Biochimica et Biophysica Acta, 981 (1989) 121–129
Elsevier

BBA 74408

Calcium chelators induce association with the detergent-insoluble cytoskeleton and functional inactivation of the transferrin receptor in reticulocytes

Evan H. Morgan

Department of Physiology, The University of Western Australia, Nedlands (Australia)

(Received 11 October 1988)

Key words: Transferrin receptor; Calcium chelator; Cytoskeleton; Reticulocyte

Incubation of reticulocytes with EDTA, EGTA (ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid) and BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid), but not with desferrioxamine B, at temperatures above 20 °C resulted in the loss of their ability to take up iron in a temperature-, time- and concentration-dependent manner. No inhibition of transferrin or iron uptake occurred if the incubations were performed at 20 °C or below. At higher temperatures, the inhibition was attributable to loss of functional transferrin receptors, not to altered affinity or endocytosis of the remaining receptors. The changes could not be reversed by washing the cells and reincubation in the presence of Ca^{2+}, Mg^{2+} or Zn^{2+}. However, they could be completely prevented by performing the initial incubation with chelators in the presence of diferric transferrin and partly prevented by the use of apotransferrin. Incubation with the chelators resulted in much less reduction in the ability of the cells to bind anti-transferrin receptor immunoglobulin than transferrin. The fate of the receptor was studied by polyacrylamide gel electrophoresis of reticulocyte membrane proteins before and after extraction with Triton X-100, and by immunological staining of Western blots for the transferrin receptor. Treatment of the cells with EDTA led to a loss of the ability of Triton X-100 to solubilize the receptor and its retention in the Triton-insoluble cytoskeletal matrix of the cells. It is concluded that incubation of reticulocytes with the chelators at temperatures above 20 °C causes an altered interaction of the transferrin receptor with the cytoskeleton. This change, which is probably due to chelation of Ca^{2+} in the cell membrane, is accompanied by an irreversible loss of the receptor's ability to bind transferrin.

Introduction

Immature erythroid cells acquire the iron required for cell division, growth and haemoglobin synthesis from plasma transferrin by receptor-mediated endocytosis [1–4]. In recent years, the structure of the transferrin receptor has been determined in considerable detail [5,6], but the mechanism by which it participates in the endocytotic process is poorly understood. In earlier investigations of iron uptake by reticulocytes, it was shown that incubation of the cells with the chelating agents EDTA, EGTA and DTPA caused a loss of their ability to bind transferrin and take up iron [7,8]. These observations led to the conclusion that removal of calcium from the cell membrane resulted in the loss or inactivation of the transferrin receptors [7]. The aim of the present investigation was to study this problem in more detail in order to determine its mechanism and, in so doing, to provide additional information on the structure and function of the transferrin receptor.

Materials and Methods

Materials

EDTA, EGTA and DTPA were obtained from Sigma Chemical (St. Louis, MO, U.S.A.), BAPTA from Fluka (Buchs, Switzerland) and desferrioxamine B from Ciba (Basle, Switzerland). They were dissolved in water and the pH of the solution adjusted to 7.4 using 1 M NaOH and the osmolality made up to 290 mosmol/kg with NaCl. The radioisotopes, ^{59}Fe and ^{125}I, were purchased from Amersham International, U.K.

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid.

Correspondence: E.H. Morgan, Department of Physiology, University of Western Australia, Nedlands, Western Australia, Perth 6009, Australia.

Isolation and labelling of proteins

Rabbit and rat transferrins were isolated from plasma and labelled with ^{59}Fe and ^{125}I as in earlier work [9,10]. The radiolabelled proteins were used in their diferric form. Nonradioactive apotransferrin was prepared by dialysing diferric transferrin for 8 h at 4°C with 0.05 M EDTA/sodium acetate buffer (pH 5.0), followed by dialysis with five changes of 0.15 M NaCl.

Transferrin receptor was isolated from placentas obtained from rats on the 19th day of pregnancy and was used to immunize a rabbit as described previously [11]. After removal of contaminating anti-transferrin antibodies by passage through a column of rat transferrin immobilized in Sepharose 4B (Pharmacia, Uppsala, Sweden), the immunoglobulin fraction of the serum was isolated by ammonium sulphate precipitation followed by ion-exchange chromatography on DEAE cellulose (DE 52, Whatman Chemical Separation, Maidstone, U.K.). A sample of this fraction was labelled with ^{125}I by the iodine monochloride method [12].

Reticulocytes were obtained from rabbits and rats with phenylhydrazine-induced anaemia [9] 4–6 days after the last dose of phenylhydrazine. The cells were washed four times with 0.15 M NaCl at 4°C prior to use in the experiments.

Incubation procedures

The majority of the experiments were performed with rabbit reticulocytes and rabbit transferrin. However, rat reticulocytes and transferrin were used when it was desired to identify the transferrin receptor immunologically after electrophoretic separation of cell membrane proteins or to study the binding of radiolabelled receptor antibodies.

The methods used to measure total transferrin and iron uptake, transferrin endocytosis and exocytosis and the numbers and affinity of membrane receptors for transferrin were as described earlier [13,14]. In most of the experiments, the reticulocytes were first incubated with a chelator, washed with 0.15 M NaCl and then incubated with radiolabelled transferrin. The reticulocytes were incubated with the chelators in NaCl solutions of osmolality 290 mosmol/kg and containing 10 mM glucose. Control samples of reticulocytes were incubated with the NaCl/glucose solution without the addition of the chelator. After these first incubations, the cells were washed three times with ice-cold 0.15 M NaCl and suspended in 0.15 M NaCl/10 mM glucose. They were then reincubated with ^{125}I,^{59}Fe-transferrin or with ^{125}I-immunoglobulin.

