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THE ROLE OF CALCIUM IN TRANSFERRIN AND IRON UPTAKE BY RETICULOCYTES

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Summary

The possible role of calcium in the uptake of transferrin and iron by rabbit reticulocytes was investigated by altering cellular calcium levels through the use of the chelating agents EDTA and ethyleneglycol-bis-(3-aminoethylether)-*N,N'*-tetraacetic acid (EGTA) and the ionophores, A23187 and X537A. Incubation of reticulocytes with EDTA or EGTA at 4°C had no effect on transferrin and iron uptake but incubation at 37°C resulted in an irreversible inhibition associated with decreased adsorption of transferrin to the cells and evidence of inactivation or loss of the transferrin receptors. Transferrin and iron uptake were also inhibited when the cells were incubated with A23187 or X537A. In the case of A23187 the action was primarily exerted on the temperature-sensitive stage of transferrin uptake and was associated with loss of cellular K^+ and decrease in cell size. The effect was greater when Ca^{2+} was added to the incubation medium than in its absence. X537A produced relatively greater inhibition of iron uptake than of transferrin uptake, associated with a reduction in cellular ATP concentration. The action of X537A was unaffected by the presence of Ca^{2+} in the incubation medium.

The results obtained with EDTA and EGTA indicate that cell membrane Ca^{2+} is required for the integrity or binding of transferrin receptors to the reticulocyte membrane. No evidence was obtained from the experiments with ionophores that an increase of cellular Ca^{2+} affects transferrin and iron uptake directly. The inhibition caused by A23187 was mainly due to a reduction in cell size resulting from increased membrane permeability to K^+ and that caused by X537A appeared to result from an inhibition of energy metabolism and ATP production.

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Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid.

Introduction

Immature erythroid cells are able to take up iron from the plasma iron-carrying protein, transferrin, and utilize it for haem synthesis [1,2]. An essential step in this process is a direct interaction between transferrin and the cell surface [3,4]. Recently, evidence has accumulated that the interaction involves entry of transferrin to membrane receptors [5,6], followed by passage of the transferrin into the cell, probably by endocytosis [7—9]. Inhibitors of microtubules inhibit transferrin uptake and iron utilization by erythropoietic cells [10]. Hence, the endocytosis of transferrin may depend on the function of microtubules or microtubule-like proteins in the cell membrane. There is abundant evicence in the literature for the involvement of calcium in endocytosis and exocytosis and in the function of microtubules [11]. The question was therefore asked, whether cellular calcium plays a role in the uptake of transferrin and iron by immature erythroid cells. This was investigated by using rabbit reticulocytes and by altering cellular calcium levels through the use of the chelating agents EDTA and EGTA and the ionophores, A23187 and X537A.

Materials and Methods

Reagents. EDTA and EGTA were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. They were dissolved in aqueous solutions and the pH was adjusted to 7.4 using NaOH. A23187, was a gift from Eli Lilly and Co., Indianapolis, Ind., U.S.A., and X537A (in the form of its bromo analoque, RO20-0006/1) was kindly provided by Hoffman-La Roche and Co., Basle, Switzerland. The ionophores were dissolved in dimethylsulphoxide at concentrations 100 times greater than those used in the incubation media. 10 μl of the solutions or 10 μl dimethylsulphoxide (controls) were pipetted into each ml of incubation solution immediately before the cell suspensions were added. Teric 12A9 was a gift from I.C.I., London, England. The radioactive isotopes, ^{59}Fe ($FeCl_3$ in 0.1 M HCl, 10—30 Ci/g), ^{125}I and ^{131}I (NaI, carrier free) and ^{45}Ca ($CaCl_2$ in aqueous solution, 10—40 Ci/g) were purchased from the Radiochemical Centre, Amersham, England.

Reticulocytes were obtained from rabbits with haemorrhagic or phenylhydrazine-induced anaemia and were washed with 0.15 M NaCl prior to use as described previously [4]. The reticulocytes were not separated from mature erythrocytes, but, for brevity, the cells from reticulocyte-rich blood will be referred to as reticulocytes. The reticulocyte count of the cell samples used varied from 18 to 84%.

Isolation and labelling of proteins. Transferrin was isolated from rabbit plasma and was labelled with ^{59}Fe and ^{125}I as in earlier work [12,13].

Incubation procedure. Reticulocytes, suspended in incubation medium to a haematocrit of approx. 50%, were mixed with an equal volume of medium containing the agents being studied. In some cases, as specified below, the cell suspension was then pre-incubated at either 4° C or 37° C for a measured period of time before addition of transferrin labelled with ^{59}Fe and ^{125}I. In other cases no preincubation was used. The cells were then incubated with the

labelled proteins at 37°C (unless specified otherwise) in a shaking incubator. Cell samples were removed, washed and radioactivity measured after differing periods of incubation, using procedures described previously [4]. The release of transferrin from reticulocytes was measured by incubating the cells with ^{125}I-labelled transferrin for 30 min at 37°C, washing them four times at 4°C in 0.15 M NaCl and then re-incubating the cells at 37°C. The release of transferrin from the cells was measured by transferring samples after differing incubation times to centrifuge tubes containing 3 ml ice-cold 0.15 M NaCl, centrifuging the tubes and measuring radioactivity in cells and supernatant solution.

