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Additive stimulatory effect of extracellular calcium and potassium on non-transferrin ferric iron uptake by HeLa and K562 cells

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Abstract

We studied the effects of Ca^{2+} and K^+ on non-transferrin iron uptake from ferric citrate complex by HeLa and K562 cells. Uptake experiments in Na-HEPES buffer (137 mM NaCl, 4 mM KCl) showed that extracellular Ca^{2+} stimulated the iron uptake. The rate of iron uptake in 4 mM Ca^{2+} was about 3–5 times higher than without Ca^{2+}. The iron uptake in K-HEPES buffer (68 mM NaCl, 75 mM KCl) with a high K^+ level was transiently stimulated during the first 10 min. The rate of iron uptake for 0.4 mM Ca^{2+} was approximately 3 times higher in K-HEPES buffer than in Na-HEPES buffer. The calcium channel blockers verapamil (50 μM) and nifedipine (5 μM) had no effect on the uptake either in control Na-HEPES buffer or after K^+ stimulation in K-HEPES buffer. The sodium channel blocker lidocaine (50 μM) also had no effect on the uptake of iron in Na-HEPES buffer as well as after K^+ stimulation. Furthermore, the iron uptake was not significantly affected when Na^+ in the Na-HEPES and K-HEPES buffers was replaced by isotonic saccharose. We conclude that extracellular calcium per se, and not intracellular calcium or Ca^{2+} transport, stimulates ferric iron uptake by both HeLa and K562 cells. A high level of extracellular K^+ also stimulates the uptake, probably via cell membrane depolarization. Na^+ is not involved in these stimulations of iron uptake. The transient K^+ effect and continuous Ca^{2+} effect seem to be additive.

Keywords: Non-transferrin iron; Iron uptake; K562 cell; HeLa cell; Calcium; Potassium

1. Introduction

Iron is indispensable for nearly all organisms. Mammalian cells usually acquire iron from the plasma iron carrier transferrin by the relatively well described mechanism of receptor-mediated endocytosis of transferrin–transferrin receptor complex [1,2]. However, there is also iron present in mammalian plasma in a non-transferrin, low-molecular-mass form. The plasma level of this low-molecular-mass form of iron is usually below 1 μM. However, sometimes it can reach values over 20 μM [3]. Most of the plasma low-molecular-mass iron is bound to citrate [4]. Cells acquire iron from low-molecular-mass sources via an alternative transferrin-independent mechanism(s) [5–19]. The process of non-transferrin iron uptake has been shown to be of physiological significance [6,10,11,20,21]. Recently, significant progress has been made in describing molecules involved in

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transport of free iron across biological membranes. It concerns molecules such as NRAMP2/DMT1 (natural resistance-associated macrophage protein 2/divalent metal transporter 1) or SFT (stimulator of Fe transport) [22]. Despite this progress, the mechanism(s) of non-transferrin iron uptake by mammalian cells has not yet been elucidated.

Studies concerning non-transferrin iron uptake by mammalian cells have shown that there are at least two uptake mechanisms, or groups of mechanisms: (1) high-affinity transport with K_m lower than 1 μM and (2) low-affinity transport with K_m around 5 μM and higher [7,23–26]. We have demonstrated previously that iron uptake from ferric citrate by both HeLa and K562 cells has the character of low-affinity transport (K_m = 16–17 μM) [27]. There are data in the literature showing that at least the low-affinity transport of non-transferrin iron is calcium-dependent [9,10,14,18,28,29]. However, it is not clear yet whether extracellular or intracellular calcium is required. Sturrock et al. [9] and Kaplan et al. [10] assume that iron uptake is affected by the intracellular calcium level. There are also data indicating that low-affinity transport of non-transferrin iron is stimulated by a high potassium level [23,25,26]. The authors suggest that the iron transport is associated with Na^+ exchange.

Despite the fact that ferric citrate represents the most relevant form of low-molecular-mass iron in mammalian plasma, there is relatively little information on the mechanism of iron uptake from this form of iron. In a previous study [19] we have demonstrated that the first step in the uptake of iron from ferric citrate by HeLa cells is the binding of ferric iron in a complex with citrate to specific binding sites in the plasma membrane. The present study deals with the effect of calcium and potassium on this iron uptake from ferric citrate by HeLa and K562 cells. We found that extracellular Ca^{2+} as well as K^+ stimulated the iron uptake. The effect of Ca^{2+} and the effect of K^+ are additive. The effect of extracellular calcium does not appear to be related to the change in the intracellular calcium level or to the transport of Ca^{2+} across the plasma membrane. The effect of K^+ appears to be related to cell membrane depolarization.

2. Materials and methods

2.1. Materials

Fetal bovine serum was obtained from J. Kysilka (Brno, Czech Republic) and $^{55}FeCl_3$ for [^{55}Fe]ferric citrate preparation was from DuPont NEN (Boston, MA, USA). All other chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cells and culture conditions

The human tumor cell lines HeLa (cervical carcinoma) and K562 (erythroleukemia) were obtained from J. Kaplan (University of Utah School of Medicine, Salt Lake City, UT, USA) and from M. Wessling-Resnick (Harvard School of Public Health, Boston, MA, USA), respectively. Cells were maintained in the basic medium supplemented with 10% fetal bovine serum (FBS medium) at 37°C in a humidified atmosphere of 5% CO_2 in air. RPMI 1640 medium containing extra L-glutamine (300 μg/ml), sodium pyruvate (110 μg/ml), HEPES (15 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) was used as a basic medium. The calcium concentration in the basic medium was approximately 0.4 mM.

