Calmodulin Antagonists Inhibit and Phorbol Esters Enhance Transferrin Endocytosis and Iron Uptake by Immature Erythroid Cells

By Darcy Hebbert and Evan H. Morgan

Seven antagonists of the calcium-binding protein calmodulin were found to inhibit iron and transferrin uptake by reticulocytes. This inhibition could be completely accounted for by inhibition of the endocytosis and exocytosis of transferrin. When four of the antagonists were tested with the nucleated erythroid cells from the liver of the fetal rat, inhibition of iron uptake was also observed but at higher concentrations than required for the same degree of inhibition with reticulocytes. The tumor promoters phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PDB) were shown to increase the rates of iron and transferrin uptake by reticulocytes and fetal liver erythroid cells by accelerating the rates of transferrin endocytosis and exocytosis. Since these substances are known to stimulate the calcium-activated enzyme protein kinase C while calmodulin antagonists are inhibitory, it is concluded that this enzyme plays an important role in the endocytosis and intracellular cycling of transferrin, and iron uptake by immature erythroid cells. However, the possibilities that calmodulin is also involved or that the inhibitory effects of the calmodulin antagonists are due to nonspecific actions on the cell membrane cannot be excluded.

THE UPTAKE of transferrin-bound iron by immature erythroid cells occurs by receptor-mediated endocytosis of the iron-transferrin complex, followed by release of the iron from the transferrin and recycling of the receptors and apotransferrin to the cell surface. The cellular mechanisms involved in these processes are poorly understood, but they must include those responsible for coated pit formation and the membrane fusion reactions that occur during endocytosis and exocytosis. Membrane fusion is believed to require calcium ions and lateral mobility of membrane proteins. In erythroid cells, such movement of membrane proteins is restricted by their interaction with elements of the cytoskeleton. Release from this restraint may be necessary before endocytosis can occur.

There are several reasons for considering the possibility that the calcium-binding protein, calmodulin, may be involved in transferrin endocytosis and iron uptake by erythroid cells. Calmodulin binds to coated vesicles and secretory vesicles and has been implicated in endocytosis and exocytosis in nonerythroid cells. In the case of erythroid cells, the concentration of calmodulin is considerably greater in reticulocytes than in mature erythrocytes. Only immature erythroid cells (reticulocytes and their nucleated precursors) normally show endocytotic activity and can take up transferrin-bound iron. Calmodulin binds to spectrin, a major component of the erythrocyte cytoskeleton. Also, a large number of reagents that possess inhibitory activity against calmodulin produce changes in red cell shape. Hence, it is possible that calmodulin modulates the functional activity of the cytoskeleton of immature erythroid cells. Such modulation may be required for the endocytosis of transferrin and iron uptake.

The aim of the present study was to investigate the above possibility by the use of several reagents that have been shown to antagonize calmodulin-mediated processes in other cellular systems. These reagents will be referred to as calmodulin antagonists. None of them are entirely specific for calmodulin, however, and several have been shown to also inhibit the calcium-activated, phospholipid-dependent protein kinase, protein kinase C, which is present in many, if not all, types of cells. This enzyme is not influenced by calmodulin but is activated by phorbol esters, such as phorbol 12β-myristate 13α-acetate (PMA), which are potent tumor promoters. Hence, additional studies were performed to determine whether transferrin and iron uptake are affected by such compounds. The results show that calmodulin antagonists do inhibit transferrin endocytosis and that phorbol esters accelerate this process. Hence, either or both of calmodulin and protein kinase C may be involved.

MATERIALS AND METHODS

Calmidozolium, penfluridol, and pimozide were obtained from Jansen Pharmaceutica (Beerse, Belgium). The other biochemical reagents were from Sigma Chemical Co (St Louis). Rat and rabbit...
transferrins were isolated from plasma and labeled with \(^{59}\text{Fe}\) and \(^{125}\text{I}\) as described previously.\(^{16,17}\)

**Cell Preparation**

Reticulocytes were obtained from rats and rabbits with phenylhydrazine-induced hemolytic anemia.\(^{17,18}\) Erythroid cells were also obtained from the livers of fetal rats on the 15th day of gestation.\(^{18}\) At this stage of fetal development, the cell preparation consists of approximately 5% pronormoblasts, 20% basophilic normoblasts, 55% polychromatophilic normoblasts, and 10% reticulocytes and mature erythrocytes.\(^{19}\) There are only about 2% hepatocytes in the final cell suspension. The cells were washed three times in Hanks and Wallace balanced salt solution and suspended in this solution at a hematocrit of 10% to 15% for the transferrin and iron uptake experiments.

