Na⁺ modulates carrier-mediated Fe²⁺ transport through the erythroid cell membrane

Andrew EGYED

National Institute of Haematology and Blood Transfusion, 1502 Budapest, P.O. Box 44, Hungary

The effects of univalent cations on Fe²⁺ uptake by rabbit reticulocytes have been studied. The rate of Fe²⁺ uptake was almost identical when measured in Na⁺-free choline chloride or KCl medium. Na⁺ but not Li⁺ inhibited Fe²⁺ uptake even at relatively low concentrations (2.5–20 mM) in these media. In contrast to this effect of extracellular Na⁺, the rate of Fe²⁺ uptake was facilitated by an increase in the intracellular Na⁺ concentration in the range 5–40 mM, suggesting that intracellular Na⁺ is required for the uptake process. This effect also appears to be specific for Na⁺. Amiloride inhibited Fe²⁺ uptake in KCl (or in choline chloride), but had little if any effect in NaCl. Therefore a Na⁺- and amiloride-sensitive and a Na⁺- and amiloride-insensitive component can be distinguished. The two components differ in maximum velocity and pH optimum, but not in their apparent affinity for Fe²⁺. The ineffectiveness of selective inhibitors excludes the involvement of the Na⁺/H⁺ or Na⁺/Ca²⁺ exchange mechanisms. To account for the results presented in this work, a Na⁺/Fe²⁺ exchange system in the erythroid cell membrane is proposed.

INTRODUCTION

Although the transport of iron through biological membranes is an obligatory step of the transferrin–cell interaction, little is known about its mechanism, regulation and kinetics. Most of the studies on transmembrane iron transport have been done on intestinal or hepatic systems [1–5]. Only recently has attention been focused on this membrane function in cells which are able to utilize transferrin iron. In our previous work a high-affinity carrier-mediated Fe²⁺ transport system was demonstrated in rabbit reticulocyte membranes [6]. Morgan [7] has also concluded that reticulocytes can transport non-transferrin-bound iron into the cytosol by a carrier-mediated process. Sturrock *et al.* [8] have suggested that in HeLa cells a carrier-mediated Ca²⁺-dependent non-transferrin iron transport system can be found. Apart from the similarity of conclusions concerning the type of transport, the differences between the experimental conditions used are profound, so the results can hardly be compared. Using a low-ionic-strength incubation medium, Morgan has found that Na⁺ inhibits Fe²⁺ uptake and he suggests that this inhibition is not specific for Na⁺ but is due to the increased ionic strength [7]. In the present work the effect of Na⁺ on Fe²⁺ uptake was re-investigated in normal-ionic-strength incubation medium.

MATERIALS AND METHODS

Chemicals

The following reagents of analytical grade were used: Hepes, amiloride and nystatin (Sigma Chemical Co., St. Louis, MO, U.S.A.); ionophore A23187 (Ely Lilly Co., Indianapolis, IN, U.S.A.); 5-(*N*-4-chlorobenzyl)-*N*-(2',4'-dimethyl)benzamil (CBDMB) and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) (gifts from Dr. E. J. Cragoe, Jr., Merck Sharp & Dohme Research Laboratories, West Point, PA, U.S.A.); ⁵⁹FeCl₃ (576–720 GBq/g of Fe³⁺) (Central Institute of Nuclear Research, Berlin, Germany). All other reagents were purchased from Reanal Fine Chemicals, Budapest, Hungary.

Reticulocytes

Rabbit reticulocytes were prepared as described earlier [9] and depleted of endogenous transferrin by the method of

Hemmaplardh & Morgan [10]. The reticulocyte content of the preparations was between 70 and 85 %.

Measurement of Fe²⁺ uptake

The incubation medium contained 20 mM-Hepes buffer, pH 7.4, 10 mM-glucose, ⁵⁹Fe²⁺–ascorbate (final Fe²⁺ concentration 18 μM; Fe²⁺/ascorbate ratio 1:44), reticulocytes (haematocrit in the incubation medium = 10), and 135 mM-KCl (KCl medium) or 135 mM-NaCl (NaCl medium) or 135 mM-LiCl (LiCl medium) or 135 mM-choline chloride (choline chloride medium). Incubations were carried out at 37 °C. The reaction was started by the addition of ⁵⁹Fe²⁺–ascorbate. The reaction was stopped by transferring aliquots of the reaction mixture into equal volumes of ice-cold 0.9 % NaCl containing 5 mM-EDTA. The cells were then separated from the medium by centrifuging these samples (10000 g, 1.5 min) through layers of 1 % BSA and 5 % dextran containing NaCl (upper layer) and dibutyl phthalate (lower layer). The ⁵⁹Fe content of the cells was then determined.