Iron uptake experiments with the cells were in most cases carried out in Na-HEPES buffer or in K-HEPES buffer. Na-HEPES buffer represents 20 mM HEPES (pH 7.4) with 137 mM NaCl and 4 mM KCl. K-HEPES buffer represents 20 mM HEPES (pH 7.4) with 68 mM NaCl and 75 mM KCl. The viability of cells in both Na-HEPES buffer and K-HEPES buffer was tested after 60 and 90 min of incubation. Nearly 100% of the cells were alive after the incubation period, independent of the Ca^{2+} concentration. The viability of cells was determined by hemacytometer counting after staining with trypan blue. Experiments testing the effect of the A 23187 ionophore were carried out in the basic medium supplemented with 20 μM ethanolamine, 20 μM ascorbic acid, 5 nM hydrocortisone, and 11 trace elements as described previously [30,31], without any source of iron added (iron-free medium).

2.3. Iron uptake measurement

For measurement of iron uptake we employed a

modified method originally used by Olakanmi et al. [16]. Cells grown in FBS medium were harvested by low-speed centrifugation (10 min at $1000\times g$) and washed twice with either the basic medium (experiments with A 23187 ionophore), or Na-HEPES buffer where Na^+ was replaced by isotonic saccharose (experiments with saccharose), or with Na-HEPES buffer (all other experiments). Washed cells were diluted with iron-free medium (experiments with A 23187) or with corresponding buffer (all other experiments), containing tested additives at relevant concentrations, to a density of 10×10^6 cells/ml and transferred to wells (50 μl per well) of 96-well polypropylene U plates (Costar, Cambridge, MA, USA). The cells were then preincubated at 37°C for 20 min in the incubator.

The iron uptake was started by the addition of 50 μl of [^{55}Fe]ferric citrate dissolved in iron-free medium (experiments with A 23187), or in Na-HEPES buffer where Na^+ was replaced by isotonic saccharose (experiments with saccharose in Na-HEPES buffer), or in Na-HEPES buffer (all other experiments in Na-HEPES buffer), or in KCl solution (all other experiments in K-HEPES buffer). The final concentration of [^{55}Fe]ferric citrate was 1 μM (80.1 MBq/μmol Fe) and the Fe/citrate molar ratio was 1:1.5. A 1.5-fold molar excess of citrate was sufficient as demonstrated previously [32]. The KCl solution produced a final KCl concentration of 75 mM (K-HEPES buffer). After the required uptake period (0–30 min) at 37°C, the iron uptake was stopped by transferring the plate to ice while simultaneously adding 100 μl of ice-cold 500 μM non-radioactive ferric citrate in basic medium (experiments with A 23187) or 1 mM non-radioactive ferric citrate in Na-HEPES buffer (all other experiments). Plates were spun at $2300\times g$ and radioactive supernatant was discarded. In order to remove iron adherent to the cell surface, cells in individual wells were washed twice with 200 μl of the same solution containing a high level of non-radioactive ferric citrate that was used for the stopping of uptake. Washing more than twice was found to be without any other effect. After washing, the cell pellets in the wells were resuspended in 100 μl distilled water and transferred to scintillation vials. Radioactivity incorporated into the cells was measured by liquid scintillation in a Beckman LS 7800 counter.

Control experiments in the absence of cells showed that the radioactivity measured for individual wells was negligible when polypropylene plates were used. As demonstrated previously [19], the saturation of iron binding to the plasma membrane is very rapid (1.5–3 min) at 37°C and thus iron binding to the plasma membrane does not significantly influence iron uptake measured during a 30-min time interval.

3. Results

3.1. Effect of Ca^{2+} on iron uptake in Na-HEPES buffer

We measured the time dependence of iron uptake from ferric citrate (1 μM [^{55}Fe]ferric citrate) in the presence of various concentrations of added $CaCl_2$ (0, 0.04, 0.4, 1.2, and 4.0 mM) by HeLa and K562 cells in Na-HEPES buffer (137 mM NaCl, 4mM KCl). The concentration of 0.4 mM represents Ca^{2+} concentration in the culture medium used for cell maintenance. When no $CaCl_2$ was added, the real Ca^{2+} concentration in the buffer probably differed from zero due to Ca^{2+} transport across the plasma membrane by living cells.

The iron uptake increased linearly during 30 min of incubation for both cell types, probably with the exception of incubation periods longer than 15 min in 4 mM $CaCl_2$. The rate of iron uptake increased with increasing concentration of Ca^{2+}. The rate of iron uptake in 4 mM $CaCl_2$ was about 3–5 times higher than it was in the buffer without $CaCl_2$ addition. The increase in the rate of iron uptake with increasing Ca^{2+} concentration was more dramatic for Ca^{2+} concentrations over 0.4 mM than for Ca^{2+} concentrations below 0.4 mM (Figs. 1 and 2). However, the increase in the rate of iron uptake in $CaCl_2$ concentrations higher than 4 mM was less pronounced than the increase for 0.4–4 mM $CaCl_2$ (data not shown).