**Incubation Procedures**

The cell suspensions were incubated at 37 °C with 3 \(\mu\text{mol/L}\) diferric transferrin labeled with \(^{59}\text{Fe}\) and \(^{125}\text{I}\). This concentration of transferrin is sufficient to saturate the transferrin receptors and give maximal rates of iron uptake by rabbit and rat reticulocytes and rat fetal liver cells.\(^{16,18,19}\) For the measurement of total uptake of transferrin and iron, 150-\(\mu\text{L}\) aliquots of the incubation suspension were removed after measured periods of incubation at 37 °C and placed on top of 170-\(\mu\text{L}\) dibutyl phthalate in a microfuge tube, which was immediately centrifuged for 30 seconds in a Beckman microfuge. The tip of the tube, containing the cell pellet, was cut off with a razor blade and counted for radioactivity. When measurements of transferrin endocytosis were to be made, the cells were incubated with the labeled transferrin at 37 °C. Then 200-\(\mu\text{L}\) aliquots of the suspension were removed at the desired incubation times, washed three times by suspension followed by centrifugation at 4 °C in 5-\(\text{mL}\) ice-cold phosphate-buffered saline, and then incubated with 400 \(\mu\text{L}\) 0.2% Pronase for 20 minutes at 4 °C in order to distinguish between internalized (endocytosed) and surface-bound transferrin.\(^{16}\) A sample of the suspension was then centrifuged through dibutyl phthalate in the microfuge as above. After slicing the tube, both cell and supernatant fractions were counted for radioactivity to give measurements of endocytosed and surface-bound transferrin.

For the measurements of transferrin exocytosis, the cells were first incubated with labeled transferrin for 30 minutes at 37 °C, washed three times in ice-cold phosphate-buffered saline, and then reincubated in Hanks and Wallace solution containing nonradioactive diferric transferrin (1 mg/mL) at 37 °C. After a given period of reincubation, 200-\(\mu\text{L}\) aliquots were transferred to 2-\(\text{mL}\) ice-cold phosphate-buffered saline containing 0.2% Pronase, mixed, incubated at 4 °C for 20 minutes, centrifuged at 1,500 \(\text{g}\) for five minutes at 4 °C, supernatant aspirated, and radioactivity counted in the cell fraction. The decrease in \(^{125}\text{I}\) radioactivity in this fraction, expressed as a percentage of that present before reincubation of the labeled cells, is a measure of the rate of exocytosis of transferrin.\(^{18}\)

The majority of the experiments were performed with reticulocytes. The cellular uptake of transferrin and iron from \(^{125}\text{I}\) transectin were calculated from the observed counts, the specific activity of the labeled transferrin, the percentage of reticulocytes present in the sample, and the hematocrit of the cell suspension. The results were expressed as nmol/mL reticulocytes, since mature erythrocytes do not take up transferrin or iron.\(^{11,19}\) The rate of uptake of iron was calculated from the slope of the iron uptake curve over the total period of incubation, while the rate of transferrin uptake was determined from the initial slope of the transferrin uptake curve, during the first two minutes of incubation. The reticulocyte count in the different experiments varied from 27% to 66%. This variation would be associated with differences in the average degree of maturity of the reticulocytes, which can explain the differences in transferrin and iron uptake found in the various experiments (compare Figs 1, 2, 4, and 5).\(^{19}\) However, the experiments performed on any one day were with one population of cells, and the results obtained with the inhibitors were compared with controls studied on that day as part of the same experiment. Each type of experiment was repeated several times, with results consistent with those of the experiments illustrated in the figures.

Transferrin and iron uptake by fetal liver erythroid cells were calculated in terms of nmol/mL cells, based on the radioactivity measurements and the hematocrit of the cell suspension.

The calmodulin antagonists and phorbol esters were dissolved in dimethyl sulfoxide at 100 to 500 times the desired final concentrations. The cell suspensions were incubated with aliquots of these solutions or the same vol of dimethylsulphoxide (controls) for ten minutes at 37 °C before the addition of the radiolabeled transferrin.