Analytical methods

Reticulocytes were determined by staining with new Methylene blue and were packed cell volume by the microhaematocrit method. Radioactivity was counted in a 2-channel γ-scintillation counter. Polyacrylamide gel electrophoresis and immunological staining of Western blots for the transferrin receptor were performed as described previously [11]. For this purpose reticulocyte membranes were prepared by haemolysing the cells in 50 vols 10 mM sodium phosphate buffer (pH 7.4), followed by centrifugation at $40000 \times g$ for 30 min at 4°C. The membranes were washed three times with this buffer using the same centrifugation conditions. For some analyses, the washed ghosts were extracted by mixing with 1% Triton X-100 for 20 min at 4°C and then centrifuged at $40000 \times g$ for 60 min at 4°C.

For electron microscopy, reticulocytes were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) postfixed in OsO_4, then were embedded in Araldite, stained, sectioned and examined in a Philips 300 electron microscope as in previous investigations [15].

Results

Chelator-induced loss of transferrin and iron uptake

Several factors which influence the ability of EDTA and related chelators to cause the loss of transferrin and iron uptake were investigated using rabbit reticulocytes. The effects of temperature were studied by incubating the reticulocytes for 1 h with 5 mM EDTA or EGTA at temperatures varying from 4 to 37°C, followed by washing three times in ice-cold 0.15 M NaCl and reincubation with radiolabelled transferrin. Preincubation with the chelators at temperatures from 4 to 20°C did not reduce transferrin or iron uptake but, with higher temperatures, there was an increasing degree of inhibition. The initial (zero-time) uptake of transferrin also diminished with preincubation temperatures above 20°C (Figs. 1A and B). The degrees of inhibition of initial and total transferrin uptake and rate of iron uptake were similar at each preincubation temperature (Fig. 1C).

The degree of inhibition of transferrin and iron uptake increased as the time of preincubation at temperatures above 20°C was raised, but there was no inhibition when the preincubation was performed for varying times up to 60 min at 20°C (Fig. 2A). The rate of onset of inhibition suggested the possibility that internalized transferrin receptors need to be transported to the outer cell membrane by exocytosis before they can be inactivated by the action of the chelators. Hence, a direct comparison was made between the rate of exocytosis of transferrin and the rate of appearance of the inhibitory effects of the chelators. Transferrin exocytosis was determined using reticulocytes which had been labelled by incubation with 3 μM ^{125}I-transferrin, washed and reincubated in the presence of 5 mM EDTA or EGTA at 37, 30 or 20°C. At 37 and 30°C, the rates of transferrin exocytosis (Fig. 2B) were similar to the rates of inactivation of iron uptake (Fig. 2A). However, at 20°C, transferrin exocytosis still proceeded, even though in-

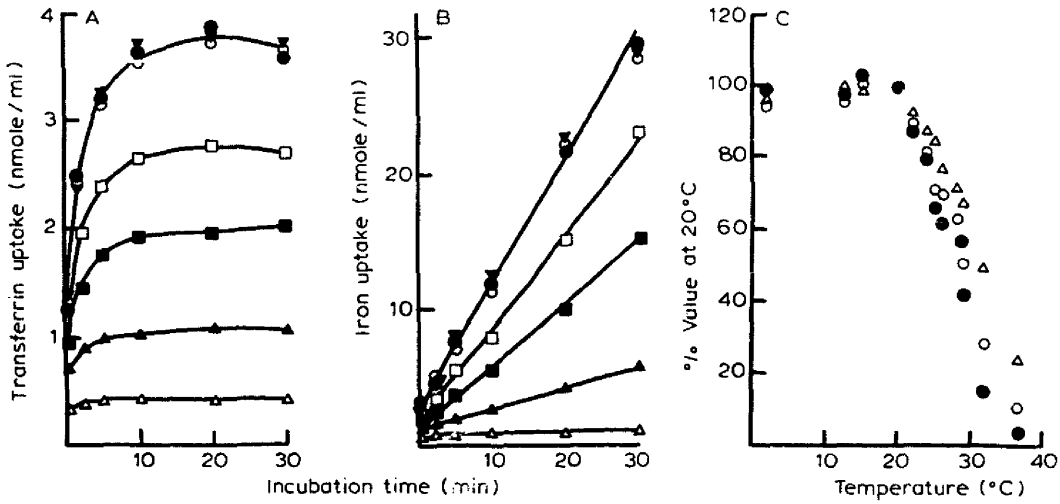

Fig. 1. Effect of temperature of preincubation with EDTA on the subsequent uptake of transferrin and iron by rabbit reticulocytes. The cells were incubated with 5 mM EDTA for 30 min, washed three times with ice-cold 0.15 M NaCl and then reincubated with ^{125}I,^{59}Fe-labelled diferric transferrin (4 μM) at 37°C in order to measure transferrin and iron uptake. A and B show transferrin and iron uptake, respectively, after preincubation with EDTA at 4 (▼), 16 (○), 20 (●), 25 (□), 29 (■), 32 (▲) and 37°C (△). The results are expressed as nmol/ml reticulocytes. C shows the effect of temperature of preincubation with EDTA on the rate of iron uptake (●), the initial (zero-time) uptake of transferrin (△) and the uptake after 30 min incubation at 37°C (○). The results are expressed as percentages of the values obtained when the temperature of preincubation with EDTA was 20°C. Very similar results to those shown in the figure were also obtained when EDTA was replaced by EGTA.

cubation of the cells with the chelators at this temperature produced no loss of transferrin or iron uptake. In subsequent experiments, a more detailed study was made of the effects of the first 10 min incubation with EDTA at 37°C. A distinct difference was observed between the exocytosis of transferrin and the development of inhibition of transferrin and iron uptake, the latter occurring considerably more slowly than exocytosis (Fig. 2C).

