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Iron and magnesium exchange via the low affinity iron transporter in rabbit erythroid cells—exchange rates and the action of valinomycin, diethylstilbestrol and protein kinase inhibitors

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Abstract

Evidence was presented previously that rabbit erythroid cells possess a low-affinity Fe^{2+} transport system which operates via the Na^+/Mg^{2+} antiport [Biochim. Biophys. Acta 1282 (1996) 163]. This was investigated further by measurements of Mg^{2+} efflux as well as Fe^{2+} uptake by the cells and by examining the inhibitory effects of valinomycin, diethylstilbestrol (DES) and protein kinase inhibitors. Mg^{2+} efflux and Fe^{2+} uptake were measured using rabbit reticulocytes and mature erythrocytes incubated in isotonic KCl or NaCl solutions. Both processes were slower in mature cells than reticulocytes. Mg^{2+} efflux into KCl solution was much lower than into NaCl solution but was stimulated by addition of Fe^{2+} to the solution. The rate of Fe^{2+}-stimulated Mg^{2+} efflux closely followed that of Fe^{2+} uptake in a one-to-one molar ratio. Valinomycin, DES and the protein kinase inhibitors all inhibited Fe^{2+} uptake from KCl solution. Valinomycin also inhibited Fe^{2+}-stimulated Mg^{2+} efflux into KCl solution but markedly stimulated the efflux into NaCl. Maximal inhibition of Fe^{2+} uptake from KCl solution required the presence of K^+, Rb^+ or Cs^+ ions with which valinomycin forms strong complexes. The results could not be explained on the basis of changes in cell membrane potential or cell volume. By contrast, the increase in Mg^{2+} efflux into NaCl solution produced by valinomycin was accompanied by cell shrinkage and production of a more negative membrane potential, either of which may be responsible for the effect. The inhibition produced by the protein kinase inhibitors indicate that phosphorylation of the transporter or an associated protein by protein tyrosine kinase is probably required to activate the transporter.

Keywords: Membrane transport; Magnesium–iron exchange; Reticulocyte; Erythrocyte; Valinomycin

1. Introduction

Ferrous iron (Fe^{2+}) is transported into rabbit and rat reticulocytes by at least two distinct mechanisms [1–5]. One, high-affinity transport, is present in reticulocytes but disappears as the cells mature into erythrocytes [2,4,6,7]. It has properties, including impaired function in reticulocytes from homozygous Belgrade rats [2,4], which show that it is mediated by divalent metal transporter 1 (DMT1) [8]. This is probably the mechanism by which iron is transported across the endosomal membrane after receptor-mediated uptake of transferrin-bound iron, the normal source of iron for developing erythroid cells [9,10].

The second transport process which has a lower affinity but higher capacity for iron transport [2,4,5] is less well defined. It persists in mature red blood cells but at a lower level of activity than in reticulocytes and is not impaired in cells from Belgrade rats [2,4]. The iron taken up is incorporated into heme but less efficiently than iron taken up from transferrin [2,5]. There is evidence that iron transport by this process is mediated by the Na^+/Mg^{2+} antiport [11]. The iron uptake is inhibited by Na^+ and stimulated by K^+ in the extracellular fluid [1,2]. The results suggest that, in Na^+-free solutions such as isotonic KCl, Fe^{2+} can substitute for extracellular Na^+ and be transported into the cell in exchange for Mg^{2+} [11]. Confirmation of this requires measurement of the rates of movement of Fe

Abbreviations: DES, diethylstilbestrol; MCHC, mean corpuscular hemoglobin concentration; PCV, packed cell volume; V_m, transmembrane potential difference

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and Mg across the cell membrane under conditions which are known to affect the rate of iron transport such as changes in iron concentration and the presence of inhibitors of iron transport. Two of the most potent inhibitors are valinomycin and diethylstilbestrol (DES) [3–5]. Moreover, there is evidence that protein phosphorylation is required for function of the Na^+/Mg^{2+} antiport [12]. Is it also required for Fe uptake?

The aim of this investigation was to investigate the mechanism of low-affinity iron transport into reticulocytes by comparing the rates of Fe^{2+} uptake and Mg^{2+} efflux and by studying the actions of valinomycin, DES and protein kinase inhibitors on Fe^{2+} uptake. The experiments were performed with freshly obtained rabbit reticulocytes and mature erythrocytes. In most cases, they were incubated in isotonic KCl solution, pH 7.0, conditions which produce maximal Fe^{2+} uptake [2]. The results showed that addition of Fe^{2+} to the KCl solution stimulated Mg^{2+} efflux from the cells at a rate proportional to that of Fe^{2+} uptake. Inhibitors of Fe uptake from KCl solution also inhibited Mg efflux. However, in NaCl solution, they had little effect on Mg efflux and, in the case of valinomycin, there was stimulation of efflux.

2. Materials and methods

2.1. Materials

Radioactive iron, ^{59}Fe ($FeCl_3$) was purchased from Dupont (Sydney, NSW, Australia). The biochemical reagents were from Sigma Chemical Company (St. Louis, MO, USA). Reticulocyte-rich blood was obtained from rabbits with phenylhydrazine-induced hemolytic anemia, 4–6 days after the last injection of phenylhydrazine [13]. The cells were washed three times in 0.155 M NaCl and then centrifuged at a packed cell volume (PCV) of approximately 0.7 at 4 °C for 30 min at 2500 × *g*. The top one-quarter of the cell layer was removed and washed once more in the final incubation solution, 0.155 M KCl, 0.155 M NaCl or 0.27 M sucrose. The cells were then suspended at a PCV of 0.3–0.4 in the same solution. The buffy coat (white cells and platelets) was removed after each centrifugation before resuspending the cells in the next washing solution. The reticulocyte count of the final cell suspensions varied from 33% to 83%. To differentiate them from suspensions containing virtually only mature erythrocytes (see below), the cells of the reticulocyte-rich cell suspensions will be referred to as reticulocytes even though they did contain some mature cells. The Mg^{2+} content of the cells was 6.05 ± 0.35 mmol/l cells (n = 5).

Mature erythrocytes, obtained from normal rabbits not treated with phenylhydrazine, were washed in the same way as reticulocytes except that the bottom one-quarter of the cells was taken after the 30-min centrifugation. The reticulocyte count of these cells varied from 1% to 3% and the Mg^{2+} content was 4.28 ± 0.16 mmol/l cells (n = 4).