Extraction of reticulocyte ghosts with Teric 12A9. Ghosts were prepared from reticulocytes by haemolysis with 15 volumes 15 mOsM sodium phosphate buffer, pH 7.4, and were centrifuged at 40 000 × *g* for 30 min at 4°C. The ghosts were washed three times in the same buffer. They were then extracted with four volumes 1% Teric 12A9 in 15 mOsM sodium phosphate buffer, pH 7.4, for 30 min at 4°C and were centrifuged at 40 000 × *g* for 60 min at 4°C to separate residue from solubilized extract. The extract was fractionated by gel filtration at 4°C on a 2.5 × 50 cm column of Sephadex G-200 eluted with a solution of 0.1% Teric 12A9 in 15 mOsM sodium phosphate buffer, pH 7.4. Teric 12A9 is a non-ionic detergent. It is a polyethoxylated (n = 9) lauryl alcohol.

Analytical methods. Reticulocytes were counted on dried smears after staining with new methylene blue. Packed cell volume was determined by the microhaematocrit procedure. The size distribution of erythrocytes was measured using a Coulter Counter, Model ZB_1. Sodium and potassium concentrations of cell suspensions were measured by flame photometry after washing the cells twice at 4°C with 0.25 M sucrose. Cellular ATP was measured by the method of Williamson and Corkey [15]. Iron concentration was determined by the procedure recommended by the International Society for Standardization in Haematology [16]. The radioisotopes, ^{59}Fe, ^{125}I and ^{131}I, were measured in a three channel gamma-scintillation counter, and ^{45}Ca was counted in a liquid scintillation counter after decolorization of erythrocytes with H_2O_2 and solubilization with Soluene (Packard Instrument Co., Downers Grove, Ill., U.S.A.).

Results

Experiments with EDTA and EGTA

In the initial experiments reticulocytes were suspended in solutions of EDTA (1 and 5 mM) and EGTA (1 and 5 mM) at 4°C for varying periods from 1 to 16 h in an attempt to deplete the cells of Ca^{2+}. The reticulocytes were then washed with 0.15 M NaCl and incubated with ^{59}Fe—^{125}I-labelled transferrin. No inhibition of either transferrin or iron uptake was observed. However, if the cells were incubated for 1 h with the chelator solutions at 37°C before washing and incubating with the labelled transferrin both transferrin and iron uptake were markedly diminished (Fig. 1). The presence of Ca^{2+} or Sr^{2+} in the solution containing the chelator, in equimolar concentration to that of the chelator prevented the inhibition caused by the chelator, whereas Mg^{2+} resulted in a return to 50—70% of the control values.

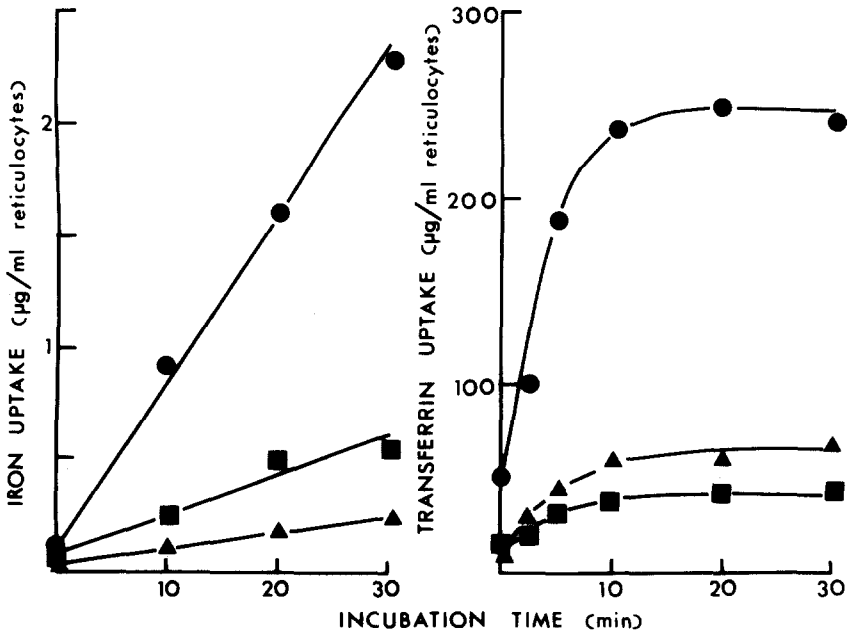

Fig. 1. Iron and transferrin uptake by rabbit reticulocytes. The cells (73% reticulocytes) were incubated for 1 h at 38°C either with no chelator (●———●) or with 1 mM EGTA (■———■) or 1 mM EDTA (▲———▲). Labelled transferrin was then added (iron concentration, 2.2 μg/ml; transferrin concentration, 3.0 mg/ml) and incubation was continued.