The effects of chelator concentration were studied using EDTA, EGTA and BAPTA. They gave very similar results (Fig. 3). 50% inhibition occurred at 1–2 μM concentrations of the chelators.

One possible cause of the inactivation of the transferrin receptors which was considered was that EDTA and

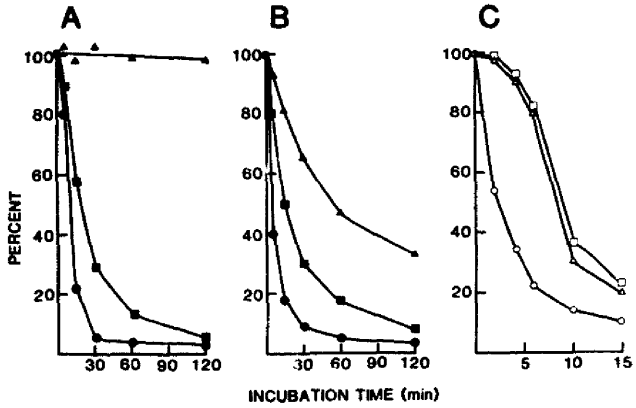

Fig. 2. (A) Effect of time of preincubation with 5 mM EDTA at 37 (●), 30 (■) or 20°C (▲) on the rate of iron uptake by rabbit reticulocytes during subsequent incubation with ^{125}I,^{59}Fe-transferrin. The results are expressed as percentages of the rate of iron uptake obtained with cells which were not preincubated with EDTA. (B) Effect of incubation temperature (●, 37°C; ■, 30°C; ▲, 20°C) on ^{125}I-transferrin release from reticulocytes during incubation in the presence of 5 mM EDTA. The cells were preincubated with ^{125}I,^{59}Fe-transferrin prior to washing and reincubation at the indicated temperatures. The results are expressed as the percentage of initial cellular ^{125}I-transferrin which remained with the cells at the indicated times. (C) Effect of time of preincubation with 5 mM EDTA at 37°C on the subsequent rate of iron uptake (□) and amount of transferrin uptake (△) when reincubated with ^{125}I,^{59}Fe-transferrin at 37°C. Results are expressed as in (A). Also shown in ^{125}I-transferrin released during reincubation at 37°C under conditions similar to (B) (○).

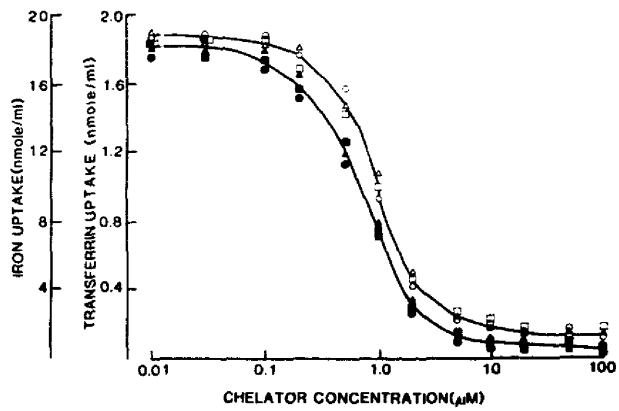

Fig. 3. Effect of chelator concentration during preincubation at 37°C for 30 min on iron uptake (●, ■, ▲) and transferrin uptake (○, □, △) by rabbit reticulocytes during subsequent incubation with 4 μM labelled transferrin for 20 min at 37°C. The chelators were EDTA (●, ○), EGTA, (■, □) and BAPTA (▲, △).

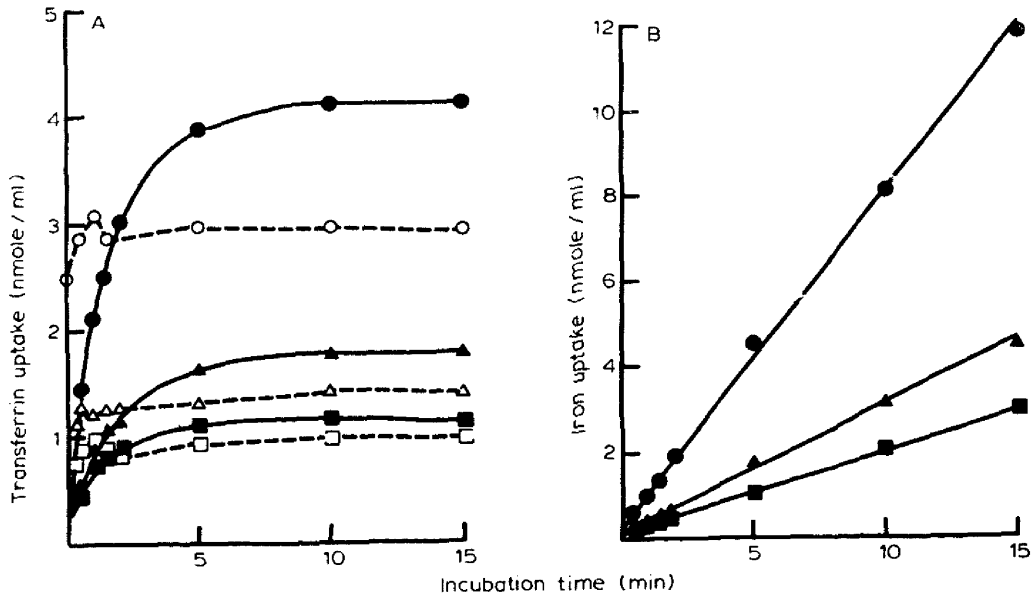

Fig. 4. Transferrin and iron uptake by rabbit reticulocytes at 37° C after preincubation with 0 (●, ○), 5 μM (▲, △) and 20 μM (■, □) EDTA for 30 min at 37° C. The results for transferrin uptake (A) show endocytosed (●, ▲, ■) and surface-bound (○, △, □) transferrin, while those for iron uptake (B) only show intracellular iron.