3.2. Effect of Ca^{2+} on iron uptake in K-HEPES buffer

Similarly, we measured the time dependence of iron uptake from ferric citrate (1 μM) in the presence of various $CaCl_2$ concentrations (0, 0.04, 0.4, 1.2, and 4.0 mM) by HeLa and K562 cells in K-HEPES

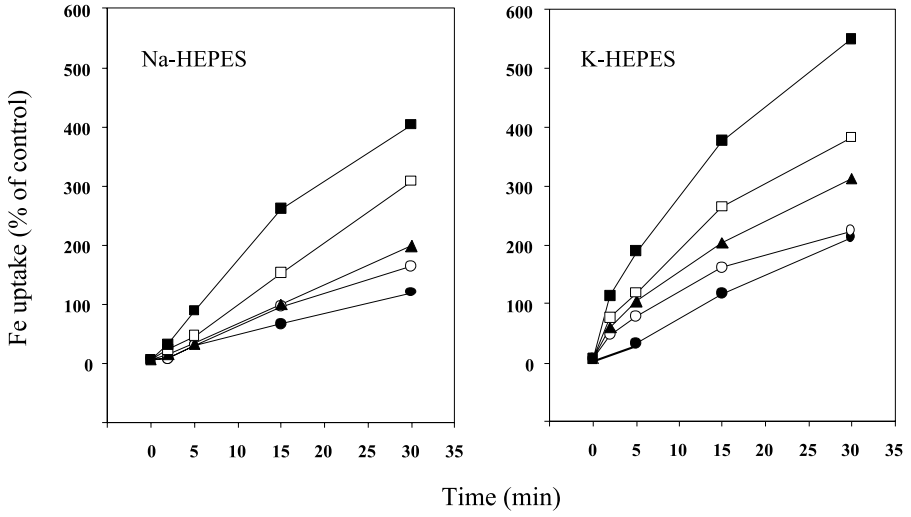

Fig. 1. Time dependence of iron uptake from [^{55}Fe]ferric citrate (1 μM) in Na-HEPES buffer and in K-HEPES buffer (see Section 2) by HeLa cells at 37°C incubated in the presence of various concentrations of added $CaCl_2$: 0 mM (●), 0.04 mM (○), 0.4 mM (▲), 1.2 mM (□), 4.0 mM (■). Each point represents the mean of at least six experimental values. S.E.M. (not shown) represents less than 15% of the mean for most of the points. The uptake within 15 min in Na-HEPES buffer with 0.4 mM $CaCl_2$ was used as a control (3.98 pmol Fe/10^6 cells).

buffer (68 mM NaCl, 75 mM KCl). KCl at a concentration of 75 mM was supposed to cause depolarization of the cell membrane.

Iron uptake in K-HEPES buffer was higher than the uptake observed in Na-HEPES buffer for both HeLa and K562 cells. The initial sections of the time dependence curves were apparently steeper than later parts. Again, the rate of iron uptake increased with increasing Ca^{2+} concentration (Figs. 1 and 2).

Detailed comparison of iron uptake in Na-HEPES buffer with the uptake in K-HEPES buffer, when 0.4 mM concentration as well as other concentrations of

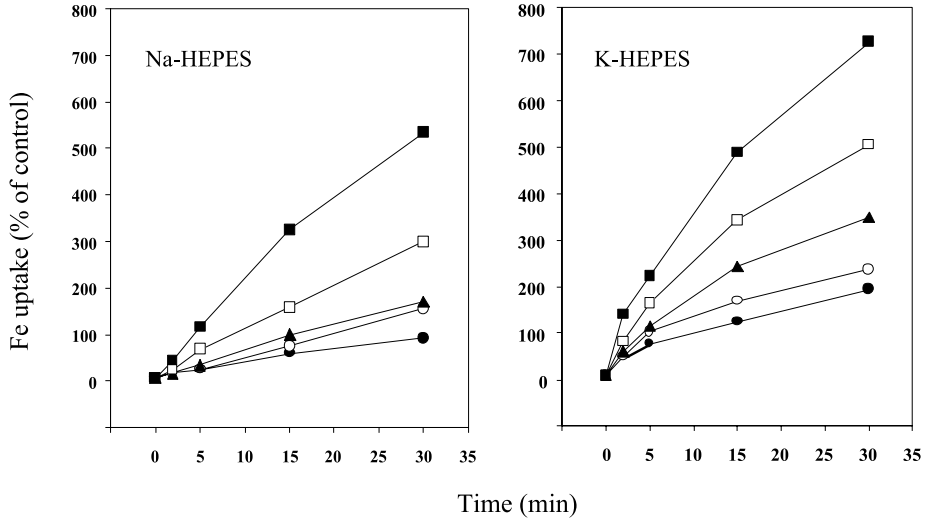

Fig. 2. Time dependence of iron uptake from [^{55}Fe]ferric citrate (1 μM) in Na-HEPES buffer and in K-HEPES buffer (see Section 2) by K562 cells at 37°C incubated in the presence of various concentrations of added $CaCl_2$: 0 mM (●), 0.04 mM (○), 0.4 mM (▲), 1.2 mM (□), 4.0 mM (■). Each point represents the mean of at least six experimental values. S.E.M. (not shown) represents less than 15% of the mean for most of the points. The uptake within 15 min in Na-HEPES buffer with 0.4 mM $CaCl_2$ was used as a control (4.02 pmol Fe/10^6 cells).