The effect of incubation of reticulocytes with EDTA and EGTA at 37°C was investigated in some detail. Fig. 1 shows the uptake of iron and transferrin by control reticulocytes and cells which had been preincubated for 1 h at 37°C with 1 mM EGTA and EDTA prior to incubation with labelled transferrrin. As has been reported previously [17] iron uptake was linear over the 30 min incubation period, but transferrin uptake occurred in three phases, an initial adsorption immediately after mixing cells and transferrin at 4°C, followed by a second phase of progressive uptake during the first 10—15 min of incubation at 37°C to reach a plateau phase when the amount of labelled transferrin on the cells remained constant. Hence, in the following presentation of results iron uptake will be expressed as the rate of uptake obtained from the linear regression through the values obtained during 30 min incubation at 37°C. Transferrin uptake will presented as (a) adsorbed transferrin, the amount bound immediately on mixing cells and transferrin, (b) rate of transferrin uptake, the rate of increase of cell-bound transferrin during the first 2—3 min of the progressive uptake phase during incubation at 37°C and (c) total transferrin uptake, the amount bound by the cells when the plateau phase had been reached. In many experiments the results for these values will be presented as percentages of the values obtained with control samples of cells, treated in the same way as the test samples but with omission of the chelators or ionophores from the incubation solutions.

When reticulocytes were incubated at 37°C with EDTA or EGTA for periods varying from 0 to 4 h before incubating with labelled transferrin decrease in the rate of iron uptake and in the three phases of transferrin uptake was observed.

As shown in Fig. 2 the degree of inhibition of the rate of iron uptake was similar to the inhibition of transferrin adsorption to the cells and the rate of transferrin uptake. A similar effect on the total uptake of transferrin was also found. Comparable results were obtained when the chelators were used at a concentration of 5 mM except that the degree of inhibition showed an even greater increase as the duration of incubation with the chelators was prolonged. By 1 h the rates of iron uptake and all aspects of transferrin uptake were less than 3% of the control values. Determination of correlation coefficients for the relationship between the rate of iron uptake and (i) transferrin adsorption, (ii) rate of transferrin uptake (iii) total transferrin uptake found in these experiments gave values of 0.833, 0.898 and 0.818, respectively. These values are all statistically significant ($P < 0.01$).

Incubation of the reticulocytes with 1 or 5 mM EGTA or EDTA at 37°C for up to 4 h produced no change in the packed cell volume of the cell suspension relative to that of the controls, indicating that there was no change in mean cell volume. Measurements of cellular concentrations of ATP, K^+ and Na^+ were also made after 1 h incubation with the chelators. The values obtained were not significantly different from the control values.

Attempts were made to reverse the inhibitory effects of incubation with EDTA and EGTA by washing the cells with 0.15 M NaCl after incubation with the chelators. However, transferrin and iron uptake were still inhibited. Samples of cells which had been incubated with EDTA for EGTA at 37°C for 1 h were washed and incubated twice at 37°C for 30 min with 0.15 M NaCl/10 mM glucose, 2.5 mM $CaCl_2$ or 2.5 mM $CaCl_2$ plus 1.0 mM $MgCl_2$ dissolved in the NaCl/glucose solution. The cells were then centrifuged and reincubated in labelled transferrin. Table I shows the results obtained with 1 mM EGTA.

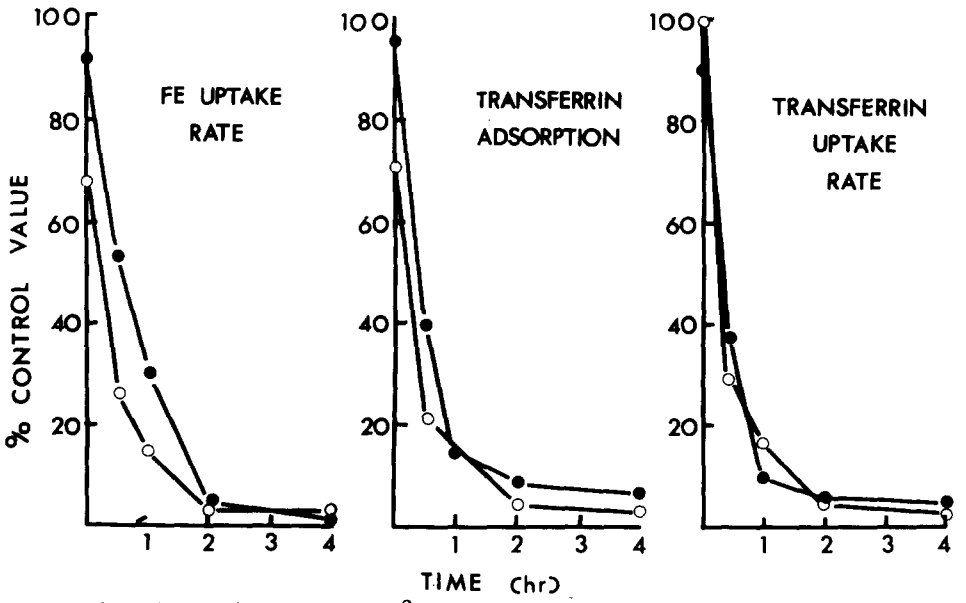

Fig. 2. Effect of time of incubation at 37°C with 1 mM EGTA (●———●) or 1 mM EDTA (○———○) on the rate of iron uptake, the adsorption of transferrin and the rate of transferrin uptake by reticulocytes.