EGTA were stimulating lipid peroxidation of the cell membranes, leading to damage to the receptors, possibly as a result of chelating Fe derived from the cells or the medium. If so, incubation of reticulocytes with the Fe-EDTA complex should produce a greater degree of inhibition than with EDTA alone. This was not observed. Instead, preincubation at 37° C for 10–60 min with Fe-EDTA (1 mM Fe, 5 mM EDTA) caused approx. 10% less inhibition of transferrin and iron uptake with each preincubation time than occurred with EDTA alone (results not shown). These results confirm earlier observations against involvement of free radicals in

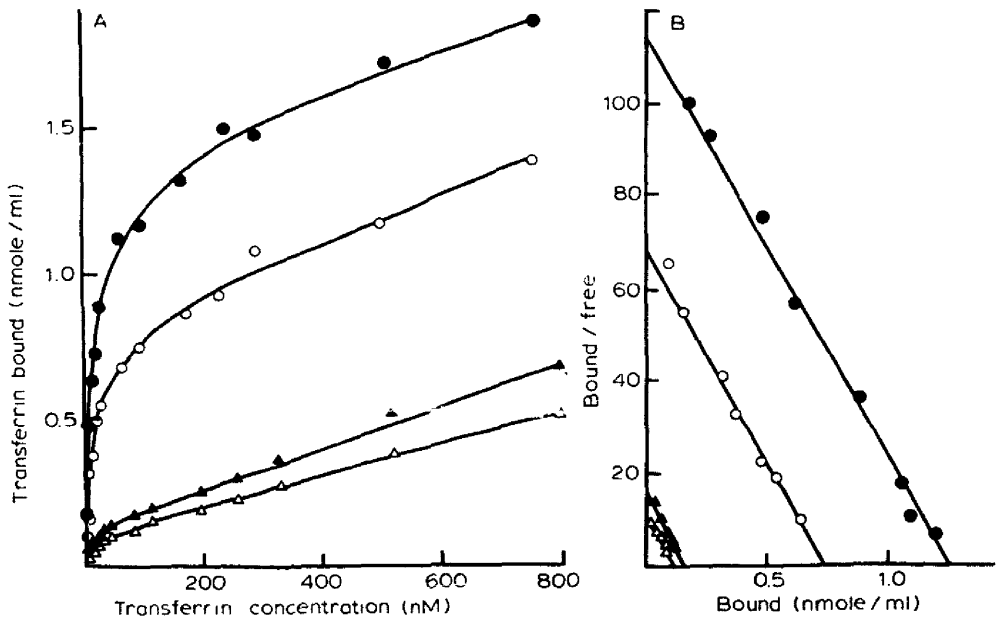

Fig. 5. (A) Effect of transferrin concentration on transferrin binding at 4° C by rabbit reticulocytes after preincubation for 15 min at 27° C with 0 (●), 5 (○), 25 (▲) and 100 μM (△) EDTA. (B) Scatchard plots of the results in (A) after correction for nonspecific binding by subtracting the nonspecific component of uptake as determined by the slopes of the linear parts of the uptake curves.

receptor inactivation, in which a variety of free-radical scavengers were found to have no effect on DTPA-induced receptor inactivation [8].

In another experiment, reticulocytes were preincubated at 37°C for 30 min with 5 mM solutions of EDTA and EGTA to which had been added equimolar amounts of $CaCl_2$. No loss of transferrin uptake resulted from this treatment.

Transferrin endocytosis and receptor affinity

The above experiments suggested that incubation of reticulocytes with the chelators causes a loss of transferrin receptors from the cells. However, the results could also be explained by inhibition of the endocytotic process or by a change in the affinity of the receptors. These possibilities were investigated by incubating rabbit reticulocytes with different concentrations of EDTA to produce varying degrees of inhibition of transferrin and iron uptake, followed by washing the cells and measuring transferrin endocytosis, iron uptake and receptor affinity for transferrin. This produced decreases in the rate of transferrin endocytosis and the steady-state levels of surface-bound and intracellular transferrin (Fig. 4A. The rates of iron uptake (Fig. 4B) were reduced to almost the same degree as those for transferrin endocytosis (indicated by the slopes of the curves during the initial phase of transferrin endocytosis in Fig. 4A).

Surface-receptor numbers and affinity for transferrin were determined by incubation at 4°C with varying concentrations of radiolabelled transferrin, using cells which had been preincubated with 5, 25 and 100 μM EDTA for 15 min at 37°C. This resulted in a marked reduction in transferrin binding (Fig. 5A) due to a reduction in the number of receptors without a change in affinity for transferrin (Fig. 5B). As determined by Scatchard analysis, the number of receptors in terms of the amount of bound transferrin was reduced from 1.23 nmol/ml reticulocytes in the control cells to 0.73, 0.16 and 0.12 nmol/ml after treatment with 5, 25 and 100 μM EDTA, respectively. The association constant for the interaction between the receptors and transferrin, K_a, varied only between $9.2 \cdot 10^7$ M^{-1}.