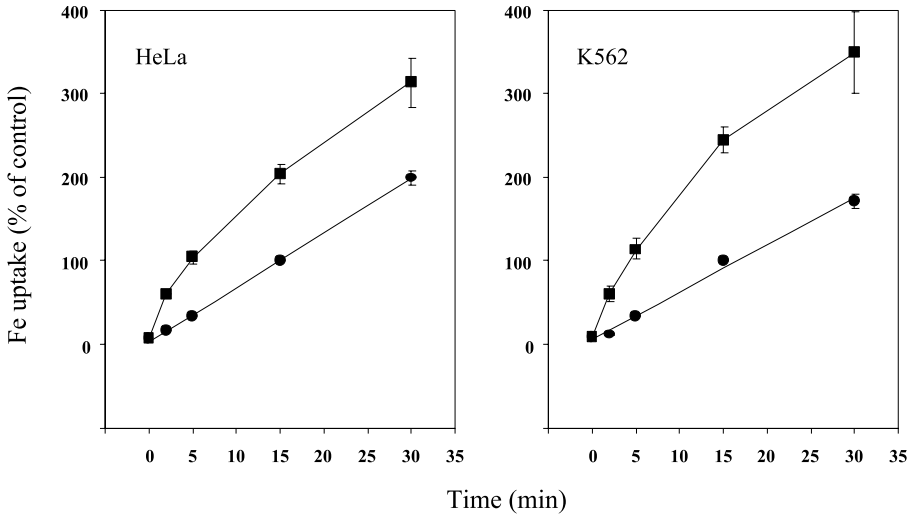

Fig. 3. Comparison of the time dependence of iron uptake from [^{55}Fe]ferric citrate (1 μM) in Na-HEPES buffer (●) and in K-HEPES buffer representing K^+ stimulation (■) by HeLa and K562 cells at 37°C in the presence of 0.4 mM $CaCl_2$. Each point represents the mean ± S.E.M. of ten experimental values. The uptake within 15 min in Na-HEPES buffer was used as a control (HeLa: 3.98 pmol Fe/10^6 cells; K562: 4.02 pmol Fe/10^6 cells).

Ca^{2+} were present, showed that after approximately the initial 10 min of a high rate of iron uptake in K-HEPES buffer the rate in K-HEPES buffer became similar to the rate in Na-HEPES buffer, i.e., the time dependence curves for both buffers became more or less parallel (Fig. 3). It appears that there were two additive effects in play, i.e., an initial short-term effect of K^+ and a continuous effect of Ca^{2+}.

3.3. Effect of calcium ionophore A 23187 on iron uptake

We tested the effect of various concentrations of the calcium ionophore A 23187 on iron uptake from ferric citrate (1 μM) in iron-free medium containing approximately 0.4 mM calcium. Preliminary fluorometric measurements showed that a 10 μM concentration of the ionophore was sufficient to produce a pronounced increase in the intracellular calcium level (data not shown). Concentrations of the ionophore up to 10 μM, i.e., concentrations usually used for calcium transport across membranes, did not change iron uptake by either HeLa or K562 cells. Higher ionophore concentrations increased the uptake significantly (Table 1). However, it is possible that the ionophore effect at high concentrations was not related to the ionophore's ability to increase the intracellular calcium level but to other effect(s) of the ionophore (see Section 4).

3.4. Effect of calcium channel blockers verapamil and nifedipine on iron uptake

Iron uptake from ferric citrate (1 μM) was assessed in the presence of the L-type calcium channel blockers verapamil (50 μM) and nifedipine (5 μM). Iron

Table 1
Effect of the calcium ionophore A 23187 on iron uptake by HeLa and K562 cells

Ionophore (μM)	Fe uptake (% of control)	
	HeLa	K562
0	100 ± 4	100 ± 8
3	104 ± 2	98 ± 3
10	104 ± 5	109 ± 5
30	147 ± 14	234 ± 27
100	233 ± 12	438 ± 33

The uptake from [^{55}Fe]ferric citrate (1 μM) was measured within 30 min of incubation at 37°C in the presence of various concentrations of A 23187 in iron-free medium containing approximately 0.4 mM calcium. Each value represents the mean ± S.E.M. of at least six experimental values. The uptake within 30 min without the ionophore was used as a control (HeLa: 5.98 pmol Fe/10^6 cells; K562: 2.86 pmol Fe/10^6 cells).

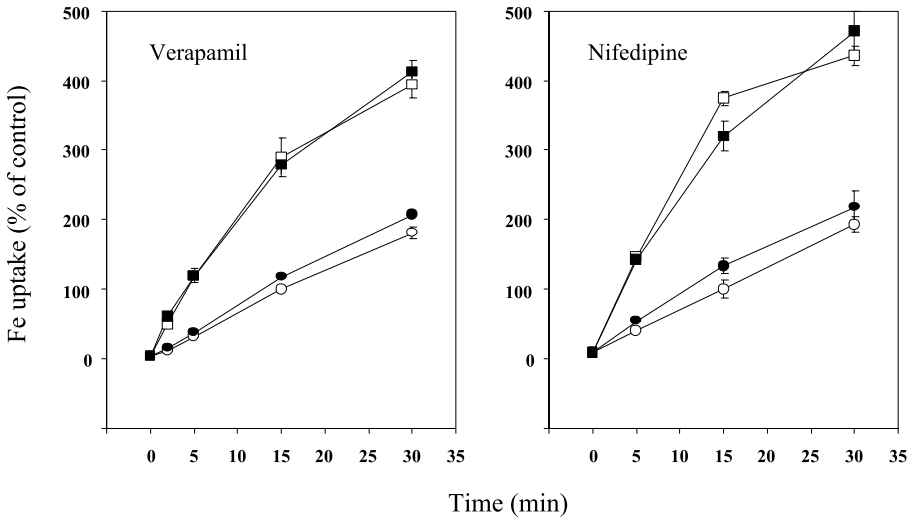

Fig. 4. Effect of 50 μM verapamil and 5 μM nifedipine on iron uptake from [^{55}Fe]ferric citrate (1 μM) in Na-HEPES buffer (●) and in K-HEPES buffer representing K^+ stimulation (■) by K562 cells at 37°C in the presence of 0.4 mM $CaCl_2$. Parallels without the inhibitor in Na-HEPES buffer (○) and in K-HEPES buffer (□) are shown. Each point represents the mean ± S.E.M. of at least four experimental values. The uptake within 15 min in Na-HEPES buffer without the inhibitor was used as a control (verapamil: 3.65 pmol Fe/10^6 cells; nifedipine: 4.15 pmol Fe/10^6 cells).

uptake by K562 cells in Na-HEPES buffer with 0.4 mM of Ca^{2+} was not significantly affected by the presence of verapamil or nifedipine. Under the stimulation of iron transport by K^+ in K-HEPES buffer with 0.4 mM Ca^{2+}, neither verapamil nor nifedipine eliminated the effect of K^+ stimulation (Fig. 4). Similar results were obtained with HeLa cells (data not shown).