2.2. Measurement of iron uptake and magnesium efflux

Iron uptake was determined by incubating 50 μl samples of the cell suspensions in 2.0 ml Hepes-buffered (pH 7.0) isotonic KCl solution (290 mOsm/kg) or NaCl solution (pH 7.4) at 37 °C for 15 min in the presence of ^{59}Fe-labelled Fe^{2+}, as in earlier work [2,4]. As shown in these earlier studies, incubation in KCl, pH 7.0 provides optimal conditions for iron uptake by the low-affinity uptake process but is strongly inhibitory of high-affinity Fe^{2+} uptake. It was also shown that iron uptake by the cells is linear for at least 30 min. Hence, the results obtained with a 15-min incubation period can be used to calculate the rate of iron uptake. After incubation, the cells were washed 3 times with ice-cold 0.155 M NaCl, followed by hemolysis in 15 mM Hepes (pH7.4) and centrifugation to separate cytosolic and membrane fractions of the cells [1,3]. These fractions were then counted for radioactivity in a γ-scintillation counter (1282 Compugamma, LKB Wallac, Sweden). The iron concentration used in most experiments was 20 μM but in some experiments, the concentration was varied as detailed below. Because this study is concerned with Fe^{2+} transport through the cell membrane, only iron uptake to the cytosolic fraction of the cells will be reported. It represented 64–79% of the total uptake. The remainder was present in the membrane fraction. The amount of iron incorporated into this fraction increased as the iron concentration in the incubation medium was raised but in no experiment did it exceed 36% of total uptake.

Mg^{2+} efflux was measured by incubating 50 μl samples of the cell suspensions in 2.0 ml Hepes-buffered KCl as for Fe^{2+} uptake or in 2.0 ml Hepes-buffered NaCl. A sample was taken at zero time, then incubation was commenced and a further four or five samples taken during the next 45 to 60 min. The samples were chilled in an ice bath for 2 min and then centrifuged at 4 °C for 8 min at 1250 × *g*. An aliquot of the supernatant solution was removed and the Mg^{2+} concentration measured by atomic absorption spectroscopy using a Varian Spectrophotometer AA-640Z instrument (Varian Australia Pty Ltd, Mulgrave, Victoria, Australia). The rate of Mg^{2+} efflux from the cells was determined by linear regression analysis of the results for Mg^{2+} concentration in the incubation solution. The Mg^{2+} content of the original cell suspension was determined by mixing 25 μl of cell suspension with 5.0 ml 10% (w/v) trichloroacetic acid followed by centrifugation at 4 °C for 15 min at 1250 × *g* and measurement of Mg in the supernatant solution.

When inhibitors were used, the cells were preincubated with the reagents for 10 min at room temperature before Fe^{2+} uptake or Mg^{2+} efflux were measured. In most experiments, iron uptake was measured only with KCl solution, not NaCl, because NaCl markedly inhibits Fe^{2+} uptake (see Refs. [2–4] and Fig. 3, below).

2.3. Analytical methods

The reticulocyte count was determined by staining blood cells with new methylene blue. Hemoglobin was determined by mixing 20 μl samples of the cell suspensions with 4 ml 0.04 % (v/v) NH_4OH solution and measurement of the absorbance at 541 nm. The assay was calibrated using standards of known hemoglobin concentration provided by the New South Wales Red Cross Blood Transfusion Service (Sydney, NSW, Australia). PCV was measured by the microhematocrit method. Osmolality was measured by freezing point depression using a Fiske One-Ten Osmometer (Fiske, Needham Heights, MA, USA). Transmembrane potential differences were determined by the method of Macey et al. [14].

The results are presented as means ± S.E. of three to five experiments. Significance of difference between means ($P<0.05$) was assessed using Student's t test.

3. Results

3.1. Magnesium efflux and iron uptake

The rate of Mg^{2+} efflux from reticulocytes into KCl solution alone was low but increased markedly when Fe^{2+} was added to the KCl. The efflux was greater when the Fe^{2+} concentration was 50 or 100 μM than when it was 20 μM, and with 50 or 100 μM Fe^{2+}, the rate was as high as that found with incubation in NaCl solution (Fig. 1A).

Fe^{2+} uptake from KCl increased as the Fe^{2+} concentration was raised (Fig. 1A), the mean values at each concentration level being approximately equal to the rate of iron-dependent Mg^{2+} efflux calculated by subtracting the values for efflux into KCl alone from those for efflux into KCl containing 20, 50 or 100 μM Fe^{2+}. When the individual values for Fe^{2+} uptake and iron-dependent Mg^{2+} efflux were compared (Fig. 2), there was a highly significant linear correlation ($P<0.0001$). The slope of the regression line (0.813) did not differ significantly from 1.0.

Fe^{2+} uptake and Mg^{2+} efflux were also measured in three experiments with mature erythrocytes. As with reticulocytes, Fe^{2+} and Mg^{2+} exchange increased as the Fe^{2+} concentration was raised but the rates were lower than in reticulocytes (Fig. 1B).

Fe^{2+} uptake and Mg^{2+} efflux from reticulocytes incubated in KCl solution containing 20 μM Fe^{2+} were both markedly inhibited by valinomycin (0.5 μM), DES (20 μM), imipramine (20 μM) and amiloride (500 μM) (Fig. 3). Iron uptake from NaCl was extremely low and was not affected significantly by the inhibitors. Mg^{2+} efflux into NaCl solution was significantly inhibited by DES although the degree of inhibition was less than that observed with KCl plus Fe^{2+}. However, imipramine and amiloride had no significant effect and efflux was greatly enhanced by valinomycin when the incubations were performed in NaCl (Fig. 3).