Irreversibility of receptor inactivation

Rabbit reticulocytes were incubated with 5 mM EDTA or EGTA for 30 min at 37°C, washed and reincubated with Hanks and Wallace balanced salt solution [16], or $CaCl_2$, $MgCl_2$, $ZnCl_2$, $CaCl_2$ plus $MgCl_2$ in NaCl/glucose solution at concentrations from 0.2 to 5 mM, for periods up to 1 h before rewashing and incubating with radiolabelled transferrin in either NaCl/glucose or Hanks and Wallace solution. No recovery of transferrin and iron uptake ability was observed.

Protection by diferric transferrin

In all of the experiments described above, the incubation with chelators was performed in the absence of added transferrin. However, when this incubation was performed in the presence of diferric transferrin,

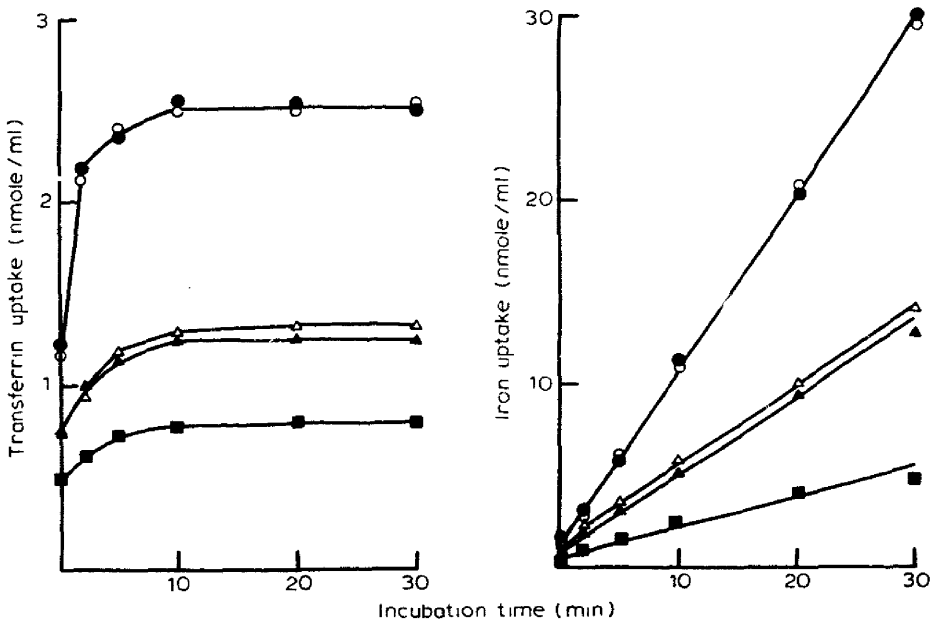

Fig. 6. Effect of the presence of diferric transferrin or apotransferrin (12.5 μM) during preincubation with 5 mM EDTA for 30 min at 37°C on the subsequent uptake of radiolabelled transferrin and iron by rabbit reticulocytes. The preincubation conditions were: no EDTA (●), EDTA alone (■), EDTA plus diferric transferrin (○), EDTA plus apotransferrin (▲), EDTA plus apotransferrin and desferrioxamine (5 mM) (△).

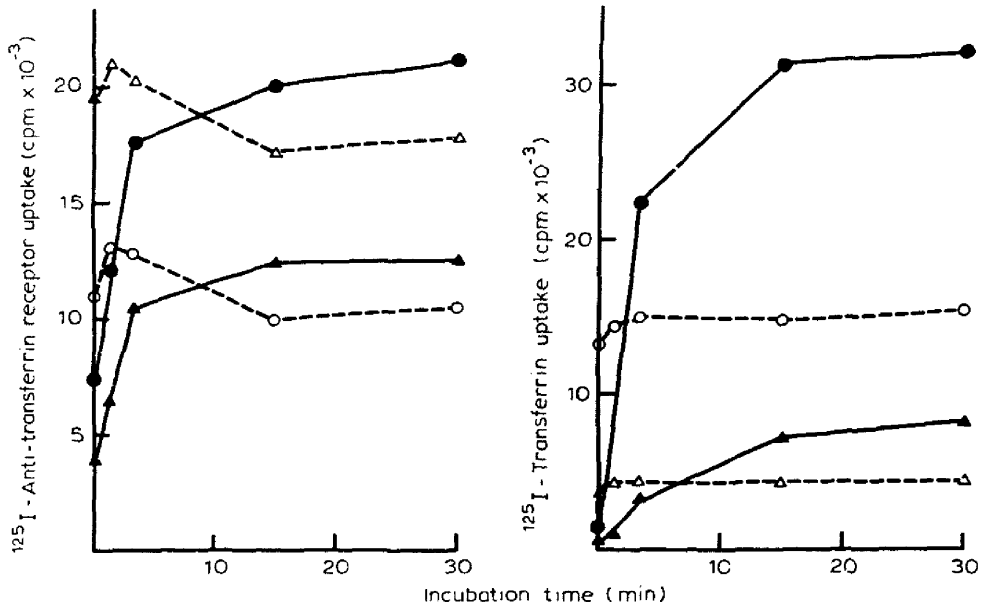

Fig. 7. Uptake of ^{125}I-labelled anti-rat transferrin receptor and ^{125}I-labelled diferric rat transferrin by rat reticulocytes after preincubation for 30 min at 37°C with 5 mM EDTA. The figures show endocytosed (●, ▲) and surface-bound (○, △) labelled proteins in control cells (●, ○) and those preincubated with EDTA (▲, △). As in all other experiments, the cells were washed three times in ice-cold 0.15 M NaCl after the preincubation in the presence or absence of EDTA and before they were incubated with the labelled proteins.

the inhibitory effect produced by EDTA was prevented (Fig. 6). Apotransferrin provided a smaller degree of protection of the cells' capacity to take up transferrin and iron. This effect of apotransferrin persisted even when desferrioxamine was present during the preincubation with apotransferrin (Fig. 6).