**Analytical Methods**

Reticulocytes were stained with new methylene blue and counted on dried smears. Radioactivity was counted in a well-type scintillation detector. Cellular ATP levels were measured by an enzymatic procedure.\(^{20}\)

**RESULTS**

The initial experiments were performed with trifluoperazine, one of the most widely used calmodulin antagonists. It inhibited the uptake of both iron and transferrin by reticulocytes in a dose-dependent manner (Fig 1). As is usually observed in this type of incubation, iron uptake by the control was linear throughout the 30-minute incubation period, while the uptake of \(^{125}\text{I}\)-transferrin occurred in three phases: an initial rapid binding immediately after mixing transferrin and the cells, a phase of progressive uptake during the first five to ten minutes of incubation at 37 °C, and a plateau level of uptake after that time. By then, a steady state had been reached, when the uptake and release of \(^{125}\text{I}\)-transferrin occurred at the same rate. The trifluoperazine reduced the rate of progressive uptake of transferrin but had little effect on the initial binding of transferrin by the cells. This suggests that its action is achieved by inhibition of

![Effect of trifluoperazine on iron (A) and transferrin uptake (B) by rabbit reticulocytes: ■ control; □, 50 \(\mu\text{mol/L}\) trifluoperazine; △, 75 \(\mu\text{mol/L}\) trifluoperazine; A, 100 \(\mu\text{mol/L}\) trifluoperazine.](image)
transferrin endocytosis, which is the process responsible for the phase of progressive uptake. The lack of an effect on the initial binding indicates that the inhibitor does not interfere with the interaction between transferrin and its receptors on the cell membrane.

Several other calmodulin antagonists were tested in addition to trifluoperazine. They were all found to have similar effects to those illustrated in Fig 1 but produced their inhibition at different concentration levels (Table 1).

Measurements of transferrin endocytosis and exocytosis showed that the antagonists inhibited both of these processes. Figure 2 illustrates the results obtained with trifluoperazine. Comparable results were observed with all of the antagonists listed in Table 1. The degree of inhibition of the rate of endocytosis, as measured by the initial rate of progressive uptake of transferrin, varied with the concentration and type of antagonists used, but in all cases it was proportional to the degree of inhibition of the rate of iron uptake (Fig 3). These results showed that the inhibition of iron uptake produced by the calmodulin antagonists is fully accounted for by inhibition of transferrin endocytosis and is not dependent on an effect on transferrin-receptor interaction or on the mechanism of iron release from transferrin.

The concentration dependence of the effects of the antagonists was determined by measuring iron uptake by rabbit reticulocytes during a 20-minute period of incubation. The results, expressed as the concentration that produced 50% inhibition (IC50) of iron uptake, are summarized in Table 1. Certain of the antagonists were also tested with rat reticulocytes and rat fetal liver erythroid cells. Rabbit and rat reticulocytes showed approximately equal sensitivity to the reagents, but higher concentrations were required to produce the same degree of inhibition of iron uptake by the fetal erythroid cells as by reticulocytes (Table 1).

The rate of transferrin endocytosis and iron uptake is reduced by inhibition of cellular energy metabolism. Hence, it is possible that the effects reported above could be due to interference with cell metabolism. To test this, ATP levels were measured in rabbit reticulocytes that had been incubated with 50 μmol/L trifluoperazine or 50 μmol/L penfluridol for five to 30 minutes at 37 °C. No reduction in ATP was found with penfluridol, and only 22% reduction was found after 30 minutes of incubation with trifluoperazine. This is insufficient to cause any more than a small fraction of the degree of inhibition of transferrin and iron uptake that was observed.21

By contrast to the action of the calmodulin antagonists, the tumor promoter, PMA, accelerated the rates of uptake of iron and transferrin by rabbit reticulocytes (Fig 4). This was associated with increased rates of transferrin endocytosis and exocytosis (Fig 5).

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**Table 1. Concentrations of Calmodulin Antagonists Required to Produce 50% Inhibition (IC50) of the Rate of Iron Uptake by Immature Erythroid Cells**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Rabbit Reticulocytes IC50 (μmol/L)</th>
<th>Rat Reticulocytes IC50</th>
<th>Rat Fetal Liver Erythroid Cells IC50 (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calmodozolium</td>
<td>*</td>
<td>17</td>
<td>35</td>
</tr>
<tr>
<td>Penfluridol</td>
<td>28</td>
<td>23</td>
<td>50</td>
</tr>
<tr>
<td>Trifluoperazine</td>
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<td>35</td>
<td>64</td>
</tr>
<tr>
<td>Pimozide</td>
<td>65</td>
<td>57</td>
<td>92</td>
</tr>
<tr>
<td>Promethazine</td>
<td>100</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>112</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Imipramine</td>
<td>102</td>
<td>—</td>
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*Hemolysis occurred at low concentrations, so that IC50 could not be measured.

