J. Biol. Chem.* **246:**1160–1167

Pfeiffer, D.R., Lardy, H.A. 1976. Ionophore A23187: The effect of H^+ concentration on complex formation with divalent and monovalent cations and the demonstration of K^+ transport in mitochondria mediated by A23187. *Biochemistry* **15:**935–943

Rifkind, J.M., Heim, J.M. 1977. Interaction of zinc with hemoglobin: Binding of zinc and the oxygen affinity. *Biochemistry* **16:**4438–4443

Scarpa, A., Brinley, F.J., Dubyak, G. 1978. Antipyrylazo III, a "middle range" Ca^{2+} metallochromic indicator. *Biochemistry* **17:**1378–1386

Simons, T.J.B. 1987. Optical probes for Zn^{2+} ions. *J. Physiol.* **394:**6P

Simons, T.J.B. 1990. An approach to the estimation of free zinc in human red blood cells. *J. Physiol.* **423:**47P

Smith, R.M., Martell, A.E. 1989. Critical Stability Constants: Second Supplement. Vol. 6. Plenum, New York

Tupper, R., Watts, R.W.E., Wormall, A. 1952. Some observations on the zinc in carbonic anhydrase. *Biochem. J.* **50:**429–432

Van Wouwe, J.P., Veldhuizen, M., DeGoeij, J.J.M., Van den Hamer, C.J.A. 1990. In vitro exchangeable erythrocytic zinc. *Biol. Trace Element Res.* **25:**57–69

Received 14 December 1990; revised 12 March 1991