Uptake of transferrin receptor antibodies

The apparent loss of transferrin receptors, as indicated by inhibition of transferrin uptake, which was produced by incubation of reticulocytes with the chelators, was investigated further using the IgG fraction of anti-rat transferrin receptor antiserum labelled with ^{125}I. These labelled antibodies were found to bind to rat reticulocytes, but not to mature rat erythrocytes, and the binding was inhibited by unlabelled anti-receptor IgG. As assessed by the ability of pronase at 4°C to release the labelled antibodies from the cells, endocytosis of the antibody by control rat reticulocytes during incubation at 37°C (Fig. 7) was comparable to that of transferrin (Fig. 3A). However, following incubation of reticulocytes with 5 mM EDTA for 30 min at 37°C, there was much less reduction in the endocytosis of antibody than of transferrin and the amount of membrane-bound antibody increased, rather than decreased, with the EDTA-treated cells when compared with the controls (Fig. 7). In six experiments of the type illustrated in Fig. 7, EDTA treatment produced 39 ± 7.2% (mean ± S.E.) reduction in total antibody uptake compared with 93 ± 1.9% reduction in transferrin uptake.

Polyacrylamide gel electrophoresis of reticulocyte membrane proteins

The results of the experiments with ^{125}I-transferrin and ^{125}I-antibody to the transferrin receptor have indicated that incubation with the chelators leads to an

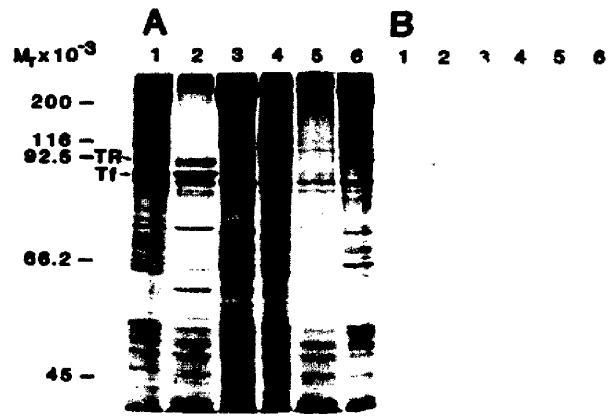

Fig. 8. (A) Polyacrylamide gel electrophoresis of rat reticulocyte membrane proteins and (B) Western blots of duplicates of the same membrane extracts after immunological staining for the transferrin receptors. Lanes 1–3 show the total membrane with Triton X-100-soluble extract and Triton X-100-insoluble residues, respectively, of control reticulocytes. Lanes 4–6 are the corresponding preparations from reticulocytes which had been preincubated for 30 min at 37°C with 5 mM EDTA before haemolysis and preparation of the membranes. The positions of the transferrin receptor (TR) and transferrin (Tf) are indicated. See the text for details.

almost complete loss of transferrin-binding ability but much less loss of the antigen which binds the antibody. That is, the loss of transferrin binding and iron uptake is due to the inactivation but not to the loss of the receptors from the cells. This possibility was investigated with rat reticulocytes and polyacrylamide gel electrophoresis of membrane proteins followed by Western blot transfer to nitrocellulose membranes and immunochemical staining for the receptor. Using ^{125}I,^{59}Fe-labelled rat transferrin, it was shown that treatment of rat reticulocytes with EDTA at 37°C resulted in an inactivation of transferrin and iron uptake similar to that observed with rabbit reticulocytes. When the reticulocyte membranes were examined by the electrophoretic technique, the retention of the receptor in the EDTA-treated cells was confirmed (Fig. 8). However, a marked difference in the solubility of the receptors in the nonionic detergent, Triton X-100, was noted between control and EDTA-treated reticulocytes. In the control cells, the receptor was almost completely solubilized by treatment with 1% Triton X-100, but, after EDTA treatment, it was no longer soluble in the detergent and was retained in the Triton-insoluble fraction of the cell membranes (Fig. 8). Similar results were obtained with rabbit reticulocyte membranes as judged by the presence or absence of the 92 kDa protein corresponding to the transferrin receptor, but no antibody was available to confirm this observation by immunological means. It was also observed with both rat and rabbit reticulocytes that the control cell membranes had a prominent band corresponding to the molecular weight of transferrin (80000), but this was absent from the EDTA-treated cells. The identity of this band as transferrin was established immunologically with rat reticulocytes.

The change in solubility of the transferrin receptor in Triton X-100, which was induced by treatment with EDTA, was confirmed using rat reticulocytes which had been incubated with ^{125}I-transferrin or ^{125}I-antibody to the transferrin receptor with or without prior treatment with EDTA. After EDTA treatment, only 4% of the ^{125}I-antibody in the cells was solubilized by 1% Triton X-100, compared with 80% in control cells. With the ^{125}I-transferrin, however, there was little change in solubility but, in this case, the total amount of labelled protein present in the treated cells was only about 10% of that in the controls (Table I).

Electron microscopy

Thin sections of rabbit reticulocytes which had been incubated for 30 min at 37°C with or without 5 mM EDTA were examined by electron microscopy to see whether the biochemical changes noted above were accompanied by any morphological changes. Particular attention was paid to the number and size of coated pits and intracellular vesicles. No differences between control and EDTA-treated cells were detected.