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**Fig 2. Effect of trifluoperazine (40 μmol/L) on iron uptake (A), transferrin endocytosis (B), and transferrin exocytosis (C) by rabbit reticulocytes. Intracellular (---) and membrane-bound (-----) transferrin and iron in control cells (●, △) and cells treated with trifluoperazine (○) are shown. There were no differences in the values for membrane-bound transferrin and iron between the control and treated cells.**

**Fig 3. Relationship between rates of iron and transferrin uptake by reticulocytes treated with varying concentrations of the calmodulin antagonists, penfluridol (■), trifluoperazine (▲), pimozide (□), promethazine (○), imipramine (●), and chlorpromazine (●). The rates of iron and transferrin uptake were expressed as the percentage of the value found in control cells incubated in the absence of the antagonists. The equation of the regression line is y = -3.35 + 0.98 x (r = .977).**
Although the effects were relatively small (Table 2), they were highly significant, and increases in the rates of iron and transferrin uptake of from 1% to 42% were observed in every one of 24 separate incubations of reticulocytes treated with PMA concentrations varying from 1 nmol/L to 1 μmol/L. In each case, the degree of change in the rate of iron uptake was similar to that in the rate of transferrin uptake. Hence, the effect on iron uptake could be attributed to an alteration in the rate of transferrin endocytosis.

One experiment was performed with rat fetal liver erythroid cells treated with PMA (1 to 100 nmol/L). Iron and transferrin uptake were accelerated to approximately the same degree as with reticulocytes (Table 2).

Another tumor promoter, phorbol 12β-13α-dibutyrate (PDB) was also tested with rabbit reticulocytes. It was found to produce 4% to 20% increase in the rates of iron and transferrin uptake when used at concentrations of 1 nmol/L to 1 μmol/L (Table 2).

**DISCUSSION**

The calmodulin antagonists used in this work inhibited iron uptake by blocking transferrin endocytosis. This was demonstrated by direct measurements of the rates of endocytosis and exocytosis (Fig 2) and by the close correlation found between the rates of iron and transferrin uptake by reticulocytes treated with varying concentrations of the drugs (Fig 3). Hence, any other mechanisms of action on iron uptake must have been quantitatively unimportant. Such mechanisms include neutralization of intravesicular pH due to the basic properties of the drugs, inhibition of cell metabolism, and interference with transferrin-receptor interaction. The latter two explanations may also be ruled out by the results of measurement of cellular ATP levels and because the binding of transferrin to outer cell membranes was not affected by the antagonists (Fig 2).

One explanation of the present results is that calmodulin has an essential role in transferrin endocytosis and exocytosis in immature erythroid cells. This is supported by the fact that the order of sensitivity of reticulocyte iron uptake to the various drugs used in this work (Table 1) is similar to that reported for calmodulin-dependent enzymes (summarized in a previous report14). Furthermore, the IC50 values for iron uptake are comparable to those for the inhibition of endocytosis or exocytosis in other cellular systems that were considered to be calmodulin-dependent processes.7,9-11 The nature of the role of calmodulin in transferrin endocytosis is unknown, but possibilities include alterations in intracellular calcium metabolism by interaction with the Ca2+-ATPase, involvement in coated-pit formation or membrane fusion reactions, and modulation of the function of the cytoskeleton. The latter may be required to increase the mobility of certain membrane proteins so that membrane fusion can occur. One interesting feature of the results is the lower sensitivity of fetal liver erythroid cells than reticulocytes to inhibition of iron uptake by the calmodulin antagonists. This could be because fetal cells
require a lower minimal level of calmodulin activity for endocytosis to occur, possibly due to the degree of interaction between cytoskeletal proteins and membrane proteins being less in the more immature cells obtained from the fetal liver than in reticulocytes.