TABLE I

EFFECT OF Ca^{2+} AND Mg^{2+} ON IRON AND TRANSFERRIN UPTAKE BY RETICULOCYTES TREATED WITH EGTA

The cells (80% reticulocytes) were incubated at 37°C for 1 h with 1 mM EGTA, washed three times with ice-cold 0.15 M NaCl. The cells were then incubated twice for 30 min at 37°C with 0.15 M NaCl/0.01 M glucose with the additions shown below, the incubation solution removed and iron and transferrin uptake measured. The results are expressed as a percentage of the values obtained with cells treated in the same way, except that EGTA was omitted from the original incubation solution.

Additions to incubation solution	Percent control value			
	Iron	Transferrin		
	Rate of uptake	Adsorption	Rate	Amount
Nil	33	27	33	31
2.5 mM $CaCl_2$	27	27	34	32
1.0 mM $MgCl_2$	18	25	33	27
2.5 mM $CaCl_2$ + 1.0 mM $MgCl_2$	20	32	23	30

Similar results were obtained with 5 mM EGTA and 1 or 5 EDTA. Calcium, magnesium or a combination of the two failed to produce any recovery of iron and transferrin uptake in these experiments.

The effect of incubation of reticulocytes with EGTA and EDTA on the binding of transferrin to membrane binding sites was investigated by extracting reticulocyte ghosts with Teric 12A9 and fractioning the extract by gel filtration. Reticulocytes were incubated with 1 mM EGTA or 1 mM EDTA for 30 min at 37°C, then washed three times with 0.15 M NaCl and reincubated with ^{125}I—^{59}Fe-labelled transferrin for 20 min at 37°C. The cells were again washed three times, haemolysed and the washed ghosts extracted with Teric 12A9. Control cells were treated in the same way except that EGTA and EDTA were omitted from the first incubation. When the extract from the control cells was fractionated by gel filtration the ^{125}I-labelled transferrin was eluted as two peaks, one just after the void volume and one at the volume expected for free transferrin (Fig. 3). The first peak contained 48% of the total ^{125}-labelled transferrin. Treatment of the cells with EGTA and EDTA resulted in a marked reduction in the relative size of the first peak, so that it contained only 3—10% of the total ^{125}I-labelled transferrin in the extract (Fig. 3).

Experiments with A23187

The ionophores A23187 and X537A were used in order to determine the effects of increased cellular Ca^{2+} on transferrin and iron uptake. In the first experiments reticulocytes were incubated with A23187 in varying concentrations for 10 min at 37°C. The cells were then cooled in an ice bath, labelled transferrin added, and incubation at 37°C commenced. As shown in Table II there was a moderate reduction in the rate of iron uptake and the rate and amount of transferrin uptake. The degree of reduction increased as the concentration of A23187 was raised. The addition of $CaCl_2$ to the incubation medium resulted in an even greater reduction in iron and transferrin uptake. This effect increased as the molar ratio of Ca^{2+}: ionophore was raised from 1 to 10. With higher ratios than this haemolysis was observed, hence, ratios above 10 were