TABLE I

Solubility of reticulocyte-bound ^{125}I-transferrin and ^{125}I-anti-transferrin receptor in 1% Triton X-100

Samples of rat reticulocytes were incubated for 30 min at 37°C without (control) or with 5 mM EDTA in 0.15 M NaCl/10 mM glucose, washed three times and then reincubated for 15 min at 37°C with ^{125}I-transferrin (Tf) or ^{125}I-anti-reticulocyte receptor immunoglobulin (A/TR). After again washing three times in 0.15 M NaCl at 4°C, they were treated with 1% Triton X-100 in 0.15 M NaCl at 4°C for 15 min then centrifuged at 40000 × *g* for 60 min at 4°C to separate supernatant and pellet fractions of the cells. Results are expressed as the cpm of ^{125}I in the cells before solubilization (total) and in the two fractions, and as percentages of the total radioactivity in each fraction.

		Control cells		EDTA-treated cells	
		^{125}I-Tf	^{125}I-A/TR	^{125}I-Tf	^{125}I-A/TR
Total	cpm	17047	18255	1710	16470
	%	100	100	100	100
Supernatant	cpm	14490	14753	1350	1641
	%	85	80	79	4
Pellet	cpm	2558	3761	347	15813
	%	15	20	21	96

Discussion

These investigations confirm earlier observations that incubation of reticulocytes with EDTA, EGTA or DTPA in the absence of transferrin leads to a loss of their ability to take up transferrin and iron [7,8] and provide evidence that this is due to an alteration in the functional activity of the transferrin receptor. It is likely that this is due to chelation of Ca^{2+}. The concentration-dependence of the inhibition of transferrin and iron uptake was similar for the three chelators, EDTA, EGTA and BAPTA (Fig. 3). They have similar affinities for Ca^{2+}. EDTA also has approximately the same affinity for Mg^{2+}. However, with the other two chelators, the calcium affinity is approx. five orders of magnitude higher than that for Mg^{2+} [17]. The chelators can also bind many other ions, including Fe^{3+}, but none of them are believed to play a role in cell membrane structure and function, and an effect due to chelation of Fe^{3+} can be ruled out by the failure of desferrioxamine to mimic the action of the other chelators. DTPA, like desferrioxamine, binds Fe^{3+} strongly, but, unlike desferrioxamine, it chelates calcium with an affinity comparable to that of the other chelators which inactivate the transferrin receptor [18]. Desferrioxamine has a low affinity for Ca^{2+} [19]. Hence, the most likely explanation for the observed effects is the chelation of Ca^{2+} and its removal from the cell membrane at a site on or in close proximity to the transferrin receptor. This im-

plies a role for Ca^{2+} in the maintenance of the integrity of the receptor. It is unlikely that the chelator-induced inactivation of the receptor is due to an involvement of Ca^{2+} in the transferrin-receptor binding reaction because the chelators were removed by washing the cells before transferrin uptake was measured, the replacement of Ca^{2+} had no effect, and because the addition of EDTA at the time of measuring transferrin and iron uptake had little effect on the processes [20].

The inactivation process involved a progressive irreversible reduction in the number of functional receptors. The remaining unaffected receptors retained their normal affinity for transferrin and ability to undergo endocytosis. The time- and temperature-dependence of receptor loss suggested that the inactivation process occurred after their externalization by exocytosis to the outer cell membrane. This is probably a consequence of the fact that the chelators cannot penetrate the cell membrane. However, exocytosis is not the only factor involved because inactivation was not apparent until some minutes after externalization of transferrin and, presumably, of the receptors had occurred (Fig. 2). Also, inactivation was completely inhibited when the temperature was lowered to 20°C, but exocytosis still proceeded at this temperature (Fig. 2). The discontinuity in the change in the degree of inactivation with lowering of incubation temperature which occurred at about 20°C (Fig. 1) is suggestive of an effect due to phase transition or separation within the membrane lipids [21,22]. It is known that changes from the liquid-crystal to the gel phase occur in erythrocyte membranes at about 20°C and these affect various membrane functions, such as viscosity, enzyme activities and transport kinetics [21,23]. Chelator-induced transferrin receptor inactivation is possibly another such property. This implies that the state of the reticulocyte membrane lipids, as well as calcium ions, affects the properties of the receptor.

The inactivation of receptor function was also markedly reduced by diferric transferrin (Fig. 6), as has previously been observed with DTPA [8]. The presence of transferrin on the receptor may block the access of the chelator to its site of action or produce a conformational change in the receptor which prevents the release of Ca^{2+} from a critical site on the receptor or its close environment in the cell membrane. Apotransferrin produced a smaller degree of protection than did diferric transferrin, possibly due to its lower affinity for the receptor than the iron-containing molecule [24]. It is unlikely that the amount of protection afforded by apotransferrin was due to the presence of a small amount of contaminating iron-transferrin because the protection was still observed when the incubation with EDTA was performed in the presence of desferrioxamine as well as apotransferrin. The cells would have quickly assimilated any iron present on the transferrin and the desferrioxamine would have prevented it acquiring iron from the incubation medium.

The experiments with ^{125}I-labelled transferrin receptor antibodies (Table I) and the electrophoretic investigations (Fig. 8) showed that the functional inactivation of the receptor produced by the chelators was not due to the loss of the receptors from the cells. Rather, it was associated with a loss of their solubility in 1% Triton X-100 and retention in the triton-insoluble cytoskeletal matrix of the cells. Extraction of cells and cell membranes with Triton X-100 has been used to separate soluble components from the cytoskeleton in erythrocytes [25,26], platelets [27–30] and many other types of cells [31–34]. Hence, treatment of reticulocytes with EDTA, EGTA and other chelators with a high affinity for Ca^{2+} appears to lead to an altered interaction of the transferrin receptor with the cytoskeleton and a concomitant irreversible loss of its ability to bind transferrin.