The data demonstrated that blockage of L-type calcium channels did not affect iron uptake, even after the stimulation of the uptake in K-HEPES buffer. Thus, an increase in the intracellular calcium level

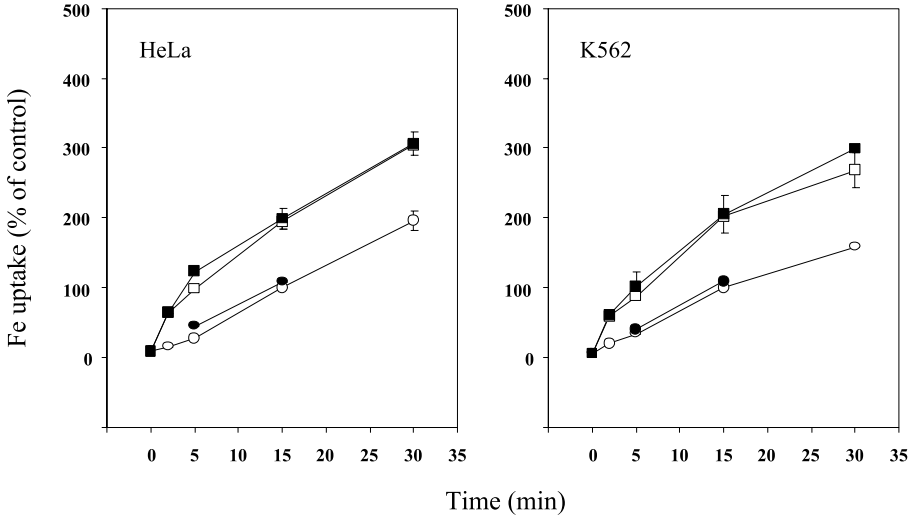

Fig. 5. Effect of 50 μM lidocaine on iron uptake from [^{55}Fe]ferric citrate (1 μM) in Na-HEPES buffer (●) and in K-HEPES buffer representing K^+ stimulation (■) by HeLa and K562 cells at 37°C in the presence of 0.4 mM $CaCl_2$. Parallels without the inhibitor in Na-HEPES buffer (○) and in K-HEPES buffer (□) are shown. Each point represents the mean ± S.E.M. of four experimental values. The uptake within 15 min in Na-HEPES buffer without the inhibitor was used as a control (HeLa: 3.66 pmol Fe/10^6 cells; K562: 4.16 pmol Fe/10^6 cells).

Table 2
Effect of NaCl replacement by isotonic saccharose on iron uptake by HeLa cells

Time (min)	Fe uptake (% of control)			
	NaCl present		NaCl replaced by isotonic saccharose	
	Na-HEPES	K-HEPES	Na-HEPES	K-HEPES
0	2 ± 0	2 ± 0	2 ± 0	2 ± 0
2	17 ± 1	60 ± 4	17 ± 2	75 ± 5
5	40 ± 4	104 ± 7	37 ± 5	125 ± 1
15	100 ± 1	204 ± 10	118 ± 12	248 ± 11

The time dependence of iron uptake from [^{55}Fe]ferric citrate (1 μM) was measured in Na-HEPES buffer (137 mM NaCl, 4 mM KCl) and in K-HEPES buffer (68 mM NaCl, 75 mM KCl) representing K^+ stimulation at 37°C in the presence of 0.4 mM $CaCl_2$ when NaCl was present and when NaCl was replaced by isotonic saccharose. Each value represents the mean ± S.E.M. of 2–3 experimental values. The uptake within 15 min in Na-HEPES buffer with NaCl was used as a control (2.85 pmol Fe/10^6 cells).

or an increased transport of Ca^{2+} across the plasma membrane through calcium channels itself did not seem to mediate the stimulatory effect of increasing levels of extracellular calcium. Furthermore, opening of at least voltage-dependent L-type Ca^{2+} channels and subsequent influx of Ca^{2+} ions resulting from depolarization by K^+ did not mediate iron uptake stimulation by K^+.

3.5. Effect of sodium channel blocker lidocaine on iron uptake

Iron uptake from ferric citrate (1 μM) was also assessed in the presence of the sodium channel blocker lidocaine (50 μM). Iron uptake by HeLa as well as by K562 cells in Na-HEPES buffer containing 0.4 mM Ca^{2+} was not affected by the presence of lidocaine and, under the stimulation of iron uptake by K^+ in K-HEPES buffer, lidocaine did not eliminate the effect of K^+ stimulation (Fig. 5).

The data demonstrated that blockage of sodium channels did not affect iron uptake. Even after the stimulation in K-HEPES buffer the uptake was not affected. Thus, it appeared that transport of Na^+ across plasma membrane through sodium channels was not involved in uptake stimulation by extracellular calcium. Neither did the opening of relevant channels and subsequent influx of Na^+ ions resulting from depolarization play any role in the iron uptake stimulation by K^+.