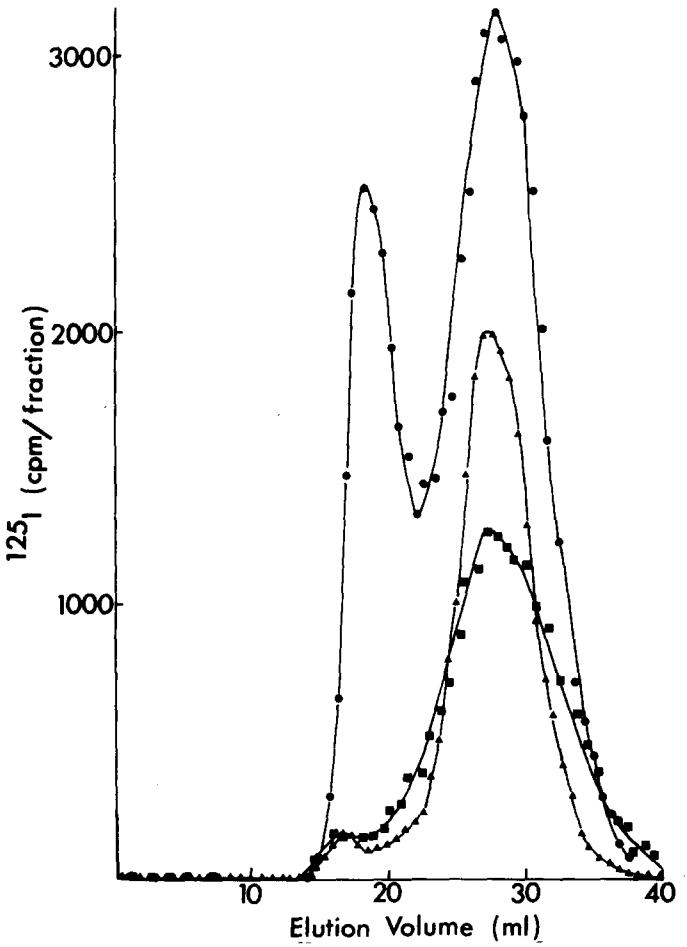

Fig. 3. Gel filtration on Sephadex G-200 of Teric 12A9 extracts of control reticulocytes (●———●) or reticulocytes which had been incubated with EDTA (▲———▲) or EGTA (■———■). The cells (81% reticulocytes) were incubated for 30 min at 37°C with no chelator or 1 mM EDTA or EGTA, then washed three times with 0.15 M NaCl and reincubated for 30 min with labelled transferrin. The cells were again washed, haemolysed, extracted with Teric 12A9 and the extract fractionated by gel filtration as described in the text.

not used in iron uptake experiments. The addition of A23187 and Ca^{2+} to the incubation medium had no significant effect on the adsorption of transferrin to the cells (Table II).

The effects of the addition of Ca^{2+}, Mg^{2+} and EDTA to the incubation medium which contained A23187 was investigated in a subsequent experiment. Because of the previously demonstrated effects of incubating reticulocytes with EDTA at 37°C on transferrin and iron uptake, in this experiment the additions to the incubation medium were made at 4°C 5 min before the labelled transferrin was added. The cell suspension was then incubated at 37°C for 5 min only. Cell samples were taken at 1-min intervals, washed and counted for radioactivity. The results are summarized in Table III. As before, the addition of Ca^{2+} increased the degree of inhibition of the rates of transferrin and iron uptake. When Mg^{2+} was also present there was only a small reduction in the

TABLE II

EFFECT OF A23187 AND Ca^{2+} ON IRON AND TRANSFERRIN UPTAKE BY RETICULOCYTES

The cells (79% reticulocytes) were incubated for 10 min at 37°C with A23187 and Ca^{2+} at the concentrations indicated, cooled in an ice bath and then incubated at 37°C with labelled transferrin (2 mg/ml). The incubation solution was 0.140 M NaCl/0.004 M KCl/0.007 M $NaHCo_3$/0.01 M glucose.

Incubation solution		Percent control value			
A23187 ($M \times 10^6$)	$CaCl_2$ ($M \times 10^5$)	Iron Rate of uptake	Transferrin		
			Adsorption	Rate	Amount
—	—	100	100	100	100
1	—	93	99	97	99
5	—	87	93	90	96
10	—	77	115	84	92
50	—	61	111	60	83
50	5	53	104	53	79
50	50	38	97	35	72

degree of inhibition. However, the presence of EDTA at 10 times the molar concentration of A23187 largely overcame the inhibitory effects of the ionophore and Ca^{2+}. This type of experiment was repeated except that the cells were preincubated at 37°C for 15 min in the presence of either EGTA or EDTA (5×10^{-4} M) before the rate of iron uptake was measured. On this occasion there was approximately a 70% inhibiton of uptake which was the same whether the chelators were used alone or in the presence of 5×10^{-5} M A23187.

During the course of the above experiments it was observed that the mean cell volume of the cell suspensions decreased during incubation with A23187 which indicated that the cells were becoming smaller. Direct measurement

TABLE III

EFFECTS OF Ca^{2+}, Mg^{2+} AND EDTA ON IRON AND TRANSFERRIN UPTAKE BY RETICULOCYTES IN THE PRESENCE OF A23187

The cells (65% reticulocytes) were incubated for 5 min at 4°C with A23187 ($5 \cdot 10^{-5}$ M) plus the addition of $CaCl_2$, $MgCl_2$ or EDTA in the concentrations shown. Labelled transferrin (2 mg/ml) was then added and incubation was continued for 5 min at 37°C, samples being taken at 1-min intervals. The incubation medium was the same as for Table II. The results are expressed as percentage of control cells incubated in the same manner except that A23187 was omitted from the solutions.

Incubation solution			Rate of uptake (percent control value)	
$CaCl_2$ ($M \times 10^5$)	$MgCl_2$ ($M \times 10^5$)	EDTA ($M \times 10^5$)	Iron	Transferrin
—	—	—	79	77
5	—	—	49	54
50	—	—	13	14
—	10	—	80	88
5	10	—	63	70
50	10	—	17	21
—	—	50	100	98
5	—	50	96	94
50	—	50	91	89

of cell size in a Coulter Counter confirmed this. These results suggested the possibility that the cells had lost intracellular K^+. Measurements of cellular K^+ and Na^+ were therefore made on cells which had been incubated with or without A23187 and Ca^{2+} at 37°C for 15 min. As shown in Fig. 4 there was a progressive loss of K^+ from the cells but little change in Na^+. The loss of K^+ was greater when the incubation medium contained Ca^{2+} as well as A23187.