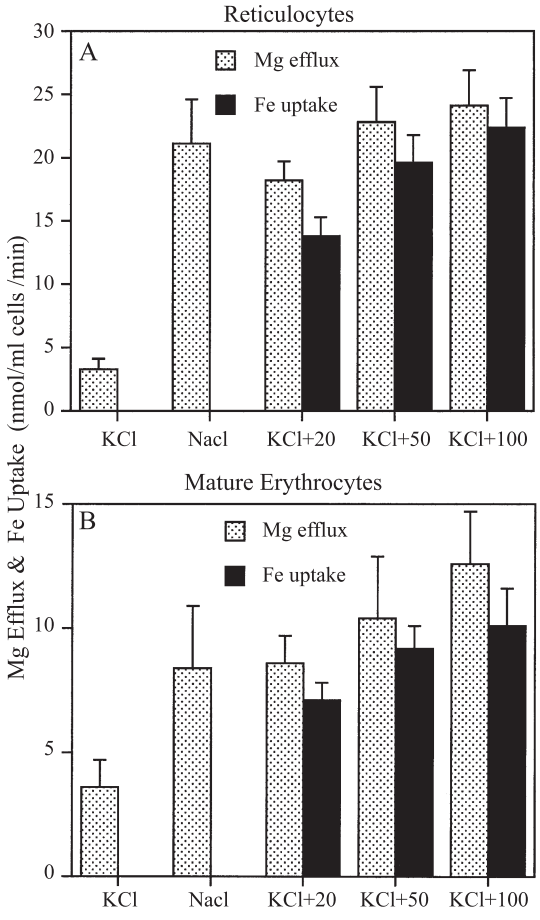

Fig. 1. Mg^{2+} efflux and Fe^{2+} uptake by rabbit reticulocytes (A) and mature erythrocytes (B). The cells were incubated in isotonic KCl without or with the addition of 20, 50 or 100 μM Fe^{2+} and in NaCl without addition of Fe^{2+} for Mg^{2+} efflux, or in KCl plus 20, 50 or 100 μM Fe^{2+} for the measurement of Fe^{2+} uptake. Each value is the mean ± S.E. of four measurements.

3.2. Action of valinomycin on iron uptake

Iron uptake from KCl solution was inhibited by valinomycin in a dose-dependent manner, maximum inhibition of more than 90% occurring at concentrations of 0.5–1.0 μM (Fig. 4). Valinomycin is an ionophore with high selectivity for K^+ [15,16]. Another ionophore with similar ion selectivity, but with lower affinity for K^+, is nonactin [16,17]. This reagent had no effect on Fe^{2+} uptake when used in the same concentration range as valinomycin (Fig. 4) and even at concentrations of 2.5, 5 and 10 μM continued to have no effect (results not shown). However, at 1 μM concentration, it did produce similar changes to the transmembrane potential difference as valinomycin (see below). It was observed previously that iron uptake from solutions of RbCl and CsCl was as efficient as from KCl but was lower when the KCl was replaced by LiCl [2]. Valinomycin was

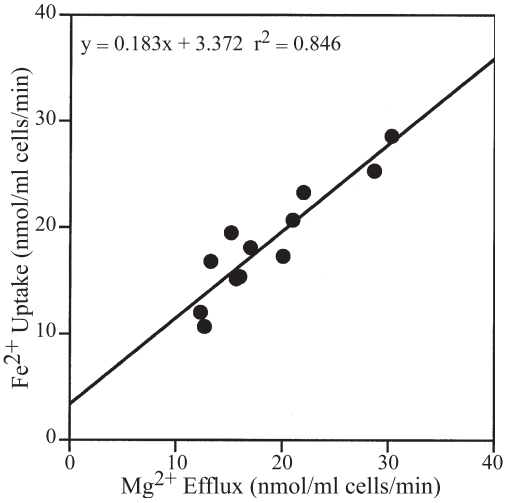

Fig. 2. Relationship between the rates of Fe^{2+} uptake and iron-dependent Mg^{2+} efflux in rabbit reticulocytes incubated in KCl solution containing 20, 50 or 100 μM Fe^{2+} solution.

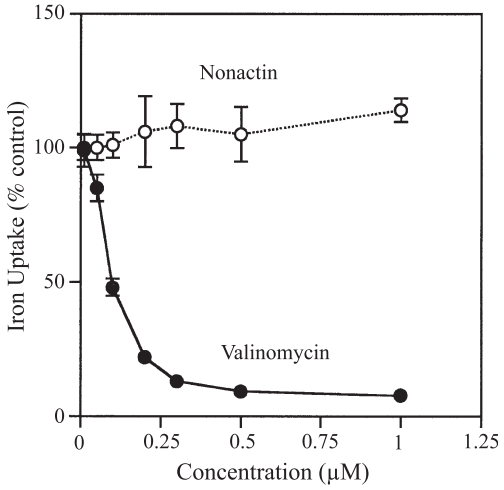

Fig. 4. Action of valinomycin and nonactin on Fe^{2+} uptake by rabbit reticulocytes. The results are presented as the percentage of the control value obtained in the absence of the reagents. Each value is the mean ± S.E. from three experiments.

found to inhibit Fe^{2+} uptake from RbCl and CsCl in a dose-dependent manner similar to that observed with KCl but had much less effect on uptake from LiCl and choline chloride solutions, especially at concentrations up to 0.3 μM (Fig. 5).

The effect of valinomycin was also influenced by the KCl concentration (Fig. 6). In cells incubated with 20 μM Fe^{2+} in isotonic sucrose solution, valinomycin stimulated iron uptake, as had been observed earlier with lower concentrations of iron [18]. When the sucrose was replaced progressively with increasing proportions of isotonic KCl, iron uptake in the absence of valinomycin increased to maximum values at about 100 μM KCl, while valinomycin inhibited uptake to a degree which increased as the KCl concentration was raised (Fig. 6).

Valinomycin increases the membrane conductance of K^+ and changes the membrane potential (V_m) of erythrocytes and reticulocytes from one dependent on the $[Cl_i^-]/[Cl_o^-]$ distribution ratio to one dependent on K^+ [18]. It therefore has different effects on V_m depending on whether the cells are suspended in KCl, NaCl or sucrose solutions. To see whether changes in V_m could be responsible for the observed effects of valinomycin on Fe^{2+} and Mg^{2+} transport,

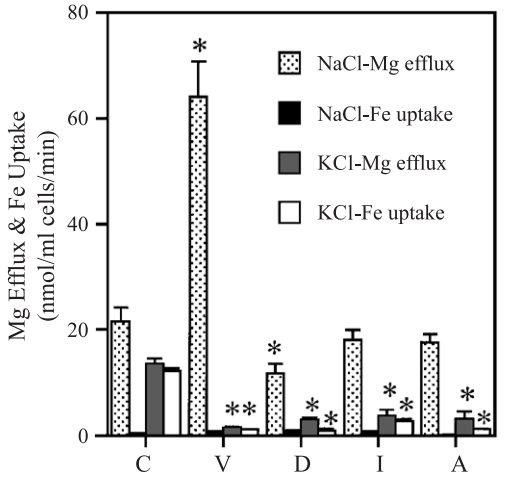

Fig. 3. Effects of inhibitors (0.5 μM valinomycin (V), 20 μM diethylstilbestrol (D), 20 μM imipramine (I) and 500 μM amiloride (A)) on the rates of Mg^{2+} efflux in rabbit reticulocytes incubated in isotonic NaCl solution or KCl solution containing 20 μM Fe^{2+} and of Fe^{2+} uptake from NaCl or KCl containing 20 μM Fe^{2+}. Also shown are the results obtained in the absence of the inhibitors (C). Each value is the mean ± S.E. of four measurements. *Significant differences ($P < 0.05$) from corresponding control (C) value.