3.6. Effect of Na^+ replacement by saccharose on iron uptake

When Na^+ in Na-HEPES buffer was replaced by isotonic saccharose, the time course of iron uptake from ferric citrate (1 μM) in the presence of 0.4 mM

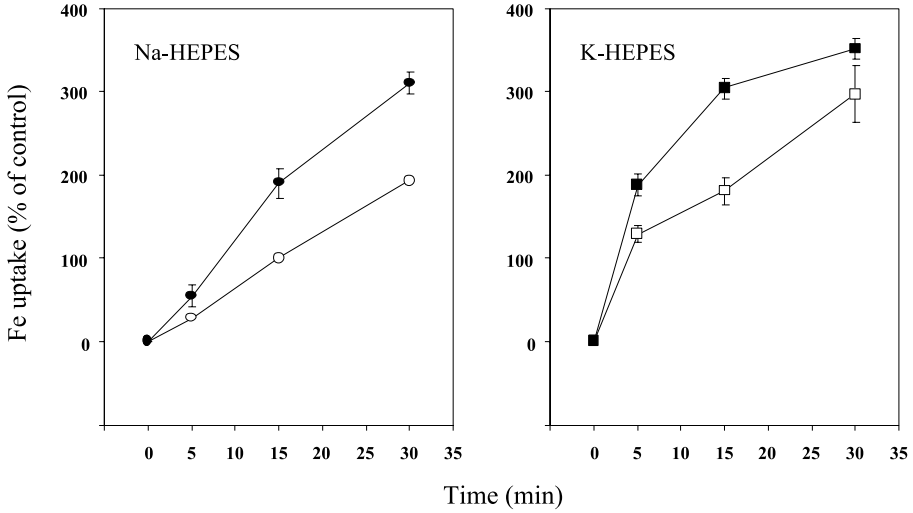

Fig. 6. Effect of 1 mM LiCl on iron uptake from [^{55}Fe]ferric citrate (1 μM) in Na-HEPES buffer (●) and in K-HEPES buffer representing K^+ stimulation (■) by K562 cells at 37°C in the presence of 0.4 mM $CaCl_2$. Parallels without LiCl in Na-HEPES buffer (○) and in K-HEPES buffer (□) are shown. Each point represents the mean ± S.E.M. of three experimental values. The uptake within 15 min in Na-HEPES buffer without LiCl was used as a control (4.10 pmol Fe/10^6 cells).

Ca^{2+} did not differ significantly from the uptake seen in regular Na-HEPES buffer. Similar data, or even rather higher uptake under sodium replacement, were obtained after uptake stimulation by K^+ in K-HEPES buffer (Table 2). This demonstrated that iron uptake was more or less independent of extracellular sodium as well as of Na^+ transport into the cells.

3.7. Effect of LiCl on iron uptake

We tested the effect of LiCl, which represented a substance increasing depolarization of the cell membrane. Iron uptake from ferric citrate (1 μM) was measured in the presence of 1 mM LiCl. The uptake by K562 cells in Na-HEPES buffer with 0.4 mM Ca^{2+} was significantly stimulated by LiCl. After the stimulation of iron transport by K^+ in K-HEPES buffer, LiCl displayed an additional stimulatory effect on the uptake (Fig. 6).

4. Discussion

Our experiments proved that calcium ions affect ferric iron transport across the plasma membrane of HeLa as well as K562 cells. An increase in the extracellular calcium level leads to an increase in iron uptake by the cells. To assess whether the increase in iron uptake is a result of the increased level of extracellular or intracellular calcium or is a result of increased calcium transport into cells, experiments with the calcium channel blockers verapamil and nifedipine as well as experiments with the calcium ionophore A 23187 were employed.

Experiments with the L-type calcium channel blockers verapamil and nifedipine in Na-HEPES buffer showed that iron uptake was not affected by the blockers at concentrations sufficient for blocking the channels. Furthermore, the presence of the calcium channel blockers did not prevent the increase of iron uptake after K^+ stimulation in K-HEPES buffer when voltage-dependent Ca^{2+} channels were opened after depolarization by K^+ (see Fig. 4). These findings support the suggestion that the intracellular calcium level, as well as calcium transport across the plasma membrane via at least L-type voltage-dependent calcium channels, do not affect iron uptake. Such a suggestion is also supported by our finding that the calcium ionophore A 23187, at a concentration (10 μM) elevating the intracellular calcium level, did not affect iron uptake. The stimulatory effect of higher ionophore concentrations (30–100 μM) on iron uptake can be explained by the ionophore's ability to bind weakly and transport iron, or by the production of some relevant changes in the plasma membrane [33,34].

Thus, we suggest that extracellular calcium per se, rather than intracellular calcium or calcium transport across the plasma membrane via calcium channels, affects the transport of ferric iron into cells. We have also shown that transport of Na^+ via sodium channels is not involved in the effect of extracellular calcium. Sturrock et al. [9] and Kaplan et al. [10] speculate that the stimulatory effect of extracellular calcium on ferric iron uptake by HeLa cells and human fibroblasts may be a consequence of an increased intracellular calcium level. However, there is insufficient experimental evidence for such a conclusion in their papers.

There is a possibility that extracellular calcium could affect the iron uptake via its interaction with the iron–citrate complex. Recently, it has been shown that calcium stimulates ferrous iron uptake from the complex with NTA due to competitive calcium binding to NTA and thus producing an increased level of free iron [35]. However, such competitive calcium binding to a chelator is probably applicable only to ferrous complexes. Similarly as in the case of NTA, stability constants for Fe^{2+} and Ca^{2+} binding to citrate are relatively comparable but ferric iron forms a much more stable complex with citrate than ferrous iron. The stability constant is about seven orders of magnitude higher [36]. Therefore, we do not suppose that extracellular calcium significantly affects iron uptake from ferric citrate by competitive binding to citrate. Furthermore, we have demonstrated previously [19] that the first step of ferric iron uptake from ferric citrate is binding of ferric citrate to specific binding sites in the plasma membrane. Free iron does not seem to be involved.