The behaviour of the transferrin receptor which was observed in the present experiments in some respects resembles that of the platelet glycoproteins IIb and IIIa which together form the fibrinogen receptor on the platelet plasma membrane and play an essential role in the aggregation of activated platelets. Like the transferrin receptor they are transmembrane glycoproteins [35], their function is affected by treatment of the cells with EDTA and they show a variable association with the platelet cytoskeleton. Platelets deprived of Ca^{2+} by incubation with EDTA at 37°C but not at 20°C irreversibly lose their ability to bind fibrinogen and to aggregate after activation with ADP [36,37]. Proteins IIb and IIIa are not associated with the Triton X-100 cytoskeletal residue of inactivated platelets but sediment with the cytoskeleton upon centrifugation after aggregation has occurred [27,29]. However, unlike the transferrin receptor, there is no evidence that treatment of platelets with EDTA changes the interaction of the platelet proteins with the cytoskeleton.

Acknowledgements

This work was supported by grants from the Australian Research Grants Scheme. The skilled technical assistance of Miss D. Hebbert and Miss B.J. Bowen is gratefully acknowledged.

References

1 Morgan, E.H. and Appleton, T.C. (1969) Nature 223, 1371–1372.
2 Sullivan, A.L., Grasso, J.A. and Weintraub, L.R. (1976) Blood 47, 133–143.
3 Hemmaplardh, D. and Morgan, E.H. (1977) Br. J. Haematol. 36, 85–96.
4 Morgan, E.H. (1981) Mol. Asp. Med. 4, 1–122.
5 Newman, R., Schneider, C., Sutherland, R., Vodinelich, L. and Greaves, M. (1982) Trends Biochem. Sci. 7, 397–400.

6 Trowbridge, I.S., Newman, R.A., Domingo, D.L. and Sauvage, C. (1984) Biochem. Pharmacol. 33, 925–940.
7 Hemmaplardh, D. and Morgan, E.H. (1977) Biochim. Biophys. Acta 468, 423–436.
8 Nunez, M.-T., Cole, E.S. and Glass, J. (1983) in Structure and Function of Iron Storage and Transport Proteins (Urushizaki, I., Aisen, P., Listowsky, I. and Drysdale, J.W., eds.), pp. 375–378, Elsevier, Amsterdam.
9 Hemmaplardh, D. and Morgan, E.H. (1974) Biochim. Biophys. Acta 373, 84–99.
10 Lim, B.C. and Morgan, E.H. (1984) J. Comp. Physiol. B. 155, 201–210.
11 Bowen, B.J. and Morgan, E.H. (1987) Blood 70, 38–44.
12 McFarlane, A.S. (1963) J. Clin. Invest. 42, 346–361.
13 Iacopetta, B.J. and Morgan, E.H. (1983) J. Biol. Chem. 258, 9108–9115.
14 Bowen, B.J. and Morgan, E.H. (1988) J. Cell. Physiol. 134, 1–12.
15 Iacopetta, B.J., Morgan, E.H. and Yeoh, G.C.T. (1983) J. Histochem. Cytochem. 31, 336–344.
16 Hanks, J.H. and Wallace, R.E. (1949) Proc. Soc. Exp. Biol. Med. 71, 196–200.
17 Tsien, R.Y. (1980) Biochemistry 19, 2396–2404.
18 Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1986) Data for Biochemical Research, 3rd Edn., Oxford University Press, Oxford.
19 Martell, A.E. and Smith, R.M. (1977) Critical Stability Constants Vol. 3, Plenum, New York.
20 Morgan, E.H. (1971) Biochim. Biophys. Acta 244, 103–116.
21 Zimmer, G. and Schirmer, H. (1974) Biochim. Biophys. Acta 345, 314–320.
22 Petit, V.A. and Edidin, M. (1974) Science 184, 1183–1185.
23 Fourcans, B. and Jain, M.K. (1974) Adv. Lipid Res. 12, 147–226.
24 Kornfeld, S. (1969) Biochim. Biophys. Acta 194, 25–33.
25 Yu, J., Fischman, D.A. and Steck, T.L. (1973) J. Supramol. Struct. 1, 233–248.
26 Burns, N.R. and Gratzer, W.B. (1985) Biochemistry 24, 3070–3074.
27 Phillips, D.R., Jennings, L.K. and Edwards, H.H. (1980) J. Cell Biol. 86, 77–96.
28 Painter, R.G. and Ginsberg, M. (1982) J. Cell Biol. 92, 565–573.
29 Rotman, A., Heldman, J. and Linder, S. (1982) Biochemistry 21, 1713–1719.
30 Zucker, M.B., Varon, D., Masiello, N.C. and Karpatkin, S. (1983) Thromb. Haemostas. 50, 848–851.
31 Ben-Ze'ev, A., Duerr, A., Solomon, F. and Penman, S. (1979) Cell 17, 859–865.
33 Braun, J., Hochman, P.S. and Unanue, E.R. (1982) J. Immunol. 128, 1198–1204.
34 Landreth, G.E., Williams, L.K. and Rieser, G.D (1985) J. Cell Biol. 101, 1341–1350.
35 Phillips, D.R. and Agin, P.P. (1974) Biochim. Biophys. Acta 352, 218–227.
36 Zueker, M.B. and Grant, R.A. (1978) Blood 52, 505–513.
37 Pidard, D., Didry, D., Kunicki, T.J. and Nurden, A.T. (1986) Blood 67, 604–611.