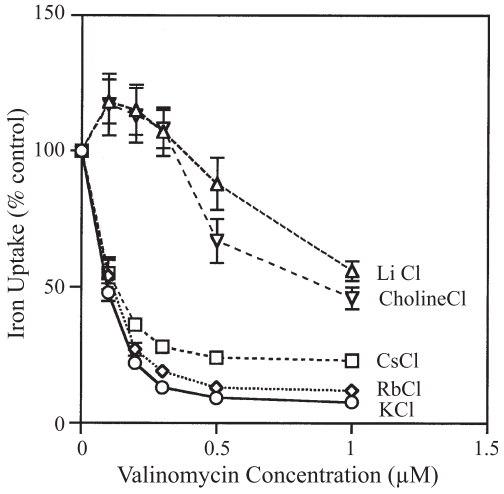

Fig. 5. Valinomycin-induced inhibition of Fe^{2+} uptake by rabbit reticulocytes incubated in isotonic media, pH 7.0, with different cation compositions. The cells were incubated with 20 μM Fe^{2+} and with varying concentrations of valinomycin in the media shown in the figure. The results are the means ± S.E. of three or four experiments with each salt.

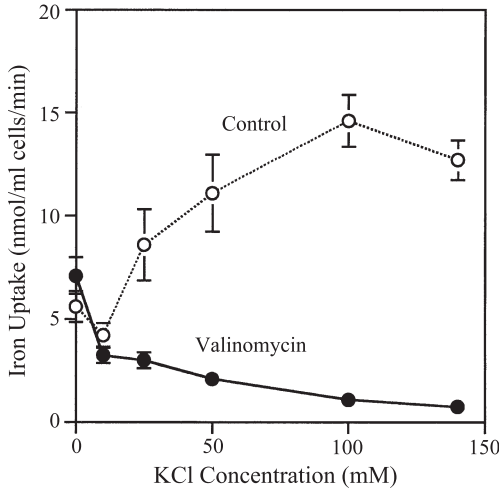

Fig. 6. Effect of KCl concentration of Fe^{2+} uptake by rabbit reticulocytes in the absence (control) or presence of valinomycin (0.5 μM). The KCl concentration was changed by mixing isotonic KCl with isotonic sucrose, in varying proportions. The Fe^{2+} concentration in the incubation media was 20 μM. The values are the means ± S.E. of three experiments.

the V_m was measured in reticulocytes suspended in isotonic sucrose which was progressively changed by replacement with increasing quantities of isotonic KCl or NaCl at pH 7.0, with or without the addition of valinomycin (0.5 μM). In the absence of valinomycin, the V_m fell from a high positive value (inside versus outside) in sucrose solution to lower values as the KCl or NaCl replaced the sucrose, reaching about −10 mV in 140 mM KCl or NaCl. In the presence of valinomycin, the mean V_m was −55 mV in sucrose solution and rose to near zero as the KCl concentration was raised but showed little change with increasing NaCl concentration (Fig. 7). Similar changes in V_m were found in two experiments using nonactin (1 μM) instead of valinomycin (results not shown).

The mechanism of interaction between valinomycin and Fe^{2+} uptake was further investigated by determining the effects of varying concentrations of valinomycin on iron uptake from KCl solutions containing increasing concentrations of Fe^{2+} (Fig. 8A). When the results were analysed by Eadie–Hofstee plots (Fig. 8B), it was found that valinomycin had very little effect on the K_m values for Fe^{2+} transport but reduced the V_{max} values (Fig. 9).

One possible effect of valinomycin, resulting from increased membrane conductance of K^+, is to change cell volume which, in turn, could affect Fe^{2+} uptake. The relative cell volume of reticulocytes was determined by measurement of hemoglobin concentration and PCV of cell suspensions and calculation of mean corpuscular hemoglobin concentration (MCHC) after incubation at 37 °C for 15 min in mixtures of isotonic sucrose and KCl solutions with or

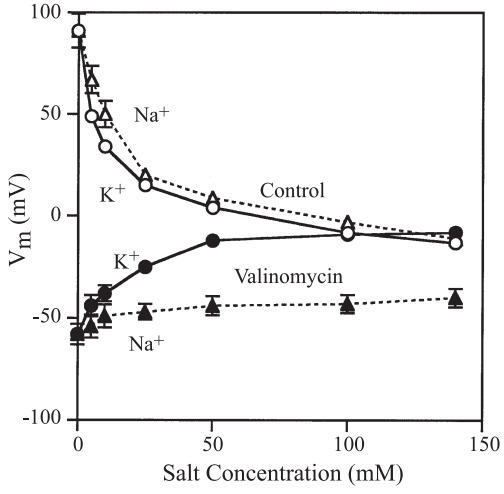

Fig. 7. Transmembrane potential difference, V_m (inside versus outside of cell), in rabbit reticulocytes incubated in isotonic KCl or NaCl mixed with isotonic sucrose in varying proportions to give the indicated salt concentrations. The incubations were performed without (control) or with 0.5 μM valinomycin. The values are the means ± S.E. of three experiments.