Modification of intracellular univalent cation composition

The ionophore nystatin was used to modify the cellular Na⁺/K⁺ ratio and to load the reticulocytes with Li⁺. The loading medium contained 30 mM-sucrose, 10 mM-Hepes, pH 7.4, and 140 mM-(KCl+NaCl) or 140 mM-(KCl+LiCl). Reticulocytes (0.1 ml) were added to 2.9 ml of ice-cold loading solution, then nystatin (12 mg/ml, dissolved in dimethyl sulphoxide within 1 h of loading) was added, while mixing vigorously, to reach a final concentration of 40 μg/ml. The cell suspension was incubated at 4 °C for 10 min, then an additional 2 ml of loading solution was added and the incubation was continued at 4 °C for another 10 min. Subsequently the cell suspension was centrifuged, the supernatant was discarded and the cell pellet was resuspended in fresh loading solution for an additional 10 min of incubation in the cold. Nystatin was removed by washing the cells three times with warm (37 °C) loading solution containing 0.5 % albumin and once with ice-cold choline chloride. A portion of the cells was then haemolysed and the haemolysate was precipitated with 5 % (w/v) trichloroacetic acid. The precipitate was centrifuged and the K⁺, Na⁺ and Li⁺ concentrations in the supernatant were determined by atomic absorption spectrophotometry.

Abbreviations used: CBDMB, 5-(*N*-4-chlorobenzyl)-*N*-(2',4'-dimethyl)benzamil; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; [Na⁺]_i and [Na⁺]_o, intracellular and extracellular Na⁺ concentrations respectively; IC₅₀, half-maximal inhibitory concentration.

Fig. 1. Fe^{2+} uptake by reticulocytes in different incubation media

The cells were incubated at 37 °C with $^{59}\text{Fe}^{2+}$ -ascorbate (18 μM) in iso-osmotic NaCl (○), LiCl (×---×), KCl (●) or choline chloride (×---×) media for the times indicated.

Fig. 2. Rate of Fe^{2+} uptake as a function of Na^+ (○) and Li^+ (●) concentrations in the incubation medium

The Na^+ and Li^+ concentrations were adjusted by adding iso-osmotic NaCl or LiCl to the incubation medium to replace an equal amount of the main electrolyte (KCl). The rate of Fe^{2+} uptake was calculated from Fe^{2+} uptake at 37 °C between 5 and 10 min. A concentration of 20 mM- Na^+ inhibited Fe^{2+} uptake by $61 \pm 14\%$ (mean $\pm$ S.D., $n = 20$) and 20 mM- Li^+ inhibited Fe^{2+} uptake by $4 \pm 5\%$ ($n = 6$); 100% = 1.85 ± 0.61 ng of Fe^{2+} /min per 10^8 reticulocytes ($n = 20$).

Analytical methods

^{59}Fe radioactivity was measured with a Beckman Biogamma Spectrometer. A Philips PU 9285 atomic absorption spectrophotometer was used for measuring the univalent cation concentrations. The osmolalities of the different solutions were checked with a Knauer Type M osmometer. pH determinations were carried out with a Beckman $\phi 40$ pH meter.

All experiments were repeated at least three (between three and eight) times with replicates. Figures show results of representative experiments, and each point represents the average of triplicate determinations. The variation between replicates was less than 5%.

Fig. 3. Kinetics of inhibition by Na^+ and activation by H^+ of Fe^{2+} uptake

A double reciprocal plot is shown. The initial rate of Fe^{2+} uptake was measured as a function of the Fe^{2+} concentration in the incubation medium. Initial rates were calculated from data obtained at 1 and 2 min of incubation at 37 °C. ●, KCl medium, pH 7.36 ± 0.01 ; ○, 2.5 mM-NaCl-containing KCl medium, pH 7.36 ± 0.01 ; ×, KCl medium, pH 7.14 ± 0.01 .