An experiment was performed to determine whether cell shrinkage and/or loss of K^+ could be the cause of the effect of A23187 and Ca^{2+} on transferrin and iron uptake by reticulocytes. The cells were incubated in a medium in which the NaCl was replaced by KCl. The inhibitory effects of A23187 and Ca^{2+} were largely overcome by this change in the composition of the incubation medium (Table IV) and no change in packed cell volume was observed.

In other experiments in which reticulocytes were incubated with ^{45}Ca it was shown that A23187 at concentrations of 10^{-5}—10^{-4} M produced a rapid uptake of Ca^{2+} by the cells. Very little uptake of Ca^{2+} occurred in the absence of A23187. Addition of EGTA to the medium at 10 times the molar concentration of Ca^{2+} completely inhibited A23187-mediated uptake of Ca^{2+}. It was also found that when reticulocytes were incubated with A23187 and ^{45}Ca for 15 min at 37°C, washed three times with ice-cold 0.15 M NaCl and reincubated in

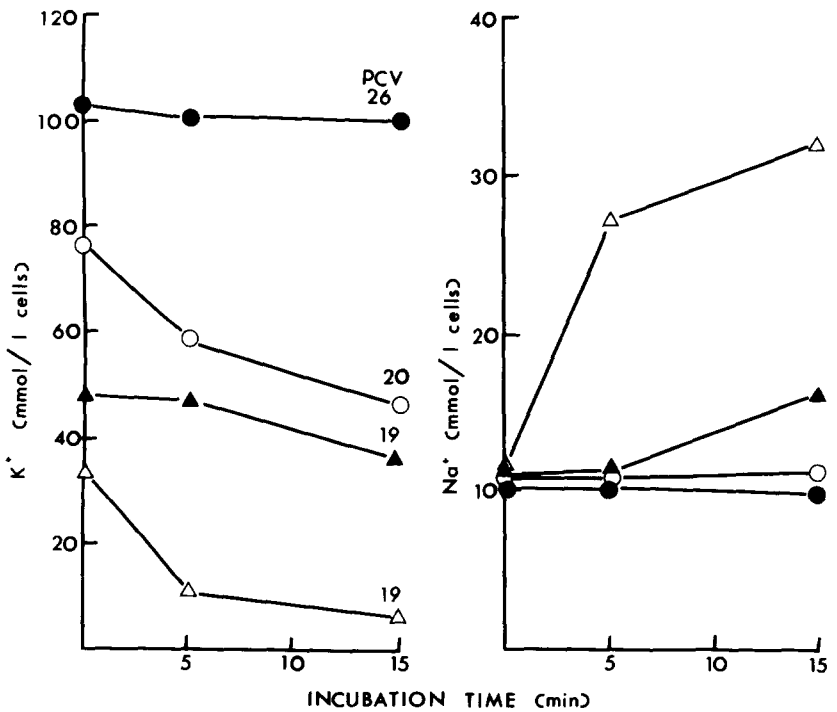

Fig. 4. Effect of A23187 and Ca^{2+} on cellular K^+ and Na^+. The cells were incubated for 5 min at 4°C and then for 15 min for 37°C in the absence of A23187 (●———●) or in the presence of 5×10^{-5} M A23187, alone (○———○) or with added $CaCl_2$, 5×10^{-5} M (▲———▲) or 50×10^{-5} M (△———△). The figure shows the results obtained immediately before the commencement of incubation at 37°C and after 5 and 15 min incubation at that temperature. The packed cell volumes of the cell suspensions at the end of the incubation time is also given.

TABLE IV

EFFECT OF KCl ON IRON AND TRANSFERRIN UPTAKE BY RETICULOCYTES IN THE PRESENCE OF A23187

The cells (90% reticulocytes) were incubated for 5 min at 4°C with A23187 ($5 \cdot 10^{-5}$ M) in 0.135 M KCl/0.02 M NaCl/0.01 M glucose plus the addition of $CaCl_2$ as indicated. Labelled transferrin was then added and the incubation was continued for 5 min at 37°C.

Incubation solution		Rate of uptake (percent control value)	
A23187 ($M \times 10^5$)	$CaCl_2$ ($M \times 10^5$)	Iron	Transferrin
—	—	100	100
5	—	102	95
5	5	99	93
5	50	81	84

the absence of the ionophore, Ca^{2+} still continued to be taken up rapidly from a Ca^{2+}-containing medium and Ca^{2+} was lost from the cells when a Ca^{2+}-free medium was used.