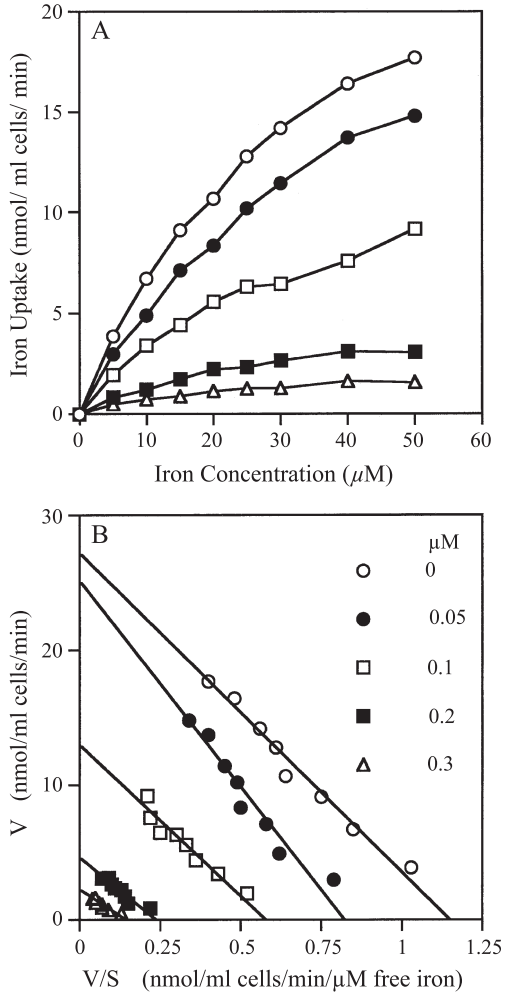

Fig. 8. (A) Effect of iron concentration on Fe^{2+} uptake by rabbit reticulocytes incubated in KCl solution in the presence of varying concentrations of valinomycin. The concentrations of valinomycin are shown in B which is an Eadie–Hoffstee plot of the results shown in A. This is a single experiment which was repeated twice more with similar results.

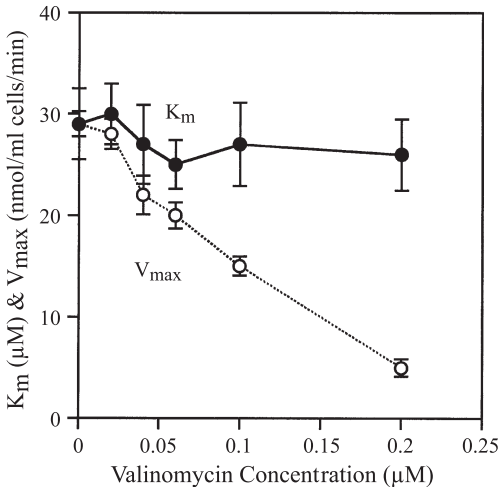

Fig. 9. Effect of valinomycin on the Michaelis–Menten constant (K_m) and maximum rate of Fe^{2+} uptake (V_{max}) calculated from three experiments of the type shown in Fig. 8. Each point is the mean ± S.E.

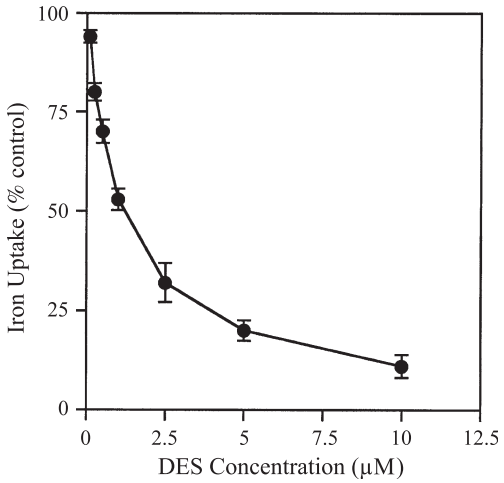

Fig. 11. Concentration-dependent inhibition of Fe^{2+} uptake by DES. Reticulocytes were incubated in KCl solution containing 20 μM Fe^{2+} and the indicated concentrations of DES. The results are shown as the means ± S.E. of three experiments.

without addition of valinomycin. These MCHC results and those of cells suspended in 0.155 M KCl before incubation were then used to calculate the relative cell volume of the incubated cells as a percentage. Valinomycin reduced the relative cell volume of reticulocytes incubated in KCl concentrations from 0 to 100 mM but not at 140 mM (Fig. 10). It also reduced the relative cell volume of reticulocytes incubated in isotonic NaCl to 66.8 ± 1.2% (n = 5).

3.3. Action of DES on Fe^{2+} uptake

DES inhibited Fe^{2+} uptake in a dose-dependent manner, near maximum inhibition occurring at 10 μM concentration (Fig. 11). Two related compounds, hexestrol and dienestrol, had almost identical effects (results not shown). When the effect of varying DES concentration on iron uptake from KCl solution containing different concentrations of Fe^{2+} was examined, it was found that DES had no significant effect of K_m for iron uptake but reduced the V_{max} values as the DES concentration was raised (results not shown).

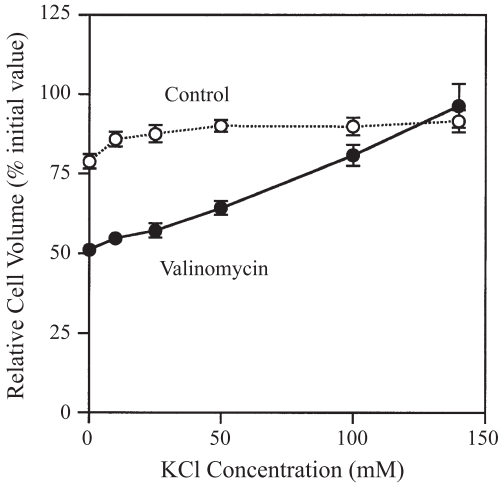

Fig. 10. Changes in relative cell volume of reticulocytes incubated for 15 min in isotonic solutions (pH 7.0) containing varying concentrations of KCl in the absence (control) or presence of 0.5 μM valinomycin. The solutions were prepared by mixing isotonic sucrose and KCl. The values (means ± S.E. of three experiments) are expressed relative to freshly obtained cells suspended in KCl and were calculated from the MCHC of the cells before and after the incubations, as described in the text.

3.4. Effects of protein kinase inhibitors on Fe^{2+} uptake

Three protein kinase inhibitors were tested, staurosporine, genistein and tyrphostin 25. They were all strong inhibitors of Fe^{2+} uptake from KCl solution (Table 1).

Table 1
Inhibition of iron uptake by protein kinase inhibitors

Inhibitor	Concentration (μM)	Iron uptake (% control value)
Staurosporine	0.1	64 ± 7.6
	0.3	42 ± 6.1
	1.0	27 ± 5.0
	2.0	22 ± 3.7
Genistein	10	100 ± 4.5
	30	99 ± 7.2
	100	40 ± 4.1
	500	29 ± 6.1
Tyrphostin 25	25	33 ± 2.3
	50	17 ± 3.2
	100	11 ± 2.9
	200	9 ± 1.7

The uptake of 20 μM Fe(II) by reticulocytes was measured using cells incubated in isotonic KCl solution (pH 7.0) in the absence (control) or presence of the inhibitors at the concentrations indicated. The results are presented as the percentage of the values obtained in the control incubations. Each value is the mean ± S.E. of four experiments.