RESULTS

Effect of extracellular Na^+ concentration ($[\text{Na}^+]_o$) on Fe^{2+} uptake

The uptake of Fe^{2+} by reticulocytes was studied in four different iso-osmotic media: NaCl, KCl, LiCl and choline chloride. As Fig. 1 shows, Fe^{2+} uptake was markedly inhibited in NaCl compared with that measured in KCl or choline chloride. Fe^{2+} uptake in LiCl was significantly less inhibited than in NaCl. Measuring Fe^{2+} uptake as a function of Na^+ or Li^+ (replacing K^+) in KCl medium yielded the results shown in Fig. 2. The difference between the effects of Na^+ and Li^+ was remarkable. Na^+ at relatively low concentrations inhibited Fe^{2+} uptake. Near-maximal inhibition could be achieved with 10–20 mM- Na^+ , whereas Li^+ had little, if any, effect in the same concentration range.

The effect of Na^+ on the initial rate of Fe^{2+} uptake in KCl medium was also measured as a function of the Fe^{2+} concentration. Using the Lineweaver–Burk transformation, the apparent kinetic parameters of the Fe^{2+} uptake process were determined. As shown in Fig. 3, 2.5 mM- Na^+ decreased the maximum velocity of Fe^{2+} uptake without affecting the apparent K_m of the carrier, indicating that the observed inhibition was of a non-competitive type.

Effect of amiloride and amiloride analogues on Fe^{2+} uptake

Amiloride, EIPA and CBDMB are inhibitors of Na^+ -related transport processes [11–14]. The effects of these inhibitors on Fe^{2+} uptake were studied in order to assess the possible involvement of known Na^+ transport processes in Fe^{2+} uptake. Amiloride, the non-selective inhibitor of Na^+ exchangers, inhibited Fe^{2+} uptake in KCl medium with a half-maximal inhibitory concentration (IC_{50}) of around 0.3 mM. In the presence of Na^+ , however, the inhibitory effect of amiloride was not significant (Fig. 4).

Both EIPA (a selective inhibitor of the Na^+/H^+ exchange) and CBDMB (a selective inhibitor of $\text{Na}^+/\text{Ca}^{2+}$ exchange) inhibited Fe^{2+} uptake by less than 10% when applied at a concentration of 20 μM , which is higher than their known IC_{50} values [11–13].

Effect of pH

The pH-dependence of the rate of Fe^{2+} uptake was also studied. The pH of the samples was measured before and after

Fig. 4. Effect of amiloride on the rate of Fe^{2+} uptake

Fe^{2+} uptake by reticulocytes was measured at 37°C in NaCl (○) and KCl (●) media in the presence of various concentrations of amiloride. Samples were taken at 5 and 10 min to determine rates of Fe^{2+} uptake. The pH of the incubation systems was 7.36 ± 0.01 .

Fig. 5. Rate of Fe^{2+} uptake as a function of the pH of the incubation medium

The effects of Na^+ and amiloride on pH-dependence are shown. The pH of the KCl incubation medium was changed by adding $0\text{--}35\ \mu\text{l}$ of $0.16\ \text{M-HCl}$ to the medium without cells. The samples were then completed with reticulocytes and incubated at 37°C with Fe^{2+} -ascorbate. Samples were taken at 5 and 10 min. The pH of the reaction mixtures was measured at the beginning and at the end of incubation. The change in pH during the incubation period was less than $0.02\ \text{pH}$ unit. Additions: ●, nil; x, $0.5\ \text{mM}$ -amiloride; ○, $20\ \text{mM}$ -NaCl.

incubation. No significant change in the pH ($< 0.02\ \text{pH}$ unit) was observed during the incubation period. In Na^+ -free medium the pH optimum of the process was found to be around 6.8. In the presence of Na^+ , the pH optimum was shifted towards an acidic pH (approx. 6.6). In Na^+ -free medium, amiloride had a strikingly similar effect on the pH-dependence of Fe^{2+} uptake to that of extracellular Na^+ (Fig. 5). The pH optima were computed by using the cubic spline curve-fitting method. In Na^+ -free medium, the pH optimum was 6.86 ± 0.1 , and in the presence of $20\ \text{mM-Na}^+$ or $0.5\ \text{mM}$ -amiloride, the pH optimum was 6.61 ± 0.07 (mean $\pm$ s.d., $n = 7$).