Experiments with X537A

The effects of X537A on transferrin and iron uptake was investigated by preincubating reticulocytes for 10 min at 4°C with different concentrations of the

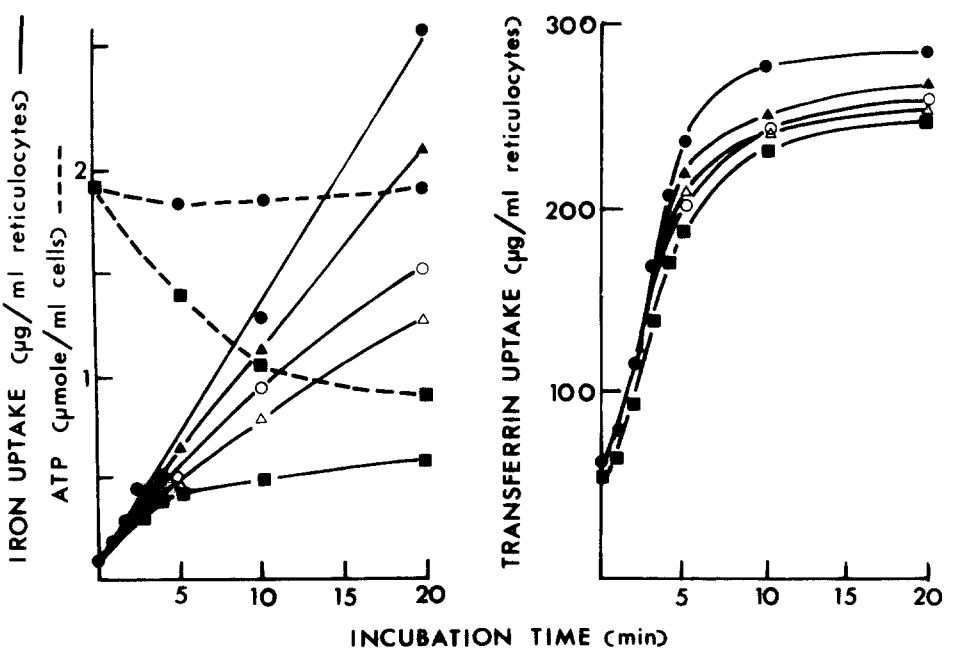

Fig. 5. Effect of X537A on iron and transferrin uptake by rabbit reticulocytes (——————) and cellular ATP concentration (- - - - -). The cells (40% reticulocytes) were incubated for 10 min at 4°C in a solution containing different concentrations of X537A. Labelled transferrin was then added and incubation continued at 37°C. The iron and transferrin concentrations were 1.5 μg/ml and 2.8 mg/ml, respectively. The X537A concentrations were: ●, 0; ▲, 10^{-6} M; ○, 10^{-5} M; △, 3×10^{-5} M; ■, 10^{-4} M.

ionophore before adding doubly labelled transferrin and continuing the incubation at 37°C. As shown in Fig. 5, X537A inhibited iron uptake, the effect increasing as the concentration of X537A was raised from 10^{-6} to 10^{-4} M. The degree of inhibition was relatively small during the first 5 min incubation with the labelled transferrin and increased with longer incubation times. X537A had relatively little effect on the initial stages of transferrin uptake, adsorption, and the first 2—3 min of progressive uptake, but did produce a slowing of uptake after that time and a lower degree of total uptake. The ATP concentration of control cells and cells incubated with 10^{-4} M X537A was measured. The level fell to 50% of that of the control after 20 min incubation (Fig. 5). The packed cell volume of the cell suspension did not change during 10 min incubation with X537A at 37°C, but there was a 10—15% increase in the packed cell volume by the end of 30 min incubation.

Addition of Ca^{2+} (0.5—2.5 mM) or EDTA (1—5 mM) produced no change in the effects of X537A on iron and transferrin uptake. When the incubation solution was changed from 0.15 M NaCl to 0.15 M KCl the degree of inhibition of iron uptake produced by 10^{-4} M X537A increased from 33% to 73%.

Discussion

The three methods used in the present work in an attempt to alter cellular calcium levels all produced inhibition of iron and transferrin uptake by the reticulocytes. However, the characteristics of the inhibition were different in the three cases. Treatment of reticulocytes with EDTA at 37°C produced a similar degree of inhibition of the rate of iron uptake and all three phases of transferrin uptake, adsorption, rate of progressive uptake and total uptake (Fig. 2). However, although A23187 resulted in the same relative inhibition of the rates of iron and transferrin uptake it had no effect on the adsorption of transferrin to the cells (Table II). In contrast to this the other ionophore used, X537A, inhibited the rate of iron uptake much more than it affected any aspect of transferrin uptake (Fig. 5). It therefore appears likely that the mechanisms responsible for the changes observed in iron and transferrin uptake in these situations are different.