4. Discussion

These experiments were performed using fresh rabbit erythroid cells without manipulation of their Mg^{2+} content, unlike many previous investigations of Mg^{2+} transport. They provide further evidence that low-affinity iron uptake by the cells is mediated by a Mg^{2+}/Fe^{2+} exchange process, probably by the Mg^{2+}/Na^{+} antiport, as discussed previously [4,11]. In support of this conclusion, Mg^{2+} efflux from the cells was found to be stimulated in a concentration-dependent manner by addition of Fe^{2+} to the KCl incubation solution or by replacement of KCl by NaCl. Moreover, the molar ratio of Fe^{2+} uptake and iron-stimulated Mg^{2+} efflux from reticulocytes into KCl solution did not differ significantly from 1.0, indicating that there was a one-to-one exchange of Mg^{2+} for Fe^{2+}.

Further support for the exchange process is derived from the effects of inhibitors and the changes which occur during maturation of reticulocytes. The potent inhibitors of low-affinity iron uptake, valinomycin, DES, imipramine and amiloride [3,4] all inhibited iron-stimulated Mg^{2+} efflux into KCl solution to almost the same degree as they inhibited Fe^{2+} uptake. However, imipramine and amiloride had no significant effect on Mg^{2+} efflux into NaCl solution at the concentrations used and valinomycin caused a large increase. With respect to these results, Mg^{2+} efflux from rat erythrocytes into a NaCl medium was previously shown to be less sensitive than efflux into KCl to inhibition by quinidine [19] which also inhibits Fe^{2+} uptake from KCl solution [3,11]. A similarly lower sensitivity to inhibition by DES, imipramine and amiloride may account for the results obtained in the present study. The explanation of these effects is unclear but they suggest that even if Fe^{2+} and Na^{+} exchange for Mg^{2+} via the same antiport, as concluded earlier [11], they probably interact with different components of the antiport. The results obtained with valinomycin are even more difficult to explain as will be discussed below.

The rate of low-affinity Fe^{2+} transport into mature rabbit [2,4,11] and rat [5] erythrocytes is lower than into reticulocytes. In the present work, iron-stimulated Mg^{2+} efflux was also shown to be lower in the mature cells. This was associated with a lower Mg^{2+} content of the cells, the values obtained being similar to those reported previously [20,21] and are in accordance with the observation that red cell magnesium concentration falls as the cells age [22]. Also, several other cation transport processes are more active in reticulocytes than erythrocytes [23].

Valinomycin produced profound inhibition of Fe^{2+} uptake and iron-stimulated Mg^{2+} efflux in KCl solution at concentrations of 0.3–0.5 μM. It is possible that the inhibition is due to a change in membrane potential because the degree of inhibition increased with increasing KCl concentration, in association with a rising V_m. However, nonactin which, like valinomycin, is a K^{+} ionophore and altered V_m in a similar manner to valinomycin, had no effect on Fe^{2+} uptake. Moreover, in 100 and 140 mM KCl, the V_m was almost the same in the presence as in the absence of valinomycin, yet the ionophore produced marked inhibition of Fe^{2+} uptake. Hence, it is unlikely that the inhibitory effect of valinomycin on Fe^{2+} uptake is due to alteration of V_m. Fe^{2+} uptake probably occurs by an electroneutral process such as the one-to-one Mg^{2+}/Fe^{2+} exchange observed in this study.

The observation that valinomycin at a concentration of 0.3 μM is inhibitory in KCl, RbCl or CsCl solutions but not in LiCl and choline chloride solutions suggests that the inhibition is due to greater membrane conductance of K^{+}, Rb^{+} and Cs^{+} compared with the other cations. Valinomycin has greater selectivity for the first three ions [15–17]. However, inhibition was observed in KCl concentrations varying from 25 to 140 mM and within this range, net movement of K^{+} could be in either direction. At lower salt concentrations, net loss of K^{+} from the cells could explain the observed decreased in cell size but cell shrinkage did not occur in 140 mM KCl even though inhibition of Fe^{2+} uptake was still observed. Hence a valinomycin-induced change in cell size cannot explain its inhibitory effect.

The most likely explanation for the inhibition produced by valinomycin in KCl solution is that it is due to the action of the ionophore–K^{+} complex rather than the free ionophore on the iron transport mechanism. This would explain why iron transport was inhibited by lower concentrations of valinomycin when the cells were incubated in the presence of K^{+}, Rb^{+} or Cs^{+} than when incubated with Li^{+} or choline^{+}, and why the inhibition was greater when the salt concentration was higher. Presumably, higher salt and valinomycin concentrations would be required to achieve inhibitory levels of the ionophore–metal complexes in the membrane when Li^{+} or choline^{+} were used than with the other three ions.

The exact mechanism by which the valinomycin–metal complex inhibits Fe^{2+} transport is unknown. It was of non-competitive nature as indicated by the change in V_{max} but not K_m values for Fe^{2+} uptake. Hence, the effect is likely to be an indirect one rather than due to interaction with the metal-binding sites of the transporter. Possibly it is due to an effect on the conformation of the transporter or on cell membrane properties which influences the function of the transporter, or on a mechanism required for activation of the carrier such as phosphorylation.

By contrast to its inhibition of iron-dependent Mg^{2+} efflux into KCl solution, valinomycin markedly stimulated Mg^{2+} efflux into NaCl. Three major differences are readily apparent with respect to the two incubation media. One is that the membrane potential (V_m) is much greater (inside negative) in isotonic NaCl than KCl. This would be expected to inhibit rather than enhance Mg^{2+} efflux. However, if the efflux occurs by an electroneutral process by exchange with Na^{+}, the change in V_m could possibly activate the transporter, resulting in the observed enhancement of Mg^{2+} efflux. The second difference is that valino-

mycin led to shrinkage of cells incubated in isotonic NaCl, but not in isotonic KCl. The change in cell volume may activate a transporter. The third difference is that valinomycin has a high affinity for K^+ but only extremely low affinity for Na^+. Reaction with K^+ produces distinct conformational changes [24] and a net positive charge on the valinomycin–K^+ complex. Either of these two effects may be required for the inhibition found in KCl solutions. Uncomplexed valinomycin is a neutral molecule which probably has greater lipophilicity and solubility in cell membranes compared with the K^+ complex, and it also has a different confirmation from the complex [24]. These properties may be responsible for the effect observed in NaCl solution. In contrast to its effect on Mg^{2+} efflux into NaCl solution, valinomycin did not significantly affect Fe^{2+} uptake. This is probably because Fe^{2+} uptake is markedly inhibited by Na^+ due to competition for the transport mechanism [5,11]. However, valinomycin has been shown to markedly stimulate Na^+ entry into rabbit erythrocytes [25]. This suggests that the increased Mg^{2+} efflux observed in the present study was due to exchange with extracellular Na^+ by activation of the antiport.