As Fig. 3 shows, lowering the pH from 7.36 towards the pH optimum by $0.22\ \text{pH}$ unit increased the maximum velocity of

Fig. 6. Effects of intracellular Na^+ (○) and Li^+ (●) concentrations on the rate of Fe^{2+} uptake

Reticulocytes were loaded with Na^+ or Li^+ using the ionophore nystatin (for details see the Materials and methods section). Fe^{2+} uptake by reticulocytes of different Na^+ and Li^+ content was measured in choline chloride medium at 37°C . The rate of Fe^{2+} uptake was calculated from data obtained after 5 and 10 min of incubation. The ordinate shows rate of Fe^{2+} uptake as a percentage of the control. Dimethyl sulphoxide without nystatin was used in the treatment of control cells. The rate of Fe^{2+} uptake by cells containing $45\ \text{mM}$ intracellular Na^+ was $158 \pm 12\%$ of the control (mean $\pm$ s.d., $n = 5$) and the rate of Fe^{2+} uptake by cells containing $50\ \text{mM}$ intracellular Li^+ was $82 \pm 6\%$ of the control ($n = 3$); $100\% = 3.15 \pm 0.47\ \text{ng}$ of Fe^{2+} /min per 10^8 reticulocytes ($n = 5$).

Fe^{2+} uptake without affecting the apparent affinity of the carrier for Fe^{2+} .

Effect of intracellular Na^+ concentration ($[\text{Na}^+]_i$) on Fe^{2+} uptake

Reticulocytes having different $[\text{Na}^+]_i$ values were prepared using the ionophore nystatin. The Na^+/K^+ ratio of the loading solution was varied from $0:140$ to $138:2$ to achieve a final $[\text{Na}^+]_i$ of 5 and $112\ \text{mM}$. The Fe^{2+} uptake was then measured in Na^+ - and K^+ -free choline chloride medium. Choline chloride incubation medium was chosen in order to decrease the leakage of Na^+ through the Na^+/K^+ pump into the incubation medium. As shown in Fig. 6, the rate of Fe^{2+} uptake was significantly affected by the changes in $[\text{Na}^+]_i$. In the lower concentration range ($[\text{Na}^+]_i = 5\text{--}40\ \text{mM}$) the rate of Fe^{2+} uptake was greatly facilitated by the increase in $[\text{Na}^+]_i$. However, no comparable effect could be observed when Na^+ was replaced with Li^+ ($[\text{Li}^+]_i$ was varied between 10 and $122\ \text{mM}$).

It should be mentioned that I failed to decrease the $[\text{Na}^+]_i$ of reticulocytes below $5\ \text{mM}$ in any of the experiments, even when an Na^+ -free loading solution was used. This may be due to intracellular compartments in reticulocytes that are not available for nystatin.

The rate of Fe^{2+} uptake by Na^+ -loaded reticulocytes ($[\text{Na}^+]_i = 53\text{--}59\ \text{mM}$) was inhibited by extracellular Na^+ , in spite of having an outward-directed Na^+ gradient. Concentrations of 2.5 and $10\ \text{mM-Na}^+$ inhibited Fe^{2+} uptake by 12 ± 4 and $63 \pm 7\%$ (means $\pm$ s.e.m., $n = 3$) respectively. Amiloride ($0.5\ \text{mM}$) also inhibited Fe^{2+} uptake by Na^+ -loaded cells ($[\text{Na}^+]_i = 53\text{--}59\ \text{mM}$) by $74 \pm 9\%$ (means $\pm$ s.e.m., $n = 3$).

It should be mentioned that all experiments in KCl medium presented in this paper were also carried out in choline chloride medium. The results obtained in both of these Na^+ -free media were essentially the same (results not shown).

DISCUSSION

The results presented in this paper suggest that Na^+ may play an important role in the translocation of Fe^{2+} through the erythroid cell membrane. Extracellular Na^+ is found to be inhibitory for Fe^{2+} uptake by rabbit reticulocytes, and this inhibition appears to be specific for this univalent cation. Near-maximal inhibition of Fe^{2+} influx can be observed at relatively low (10–20 mM) values of $[\text{Na}^+]_o$ (Figs. 1 and 2).