Experiments with K-HEPES buffer showed that not only calcium ions but also potassium ions affected ferric iron transport into the cells. A high potassium level (75 mM) stimulates the uptake of iron. Such a K^+ level is commonly used for depolarization

of the cell membrane by the opening of voltage-dependent channels. Opening of voltage-dependent channels leads to a significant influx of ions such as Ca^{2+} or Na^{+} into the cell. However, the effect of calcium transport across the plasma membrane via L-type calcium channels was excluded in the experiments with the calcium channel blockers discussed above. To assess whether an increase in the intracellular Na^{+} level (due to the opening of voltage-dependent Na^{+} channels) or Na^{+} transport into the cell per se are involved, we employed the Na^{+} channel blocker lidocaine.

The employment of lidocaine at a concentration sufficient to block Na^{+} channels showed that the blocker did not prevent iron uptake stimulation by K^{+} in K-HEPES buffer. Neither did lidocaine affect iron uptake in Na-HEPES buffer (see Fig. 5). To decide whether Na^{+} is somehow involved in ferric iron uptake, we tested the effect of Na^{+} replacement by isotonic saccharose in both Na-HEPES and K-HEPES buffers. It was found that Na^{+} replacement did not significantly change the uptake (see Table 2).

Thus, we suggest that Na^{+} is not involved in ferric iron transport into the cells or in the stimulation of ferric iron transport by K^{+}. Low-affinity ferrous iron uptake by erythroid cells shares some similarities with ferric iron uptake by HeLa and K562 cells, i.e., the stimulation by K^{+} and Li^{+}. However, there is a discrepancy concerning Na^{+} effects. Ferrous iron uptake by erythroid cells is stimulated by intracellular sodium and inhibited by extracellular sodium. Thus it is suggested that the iron uptake is associated with Na^{+} exchange [23,25,26]. This discrepancy could reflect differences in the experimental systems employed.

Iron uptake stimulation by K^{+} occurred during the initial 10 min in K-HEPES buffer. Subsequently, the rate of iron uptake in K-HEPES buffer became similar to the rate of iron uptake in Na-HEPES buffer (see Fig. 3). The timing of the K^{+} stimulation corresponds with short-term membrane depolarization and opening of voltage-dependent channels by K^{+}. In a confirming experiment (data not shown) where the cells were washed and preincubated in K-HEPES buffer instead of in Na-HEPES buffer, subsequent K^{+} stimulation was not observed. Thus, we suggest that the stimulation of iron uptake by a high potassium level is a result of depolarization of the cell membrane and we propose that a certain degree of depolarization can also be achieved by extracellular LiCl. This could explain the iron uptake stimulation by LiCl.

In summary, we can conclude that extracellular calcium per se stimulates ferric iron uptake by Hela as well as K562 cells. Intracellular calcium or Ca^{2+} transport across the plasma membrane via calcium channels do not seem to affect the iron uptake. A high extracellular level of K^{+} also stimulates ferric iron uptake. Iron uptake stimulation in this case seems to be a consequence of plasma membrane depolarization by K^{+}. Na^{+} is not involved in the stimulation seen after membrane depolarization or in the stimulation by calcium. Thus, we consider two different mechanisms of ferric iron uptake stimulation: (1) short-term potassium stimulation resulting from transient plasma membrane depolarization and (2) continuous calcium stimulation resulting from the presence of extracellular calcium. The effects of potassium stimulation and calcium stimulation appear to be more or less additive. When speculating about the nature of the ferric iron transport mechanism, one can imagine the possible involvement of a calcium-dependent ion channel.

Acknowledgements

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References

[1] Dautry-Varsat, A. Ciechanover, H.F. Lodish, pH and the recycling of transferrin during receptor-mediated endocytosis, Proc. Natl. Acad. Sci. USA 80 (1983) 2258–2262.
[2] R.D. Klausner, G. Ashwell, J. van Renswoude, J.B. Harford, K.R. Bridges, Binding of apotransferrin to K562 cells: explanation of the transferrin cycle, Proc. Natl. Acad. Sci. USA 80 (1983) 2262–2266.
[3] Hershko, T.E.A. Peto, Non-transferrin plasma iron, Br. J. Haematol. 66 (1987) 149–151.
[4] M. Grootveld, J.D. Bell, B. Halliwell, O.I. Aruoma, A. Bamford, P.J. Sadler, Non-transferrin-bound iron in plasma or