DES, like valinomycin, blocked Fe^{2+} uptake in a non-competitive manner. DES is known to inhibit several ion-motive ATPases [26–28] as are quercetin [26] and oligomycin [29], both of which are also potent inhibitors of low-affinity iron transport [3,4,11]. This suggests that the transporter may depend on ATPase activity for its function. Mg^{2+} efflux is markedly impaired in metabolically depleted human erythrocytes with low ATP levels [30,31], as is Fe^{2+} uptake by rabbit cells [11]. However, whether the hydrolysis of ATP is required to drive an active transport process is unknown [32]. Phosphorylation of a 230-kDa membrane protein is associated with increased Mg^{2+} transport out of chicken red cells and both the phosphorylation and Mg^{2+} transport are inhibited by amiloride [12]. The results presented here on the effects of protein kinase inhibitors indicate that phosphorylation is also required for low-affinity Fe^{2+} transport. Although no inhibitors are absolutely specific for particular types of protein kinase, genistein and tyrphostins are believed to show a high level of specificity for tyrosine protein kinase [33,34] which suggests that this kinase is involved. Staurosporine, a non-specific protein kinase inhibitor, was previously shown to block Mg^{2+}/Na^+ exchange in rat erythrocytes [35].

The function of the low-affinity Fe^{2+} transport mechanism is unclear. The process can transport other divalent transition metals in addition to Fe^{2+} (Mn^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}) [4]. Possibly its primary function is to transport one or more of these metals rather than Fe^{2+}. However, this is unlikely for at least three reasons. (1) The transport of all these metals into erythroid cells is inhibited by extracellular Na^+ [4], the main cation of extracellular fluid. (2) The transport process has a low affinity for all of the metals [4] and for optimal function requires concentrations of the metals much higher than those present in extracellular fluid in vivo. (3) The rate of iron uptake is far greater than that of iron uptake from the physiological source, transferrin [3,5] and hence greatly exceeds that required for hemoglobin synthesis; moreover, low-affinity transport persists in mature erythrocytes which have little or no requirement for iron uptake.

It is possible that the function of the low-affinity transporter lies in the export of one or more transition metals to help maintain cellular homeostasis or for transport across cellular barriers in the body. There is evidence that Mg^{2+} transport can reverse its direction, leading to Mg^{2+} uptake and Na^+ efflux [35]. Mn^{2+} can replace Na^+ in the exchange process and be transported in exchange for Mg^{2+} in both directions across rat erythrocyte cell membranes [19]. Also, Mn^{2+} efflux from rabbit erythroid cells is stimulated by extracellular Fe^{2+} as well as by Mn^{2+}, Mg^{2+} and Na^+ [36]. Hence, the physiological role of the transporter may be to export one or more of these metals. Iron export is not an important function in erythroid cells but it is important in other types of cells, for example, intestinal mucosal cells, hepatocytes, macrophages and placental trophoblast cells. One cellular iron exporter has been identified and called IREG1 [37], ferroportin1 [38] and MTP1 [39]. However, its mechanism of function and whether it is the only iron exporter is unknown. Also, Na^+-dependent Mg^{2+} efflux has been described in many cell types as well as erythroid cells [40]. Hence, the possibility remains that efflux of Fe^{2+} or other transition metals is mediated by this transporter. With respect to iron, dietary deficiency of Mg^{2+} in rats has been found to lead to elevated plasma and hepatic iron levels [41], suggesting that interaction between iron and magnesium in the absorptive process and enhanced iron absorption had occurred.

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References

[1] A. Egyed, Na^+ modulates carrier-mediated Fe^{2+} transport through the erythroid cell membrane, Biochem. J. 275 (1991) 635–638.
[2] L.L. Hodgson, E.A. Quail, E.H. Morgan, Iron transport mechanisms in reticulocytes and mature erythrocytes, J. Cell. Physiol. 162 (1995) 181–190.
[3] D.L. Savigni, E.H. Morgan, Use of inhibitors of ion transport to differentiate iron transporters in erythroid cells, Biochem. Pharmacol. 52 (1996) 371–377.
[4] D.L. Savigni, E.H. Morgan, Transport mechanisms for iron and other transition metals in rat and rabbit erythroid cells, J. Physiol. 508 (1998) 837–850.
[5] E.H. Morgan, Mechanisms of iron transport into rat erythroid cells, J. Cell. Physiol. 186 (2001) 193–200.
[6] A. Egyed, Carrier mediated iron transport through erythroid cell membranes, Br. J. Haematol. 68 (1988) 483–486.