This finding is in conflict with the results of Morgan [7], who concluded that the inhibitory effect of Na^+ on Fe^{2+} uptake was not specific for Na^+ . This contradiction may be due to the substantially different experimental conditions. The most profound difference is that Morgan used a low-ionic-strength medium (iso-osmotic sucrose), which strongly affects the transport characteristics of the erythroid cell membrane [15–17].

The involvement of Na^+ in the Fe^{2+} transport is indicated also by the effect of amiloride on Fe^{2+} uptake. Amiloride is known as a non-selective inhibitor of many Na^+ -related exchange pathways [18]. As Fig. 4 shows, amiloride inhibits Fe^{2+} uptake with an IC_{50} of around 300 μM . The effects of two potent and selective amiloride analogues (EIPA and CBDMB) on Fe^{2+} uptake have also been tested at concentrations up to 20 μM , and the maximal inhibition observed was less than 10%. Since the known IC_{50} values of EIPA for Na^+/H^+ exchange are in the range 0.1–1 μM , and those of CBDMB for $\text{Na}^+/\text{Ca}^{2+}$ exchange are between 3 and 8 μM [11–14], these findings indicate that the Na^+/H^+ and $\text{Na}^+/\text{Ca}^{2+}$ exchange pathways are not likely to be involved in the translocation of Fe^{2+} . Fig. 4 also shows that, in NaCl medium, amiloride has no significant effect on the rate of Fe^{2+} uptake, and the maximal inhibition caused by amiloride is the same as that caused by Na^+ , suggesting that the Fe^{2+} uptake process has a Na^+ /amiloride-sensitive component and a Na^+ /amiloride-insensitive one. The Na^+ /amiloride-insensitive component is represented by the Fe^{2+} uptake measured in the presence of Na^+ or amiloride.

The existence of these two components is also suggested by the results obtained when the pH-dependence of the rate of Fe^{2+} uptake was studied in KCl medium in the presence and absence of Na^+ or amiloride. In Na^+ -free medium, the pH optimum of the process is around 6.8. In the presence of Na^+ or amiloride, not only can the inhibition of Fe^{2+} uptake be observed, but the pH optimum is apparently lowered by about 0.2 pH unit (Fig. 5). Since the difference between the rate of Fe^{2+} uptake in the Na^+ -free medium and that measured in the presence of amiloride or Na^+ is so great, this experiment cannot show whether there is a shift in the pH optimum of a single carrier system, or whether a Na^+ /amiloride-insensitive system is always present, but is hidden by the sensitive component when Na^+ is absent.

Kinetic analysis of Na^+ inhibition of Fe^{2+} uptake revealed that Na^+ decreases the maximum velocity without affecting the apparent K_m of the carrier (Fig. 3). It is unlikely, though not impossible, that there are two different carrier systems in the cell membrane with the same affinity for Fe^{2+} . Therefore I suggest that the Na^+ /amiloride-sensitive and Na^+ /amiloride-insensitive components are two states of a single carrier system.

In contrast with the inhibitory effect of extracellular Na^+ , the rate of Fe^{2+} uptake is facilitated by an increase in $[\text{Na}^+]_i$ in the range 5–40 mM. Intracellular Li^+ has no effect on the rate of Fe^{2+} uptake. (Fig. 6). Although all of my efforts have failed to decrease $[\text{Na}^+]_i$ below 5 mM, the slope of the initial phase of the curve allows the presumption that intracellular Na^+ is required for the uptake of Fe^{2+} . It should be mentioned that extracellular Na^+ inhibits Fe^{2+} uptake even when the Na^+ concentration

gradient is outward-directed, and both the activation and the inhibition of Fe^{2+} uptake appear to be specific for Na^+ .

The inhibition of Fe^{2+} uptake by Na^+ in the medium and its activation by an elevated $[\text{Na}^+]_i$ cannot be explained by an acidification of the cytosol caused by a Na^+/H^+ -exchanger-mediated H^+ influx since: (1) Li^+ , which is able to substitute for Na^+ in the Na^+/H^+ exchange process [19,20], has no significant effect on Fe^{2+} uptake (Figs. 2 and 6), and (2) EIPA, the powerful and selective inhibitor of the Na^+/H^+ exchanger [11], has little, if any, effect on Fe^{2+} uptake at concentrations 20–200 times higher than its known IC_{50} .