EDTA and EGTA. The effects of EGTA and EDTA on iron and transferrin uptake by reticulocytes were markedly dependent on temperature. Whereas incubation with the chelators at 4°C for 16 h had no effect, incubation at 37°C for 30 min was sufficient to greatly inhibit uptake (Fig. 2). Adsorption of transferrin to the cells was inhibited to the same degree as the rates of iron and transferrin uptake. This suggested that the basic mechanism was the loss or inhibition of transferrin binding to the cell membrane, possibly at specific binding or receptor sites. The experiment in which Teric 12A9 extracts were prepared from reticulocyte which had been incubated with labelled transferrin was undertaken to investigate this possibility. It has been shown previously that when such extracts prepared with Teric 12A9 or Triton X-100 are examined by gel filtration the labelled transferrin elutes in two peaks, the earlier one having the characteristics expected of a complex between transferrin and a cell membrane receptor [5,6]. The fact that treatment of reticulocytes with EGTA or EDTA greatly reduced the amount of transferrin which eluted in the early peak

(Fig. 3) supports the concept that the mechanism of action of EGTA and EDTA is to cause a loss of receptor function. This could have resulted from chelation of divalent cations necessary for the binding of transferrin to the receptors, to denaturation of the receptors or to loss of receptors from the cell membrane. The inhibitory effect of prior treatment with the chelators was not reversed by washing the cells with 0.15 M NaCl or by incubating them in solutions free of the chelators but containing Ca^{2+} or Mg^{2+}. Hence, the observed effect seemed to be due to more than just a simple loss of ionized divalent cations. More probably it was the result of an irreversible denaturation or loss of the transferrin receptors which was in turn due to removal of the cations. The ability of EDTA and EGTA to cause the release of proteins from erythrocyte membranes is well established [18—20]. Possibly the transferrin receptor is one such protein.

A23187. The inhibiton of iron and transferrin uptake by reticulocytes which was caused by A23187 differed from that due to treatment with EDTA and EGTA because there was no inhibition of transferrin adsorption to the cells (Table II). Hence, it is unlikely that the inhibition was due to loss of functional transferrin receptors. More probably, it was due to inhibition of endocytosis, the second stage in transferrin and iron uptake [7—9]. This would account for the observation that the inhibition of the rate of iron uptake was proportional to that of the rate of transferrin uptake (Tables II, III, and IV).

The mechanism of action of A23187 on transferrin uptake by reticulocytes is probably dependent on its ability to act as an ionophore for Ca^{2+}, transporting the Ca^{2+} across the cell membrane. Thus, its inhibitory effect was increased by adding Ca^{2+} to the incubation medium (Tables II and III) and was decreased in the presence of EDTA (Table III) which was found to inhibit A23187-mediated transport of Ca^{2+} into the cells. The changes which were observed in reticulocyte cell size and electrolyte composition after incubation with A23187 (Fig. 4) may also be ascribed to its ionophore properties leading to the accumulation of Ca^{2+} in the cell membrane. This has been shown to cause an increase in the permeability of the erythrocyte membrane to K^+ [22—24]. Even in those cells which were incubated in a medium containing A23187 but no Ca^{2+} the effects on cell size and K^+ content were probably due to a redistribution of cellular Ca^{2+} to critical sites on the cell membrane [23].

As stated above, A23187 probably blocks transferrin and iron uptake by inhibiting endocytosis. The likely major cause of this inhibition is the reduction in cell size which was observed. However, several other possible causes should also be considered. These include a direct effect of the ionophore itself or of Ca^{2+} on the cell membrane, increased membrane permeability to K^+, altered K^+ or Na^+ concentration gradients or rates of flux across the membrane potential. The results obtained in the experiment in which the NaCl of the incubation medium was replaced with KCl (Table IV) indicate that these factors can contribute little to the inhibitory effect. In this context it should be noted that Wise and Archdeacon [25,26] found that prolonged incubation of reticulocytes with ouabain leads to a moderate inhibition of iron uptake and suggested that iron uptake is linked to Na^+ flux. However, they did not exclude the possibility that alterations of cell size could have been responsible for the observed effect. Changes in cell size induced simply by varying the osmolarity

of the incubation medium have been shown to alter the uptake of transferrin and iron by reticulocytes [27].

X537A. The effect of this ionophore on iron and transferrin uptake was different from that of A23187. Thus, it produced a much greater degree of inhibition of iron uptake than of transferrin uptake, and its effect was not altered by the presence of Ca^{2+} or EDTA in the incubation medium. In addition, there was little alteration in cell size. The changes produced by X537A are similar to those observed when reticulocytes are incubated with inhibitors of cellular metabolism [28]. In such cases the rate of iron uptake is closely correlated with the cellular ATP concentration and inhibition of the rate of iron uptake does not occur until the incubation has proceeded for about 5 min by which time the ATP concentration has fallen significantly [29]. This would explain why there was little inhibition during the first 4—5 min of incubation with X537A (Fig. 5).

Although X537A is a divalent cation ionophore it is not as specific for divalent cations as A23187 and will also transport monovalent cations and some primary amines [30]. It has been shown to differ from A23187 in its action on several types of cells [31—33]. It is not known whether its action on reticulocytes is dependent on its ionophore properties.

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