[7] E.H. Morgan, Membrane transport of non-transferrin-bound iron by reticulocytes, Biochim. Biophys. Acta 943 (1988) 428–439.
[8] H. Gunshin, B. Mackenzie, U.V. Berger, Y. Gunshin, M.F. Romero, W.F. Boron, S. Nussberger, J.L. Gollan, M.A. Hediger, Cloning and characterization of a mammalian proton-coupled metal-ion transporter, Nature 388 (1997) 482–488.
[9] M.D. Fleming, C.C. Trenor, M.A. Su, D. Foernzler, D.R. Bier, W.F. Dietrich, N.C. Andrews, Microcytic anaemia mice have a mutation in *Nramp2*, a candidate iron transporter gene, Nat. Genet. 16 (1997) 383–386.
[10] M.D. Fleming, M.A. Romano, M.A. Su, L.M. Garrick, M.D. Garrick, N.C. Andrews, *Nramp2* is mutated in the anemic Belgrade (*b*) rat: evidence of a role for *Nramp2* in endosomal iron transport, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 1148–1153.
[11] L.M. Stonell, D.L. Savigni, E.H. Morgan, Iron transport into erythroid cells by the Na^+/Mg^{2+} antiport, Biochim. Biophys. Acta 1282 (1996) 163–170.
[12] T. Günther, J.V. Vormann, Probable role of protein phosphorylation in the regulation of Mg^{2+} efflux via Na^+/Mg^{2+} antiport, Mag. Bull. 8 (1986) 307–309.
[13] D. Hemmaplardh, E.H. Morgan, The mechanism of iron exchange between synthetic iron chelators and rabbit reticulocytes, Biochim. Biophys. Acta 373 (1974) 84–99.
[14] R.I. Macey, J.S. Adorante, F.W. Orme, Erythrocyte membrane potentials determined by hydrogen ion distribution, Biochim. Biophys. Acta 512 (1978) 284–295.
[15] B.C. Pressman, E.J. Harris, W.S. Jagger, J.H. Johnson, Antibiotic-mediated transport of alkali ions across lipid barriers, Proc. Natl. Acad. Sci. U. S. A. 58 (1967) 1949–1956.
[16] B.C. Pressman, Biological applications of ionophores, Annu. Rev. Biochem. 45 (1976) 501–530.
[17] D.H. Haynes, B.C. Pressman, Two-phase partition studies of alkali cation complexation by ionophores, J. Membr. Biol. 18 (1974) 1–21.
[18] E.A. Quail, E.H. Morgan, Role of membrane surface potential and other factors in the uptake of non-transferrin-bound iron by reticulocytes, J. Cell. Physiol. 159 (1994) 238–244.
[19] J.-C. Féray, R. Garay, A one-to-one Mg^{2+}: Mn^{2+} exchange in rat erythrocytes, J. Biol. Chem. 262 (1987) 5763–5768.
[20] G.A. Charbon, M.H. Hoekstra, Mineral content in plasma and blood cells of various species, Acta Physiol. Pharmacol. Neerl. 10 (1962) 209–214.
[21] L.S. Valberg, R.T. Card, E.J. Poulson, J. Syivek, The metal composition of erythrocytes in different species and its relationship to the lifespan of the cells in the circulation, Comp. Biochem. Physiol. 15 (1965) 347–359.
[22] P.W. Flatman, The control of red cell magnesium, Mag. Res. 1 (1988) 5–11.
[23] C. Brugnara, L. de Franceschi, Effect of cell age and phenylhydrazine on the cation transport properties of rabbit erythrocytes, J. Cell. Physiol. 154 (1993) 271–280.
[24] D.G. Davis, D.C. Tosteson, Nuclear magnetic resonance studies of the interactions of anions and solvent with cation complexes of valinomycin, Biochemistry 14 (1975) 3962–3969.
[25] N. Escobales, J. Figueroa, Na^+/Na^+ exchange and Na^+/H^+ antiport in rabbit erythrocytes: two distinct transport systems, J. Membr. Biol. 120 (1991) 41–49.
[26] B.J. Bowman, S.E. Mainyer, K.E. Allen, C.W. Slayman, Effects of inhibitors on the plasma membrane and mitochondrial adenosine triphosphatases of *Neurospora crassa*, Biochim. Biophys. Acta 512 (1978) 13–28.
[27] M.W. McEnery, P.L. Pedersen, Diethylstilbestrol, a novel F_o-directed probe of the mitochondrial proton ATP-ase, J. Biol. Chem. 261 (1986) 1745–1752.
[28] P.L. Pedersen, E. Carafoli, Ion motive ATPases: I. Ubiquity, properties, and significance to cell function, TIBS 12 (1987) 146–150.
[29] R. Whittam, K.P. Wheeler, A. Blake, Oligomycin and active transport reactions in cell membranes, Nature 203 (1964) 720–724.
[30] H. Lüdi, H.J. Schatzmann, Some properties of a system for sodium-dependent outward movement of magnesium from metabolising human red blood cells, J. Physiol. 390 (1987) 367–382.
[31] E.J. Frenkel, M. Graziani, H.J. Schatzmann, ATP requirement of the sodium-dependent magnesium extrusion from human red blood cells, J. Physiol. 414 (1989) 385–397.
[32] P.W. Flatman, Mechanisms of magnesium transport, Annu. Rev. Physiol. 53 (1991) 259–271.
[33] T. Akiyama, J. Ishida, S. Nakagawa, H. Ogawara, S.-I. Watanabe, N. Itoh, M. Shibuya, Y. Fukomi, Genistein, a specific inhibitor of tyrosine-specific protein kinases, J. Biol. Chem. 262 (1987) 5592–5595.
[34] A. Levitzki, A. Gazit, Tyrosine kinase inhibition: an approach to drug development, Science 267 (1995) 1782–1787.
[35] T. Günther, J. Vormann, Reversibility of Na^+/Mg^{2+} antiport in rat erythrocytes, Biochim. Biophys. Acta 1234 (1995) 105–110.
[36] A.C.G. Chua, L.M. Stonell, D.L. Savigni, E.H. Morgan, Mechanisms of manganese transport in rabbit erythroid cells, J. Physiol. 496 (1996) 99–112.
[37] A. McKie, P. Marciani, A. Rolfs, K. Brennan, K. Wehr, D. Barrow, S. Muret, A. Bomford, T.J. Peters, F. Farzaneth, M.A. Hediger, M.W. Hentze, R.J. Simpson, A novel duodenal iron-regulated transporter IREG1, implicated in the basolateral transfer of iron to the circulation, Mol. Cell 5 (2000) 299–309.
[38] A.D. Donovan, A. Brownlie, Y. Zhou, J. Shepard, S.J. Pratt, J. Moynihan, B.H. Paw, A. Drejer, B. Barut, A. Zapata, T.C. Law, C. Brugnara, S.E. Lux, G.S. Pinkus, J.L. Pinkus, P.D. Kingsley, J. Palls, M.D. Fleming, N.C. Andrews, L.I. Zon, Positional cloning of zebrafish *ferroportin1* identifies a conserved vertebrate iron exporter, Nature 403 (2000) 776–781.
[39] S. Abboud, D.J. Haile, A novel mammalian iron regulated protein involved in intracellular iron metabolism, J. Biol. Chem. 275 (2000) 19906–19912.
[40] P.W. Flatman, Magnesium transport across cell membranes, J. Membr. Biol. 80 (1984) 1–14.
[41] M. Kimura, K. Yokoi, Iron accumulation in tissues of magnesium-deficient rats with dietary iron overload, Biol. Trace Elem. Res. 51 (1996) 177–197.