The most likely role of Na^+ in Fe^{2+} translocation is to act as a counter-ion of the bivalent metal in order to maintain charge balance. An $\text{Na}^+/\text{Fe}^{2+}$ exchange system in the erythroid cell membrane is therefore postulated which mediates the translocation of Fe^{2+} through the membrane into the cytosol, in exchange for the simultaneous transport of Na^+ in the opposite direction. Amiloride or Na^+ at the Fe^{2+} side of the membrane decreases the rate of Fe^{2+} transport.

Further investigation is needed to confirm the postulated exchange process, to elucidate its mechanism and to determine the $\text{Na}^+/\text{Fe}^{2+}$ coupling ratio.

I thank Professor G. Gárdos and Dr. A. Enyedi for their helpful discussions and critical comments. I am grateful to Dr. H. Tegzes of the Institute of Agricultural Quality Control, Budapest, Hungary, for the atomic absorption measurements. The excellent technical assistance of Miss. A. Thaly is gratefully acknowledged. The work was supported by the Hungarian Academy of Sciences (OTKA-968).

REFERENCES

- Marx, J. J. M. & Aisen, P. (1981) *Biochim. Biophys. Acta* **649**, 297–304
- Simpson, R. J. & Peters, T. J. (1987) *Biochim. Biophys. Acta* **898**, 181–186
- Stremmel, W., Lotz, G., Niederau, C., Teschke, R. & Strohmeyer, G. (1987) *Eur. J. Clin. Invest.* **17**, 136–145
- Wright, T. L., Brissot, P., Ma, W.-L. & Weisiger, R. A. (1986) *J. Biol. Chem.* **261**, 10909–10914
- Wright, T. L., Fitz, J. G. & Weisiger, R. A. (1988) *J. Biol. Chem.* **263**, 1842–1847
- Egyed, A. (1988) *Br. J. Haematol.* **68**, 483–486
- Morgan, E. H. (1988) *Biochim. Biophys. Acta* **943**, 428–439
- Sturrock, A., Alexander, J., Lamb, J., Craven, C. M. & Kaplan, J. (1990) *J. Biol. Chem.* **265**, 3139–3145
- Egyed, A. (1973) *Biochim. Biophys. Acta* **304**, 805–813
- Hemmaplardh, D. & Morgan, E. H. (1974) *Biochim. Biophys. Acta* **373**, 84–99
- Vigne, P., Frelin, C., Cragoe, E. J., Jr. & Lazdunski, M. (1983) *Biochem. Biophys. Res. Commun.* **116**, 86–90
- Hunyady, L., Sarkadi, B., Cragoe, E. J., Jr., Spät, A. & Gárdos, G. (1987) *FEBS Lett.* **225**, 72–76
- Zhuang, Y.-X., Cragoe, E. J., Jr., Shaikewitz, T., Glaser, L. & Cassel, D. (1984) *Biochemistry* **23**, 4481–4488
- Günther, T., Vormann, J., Cragoe, E. J., Jr. & Höllriegel, V. (1989) *Magnesium Bull.* **11**, 103–107
- Wilbrandt, W. & Schatzmann, H. J. (1960) in *Regulation of the Inorganic Ion Content of Cells* (Wolstenholme, G. E. W. & O'Connor, C. M., eds.), pp. 34–52, Churchill, London
- Passow, H. (1964) in *The Red Blood Cell* (Bishop, C. & Surgenor, D. M., eds.), pp. 71–145, Academic Press, London
- LaCelle, P. L. & Rothstein, A. (1966) *J. Gen. Physiol.* **50**, 171–188
- Benos, D. J. (1988) in *Na^+/H^+ Exchange* (Grinstein, S., ed.), pp. 121–136, CRC Press, Florida
- Paris, S. & Pouyssegur, J. (1983) *J. Biol. Chem.* **258**, 3503–3508
- Parker, J. C. (1986) *Curr. Top. Membr. Transp.* **26**, 101–114