serum from patients with idiopathic hemochromatosis. Characterization by high performance liquid chromatography and nuclear magnetic resonance spectroscopy, J. Biol. Chem. 264 (1989) 4417–4722.
[5] P. Brissot, T.L. Wright, W.-L. Ma, R.A. Weisiger, Efficient clearance of non-transferrin-bound iron by rat liver. Implications for hepatic iron loading in iron overload states, J. Clin. Invest. 76 (1985) 1463–1470.
[6] P. Basset, Y. Quesneau, J. Zwiller, Iron-induced L1210 cell growth: evidence of a transferrin-independent iron transport, Cancer Res. 46 (1986) 1644–1647.
[7] E.H. Morgan, Membrane transport of non-transferrin-bound iron by reticulocytes, Biochim. Biophys. Acta 943 (1988) 428–439.
[8] T.L. Wright, J.G. Fitz, R.A. Weisiger, Non-transferrin-bound iron uptake by rat liver. Role of membrane potential difference, J. Biol. Chem. 263 (1988) 1842–1847.
[9] Sturrock, J. Alexander, J. Lamb, C.M. Craven, J. Kaplan, Characterization of a transferrin-independent uptake system for iron in HeLa cells, J. Biol. Chem. 265 (1990) 3139–3145.
[10] J. Kaplan, I. Jordan, A. Sturrock, Regulation of the transferrin-independent iron transport system in cultured cells, J. Biol. Chem. 266 (1991) 2997–3004.
[11] Richardson, A. Baker, The uptake of inorganic iron complexes by human melanoma cells, Biochim. Biophys. Acta 1093 (1991) 20–28.
[12] S. Hamazaki, J. Glass, Non-transferrin dependent ^{59}Fe uptake in phytohemagglutinin-stimulated human peripheral lymphocytes, Exp. Hematol. 20 (1992) 436–441.
[13] M.E. Conrad, J.N. Umbreit, R.D.A. Peterson, E.G. Moore, K.P. Harper, Function of integrin in duodenal mucosal uptake of iron, Blood 81 (1993) 517–521.
[14] R.S. Inman, M. Wessling-Resnick, Characterization of transferrin-independent iron transport in K562 cells. Unique properties provide evidence for multiple pathways of iron uptake, J. Biol. Chem. 268 (1993) 8521–8528.
[15] K. Kriegerbecková, L. Döpper, B. Scheiber, J. Kovář, H. Goldenberg, Non-transferrin iron uptake by HeLa cells cultured in serum-free media with different iron sources, Eur. J. Clin. Chem. Clin. Biochem. 33 (1995) 791–797.
[16] O. Olakanmi, J.B. Stokes, S. Pathan, B.E. Britigan, Polyvalent cationic metals induce the rate of transferrin-independent iron acquisition by HL-60 cells, J. Biol. Chem. 272 (1997) 2599–2606.
[17] J.G. Parkes, N.F. Olivieri, D.M. Tempelton, Characterization of Fe^{2+} and Fe^{3+} transport by iron-loaded cardiac myocytes, Toxicology 117 (1997) 141–151.
[18] E. Baker, S.M. Baker, E.H. Morgan, Characterization of non-transferrin-bound iron (ferric citrate) uptake by rat hepatocytes in culture, Biochim. Biophys. Acta 1380 (1998) 21–30.
[19] J. Musílková, K. Kriegerbecková, J. Krůšek, J. Kovář, Specific binding to plasma membrane is the first step in the uptake of non-transferrin iron by cultured cells, Biochim. Biophys. Acta 1369 (1998) 103–108.
[20] P.A. Seligman, J. Kovar, R.B. Schleicher, E.W. Gelfand, Transferrin-independent iron uptake supports B lymphocyte growth, Blood 78 (1991) 1526–1531.
[21] R.M. Graham, E.H. Morgan, E. Baker, Ferric citrate uptake by cultured rat hepatocytes is inhibited in the presence of transferrin, Eur. J. Biochem. 253 (1998) 139–145.
[22] N.C. Andrews, M.D. Fleming, H. Gunshin, Iron transport across biologic membranes, Nutr. Rev. 57 (1999) 114–123.
[23] Egyed, Na^+ modulates carrier-mediated Fe^{2+} transport through the erythroid cell membrane, Biochem. J. 275 (1991) 635–638.
[24] T. Akompong, R.S. Inman, M. Wessling-Resnick, Phorbol esters stimulate non-transferrin iron uptake by K562 cells, J. Biol. Chem. 270 (1995) 20937–20941.
[25] L.L. Hodgson, E.A. Quail, E.H. Morgan, Iron transport mechanisms in reticulocytes and mature erythrocytes, J. Cell. Physiol. 162 (1995) 181–190.
[26] 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.
[27] J. Kovář, L.C. Kühn, V. Richardson, C. Seiser, K. Kriegerbecková, J. Musílková, The inability of cells to grow in low iron correlates with increasing activity of their iron regulatory protein (IRP), In Vitro Cell. Dev. Biol. 33 (1997) 633–639.
[28] T.L. Wright, P. Brissot, W.-L. Ma, R.A. Weisiger, Characterization of non-transferrin-bound iron clearance by rat liver, J. Biol. Chem. 261 (1986) 10909–10914.
[29] Barisani, C.L. Berg, M. Wessling-Resnick, J.L. Gollan, Evidence for a low K_m transporter for non-transferrin-bound iron in isolated rat hepatocytes, Am. J. Physiol. 269 (1995) G570–G576.
[30] J. Kovář, Growth-stimulating effect of ferric citrate on hybridoma cells: Characterization and relation to transferrin function, Hybridoma 7 (1988) 255–263.
[31] J. Kovář, F. Franěk, Growth-stimulating effect of transferrin on a hybridoma cell line: relation to transferrin iron-transporting function, Exp. Cell Res. 182 (1989) 358–369.
[32] L. Brunner-Döpper, K. Kriegerbeckova, J. Kovář, H. Goldenberg, Pitfalls in assessing specificity and affinity of non-transferrin-bound iron uptake, Anal. Biochem. 261 (1998) 128–130.
[33] B.C. Pressman, Biological applications of ionophores, Annu. Rev. Biochem. 45 (1976) 501–530.
[34] S. Chakraborti, S.K. Batabyal, J.R. Michael, T. Sanyal, Role of membrane associated serine esterase in the activation of phospholipase A2 by calcium ionophore (A23187) in pulmonary arterial smooth muscle cells, Mol. Cell. Biochem. 130 (1994) 121–127.
[35] J. Mwanjewe, R. Martinez, P. Agrawal, S.E. Samson, M.D. Coughlin, P. Brassard, A.K. Grover, On the Ca^{2+} dependence of non-transferrin-bound iron uptake in PC12 cells, J. Biol. Chem. 275 (2000) 33512–33515.
[36] S. Kotrlý, L. Šůcha, Handbook of Chemical Equilibria in Analytical Chemistry, in: R.A. Chalmers, M. Masson (Eds.), Ellis Horwood Series in Analytical Chemistry, Ellis Horwood, Chichester, 1985, pp. 142–208.