At least two other explanations for the inhibitory effects of calmodulin antagonists on iron uptake must be considered: (1) a nonspecific effect on the cell membrane, possibly related to the ability of the reagents to stabilize the red cell membrane against osmotic lysis and (2) inhibition of protein kinase C. The first of these explanations cannot be completely excluded because of the known significant correlation between the relative potencies of these drugs as calmodulin inhibitors and as membrane-stabilizing agents. They are all hydrophobic molecules that may react in a relatively nonspecific manner with the lipid bilayer of cell membranes or with hydrophobic regions of proteins. Such interactions are probably the basis of their ability to inhibit calmodulin and protein kinase C, and may be responsible for their action on membrane stability. However, Brewer, and Nelson et al. have argued that the latter effects are due to interference with the role of calmodulin as a modulator of cytoskeletal function rather than to a direct interaction with the lipid bilayer of the cell membrane. Their arguments, although based on several experimental observations, are not yet conclusive, and the question of whether calmodulin is involved in transferrin endocytosis must remain unresolved until a precise role for calmodulin in this process has been determined.

Trifluoperazine, chlorpromazine, imipramine, and calmidazolium have all been shown to inhibit protein kinase C, while PMA and PDB stimulate the enzyme. Hence, the present results suggest that protein kinase C plays an important role in transferrin endocytosis and iron uptake by erythroid cells and that inhibition of the enzyme may be the mechanism by which calmodulin antagonists block iron uptake. Protein kinase C has been implicated in endocytosis and exocytosis in nonerythroid cells, the phosphorylation of insulin and epidermal growth factor receptors, a cytoskeletal protein (vinculin), and an insulin secretory granule membrane protein. Moreover, PDB and PMA have recently been shown to induce transferrin-receptor phosphorylation and internalization in two transformed human cell lines (HL60 cells, a promyelocytic leukemic cell line, and K562 cells). In the latter case, it was reported that the internalization of surface membrane receptors was not associated with changes in the rates of recycling of transferrin or iron uptake by the cells. Undoubtedly, there are many differences in the metabolism of transferrin and iron by transformed cells and by normal erythroid cells as used in the present work. One of these differences appears to be the effect of phorbol esters on the rate of transferrin endocytosis and recycling. However, the results with the transformed cells adds credence to the hypothesis that phosphorylation of the transferrin receptor is involved in the process of transferrin endocytosis in immature erythroid cells and that phorbol esters accelerated endocytosis by increasing the activity of protein kinase C. Alternatively, it is possible that the phorbol esters exert their action on iron uptake by altering the function of the cytoskeleton or of nonreceptor membrane proteins that have a role in endocytosis, probably through phosphorylation reactions catalysed by protein kinase C.

Certain other experimental results are of interest in the context of the present work. First, the microtubule inhibitor, vinblastine, has been shown to inhibit transferrin and iron uptake by reticulocytes and more recently to inhibit calmodulin activity. Possibly its mode of action on iron uptake is through the latter effect (or even by inhibition of protein kinase C rather than by inhibition of microtubule function, as was proposed earlier). Second, only three types of stimuli have been shown to accelerate transferrin endocytosis and iron uptake by reticulocytes, namely, certain monoclonal antibodies to the transferrin receptor, inhibition of heme synthesis, and the phorbol esters. All three may act through a common mechanism, stimulation of protein C. If so, it would appear that interaction of an antibody with the transferrin receptor is able to stimulate the activity of the enzyme and free intracellular heme inhibits it. Exogenous hemin has been found to inhibit transferrin endocytosis, an observation that is in keeping with the latter suggestion and explains its well-established ability to block iron uptake.

The effects on iron uptake by both types of drugs used in the present work (calmodulin antagonists and phorbol esters) may be explained by a common mechanism acting via protein kinase C. However, the possibility that calmodulin or nonspecific effects on the cell membrane are also involved cannot be excluded. Further studies on the functions of these two calcium-binding proteins and of Ca<sup>2+</sup> itself are required to resolve this question and should provide valuable information on receptor function, endocytosis, and the mechanism of iron uptake by cells.

REFERENCES
2. Iacopetta BJ, Morgan EH, Yeoh GCT: Receptor-mediated endocytosis of transferrin by developing erythroid cells from the fetal rat liver. J Histochem Cytochem 31:336, 1983
38. Klausner RD, Harford J, van Renswoude J: Rapid internalization of the transferrin receptor in K562 cells is triggered by ligand binding or treatment with a phorbol ester. Proc Natl Acad Sci USA 81:3005, 1984
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