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Mediation of Iron Uptake and Release in Erythroid Cells by Photodegradation Products of Nifedipine

*Donna L. Savigni and Evan H. Morgan**

DEPARTMENT OF PHYSIOLOGY, THE UNIVERSITY OF WESTERN AUSTRALIA, NEDLANDS, PERTH, WESTERN AUSTRALIA 6907, AUSTRALIA

ABSTRACT. The effects of five Ca^{2+} channel antagonists on iron uptake by erythroid cells were investigated using rabbit reticulocytes and erythrocytes, and transferrin-bound iron and non-transferrin-bound iron (Fe(II)). All of the antagonists except nifedipine inhibited iron uptake, but only at relatively high concentrations (10–100 μM). Nifedipine markedly stimulated the uptake of Fe(II) but not transferrin-bound iron, but only after it had been photodegraded to its nitrosophenylpyridine derivative. This compound was found to mediate Fe(II) exchange between the cytosol and extracellular medium in both directions with both reticulocytes and erythrocytes, but not by the known iron transport processes. The effect could be reversed by washing the cells with ice-cold NaCl solution. It appeared to be relatively specific for Fe(II) since photodegraded nifedipine had little effect on the uptake of Fe(III) or Mn^{2+}. It is suggested that the nitrosopyridine derivative of nifedipine can act as an Fe(II) ionophore and may be of use as an adjuvant in chelator therapy with desferrioxamine in conditions of iron overload. BIOCHEM PHARMACOL 51;12:1701–1709, 1996.

KEY WORDS. iron transport; erythroid cells; Ca^{2+}antagonists; photodegraded nifedipine; nitrosophenyl pyridine

Iron uptake by erythroid cells can occur by at least three saturable transport mechanisms, one involving transferrin-bound iron and the other two, non-transferrin-bound Fe(II) [1–3]. The uptake of transferrin-bound iron is dependent on transferrin receptor-mediated endocytosis, and in erythroid cells it occurs only in immature cells such as the erythroblasts of the bone marrow or circulating reticulocytes [1]. During this process, the iron is released from transferrin within endosomes and then passes through the lining membrane of these organelles into the cytosol. The uptake of non-transferrin-bound iron may occur by a high and low affinity mechanism of iron transport across the plasma membrane. The high affinity mechanism has a low K_m (about 0.2 mM in rabbit reticulocytes) and, like the receptor-mediated process, occurs only in immature erythroid cells. It can be demonstrated most clearly by incubation of the cells with ^{59}Fe-labelled Fe(II) in media of low ionic strength, such as isotonic sucrose [2]. The low affinity process has a higher K_m (15 μM in rabbit cells) and is present in both mature and immature erythroid cells, although it is more active in reticulocytes than in mature erythrocytes. This process is most active in Na^+-free electrolyte media such as isotonic KCl or RbCl, and, like high-affinity transport, is inhibited by NaCl [3].

Calcium affects iron uptake by erythroid cells in a variety of ways. Depletion or increases of cellular Ca^{2+} both suppress the uptake of transferrin-bound iron by inhibition of receptor-mediated endocytosis of transferrin [4, 5]. Ca^{2+} also inhibits iron uptake by both of the non-transferrin-bound iron transport mechanisms ([2] and unpublished observations). Moreover, Fe(II) can block voltage-gated Ca^{2+} currents [6]. Hence, the question was asked whether either of the non-transferrin-bound iron transport pathways involves Ca^{2+} channels and, if so, whether Fe(II) transport is inhibited by Ca^{2+} channel antagonists. Although these drugs are best known for their effects on voltage-gated L-type Ca^{2+} channels [7], they can also inhibit other types of Ca^{2+} channels as well as the membrane transport of other substances, such as nucleosides, in erythrocytes [8, 9]. Five calcium channel antagonists were studied. With the exception of nifedipine, they all inhibited iron uptake, but only at relatively high concentrations. Nifedipine, by contrast, accentuated the uptake of non-transferrin-bound but not transferrin-bound iron even at low concentrations. The mechanism of this effect was investigated in some detail and was shown to be dependent on photodegradation of nifedipine.

MATERIALS AND METHODS

Materials

Iron-59 ($FeCl_3$) and ^{54}Mn ($MnCl_2$) were purchased from Amersham International (Amersham, U.K.). Rabbit transferrin was isolated from plasma and labelled with ^{59}Fe as in

* Corresponding author. Tel. 61-9-380-3320; FAX 61-9-380-1025.
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earlier studies [10]. The Ca^{2+} antagonists were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). They were dissolved in DMSO at 200 times the desired final concentrations and were stored at −20°.

Cells

Reticulocyte-rich blood was obtained from rabbits with phenylhydrazine-induced haemolytic anaemia [10] 4–6 days after the last injection of phenylhydrazine. Blood with a low reticulocyte count was obtained from untreated rabbits. The cells from the blood samples were washed and centrifuged as described previously [10]. The cells from anaemic rabbits contained 40–70% reticulocytes and those from untreated rabbits, 1–3% reticulocytes. For brevity, they will be referred to as reticulocytes and erythrocytes, respectively.

Iron Solutions

The radioactive iron solutions used in most experiments were either ^{125}I-^{59}Fe-labeled differic rabbit transferrin [10] or Fe(II)–sucrose, prepared by mixing $^{59}FeCl_3$ and $^{56}FeSO_4$ in a ratio of approximately 1:100 and diluting with 0.27 M sucrose to the desired concentration. The iron–sucrose solution was prepared just before commencing the cell incubations and was used between 5 and 15 min after preparation. As shown by its reaction with α,α′-bipyridine, the iron remains in the ferrous state during this time and the subsequent period of incubation with the cells. Also, due to the very rapid rate of self-exchange of Fe(II) and Fe(III) that occurs in aqueous solutions, all of the iron would be uniformly labelled with ^{59}Fe by the time the incubations were begun. For one set of experiments $^{56}FeCl_3$ was substituted for $^{56}FeSO_4$ in this solution so that the iron was present as Fe(III). It was used either in this form or after addition of 100-fold molar excess of sodium citrate.

Measurement of Iron Uptake

Samples of the cell mixtures (usually 0.1 mL at a packed cell volume of 30–40%) were suspended in 1.9 mL of the incubation media buffered to the desired pH with 4 mM HEPES. The media were isotonic (290–300 Osmol/kg) solutions of sucrose, NaCl, or KCl. Ten microliters DMSO (controls) or the calcium antagonists in 10 μL DMSO were then added to the cell suspensions which were mixed and allowed to stand for 10 min at room temperature. After this, 0.1 mL of ^{59}Fe-labelled solutions of Fe_2-transferrin or Fe(II) or, in one set of experiments, Fe(III), was added, mixed with the cells, and incubated in an oscillating water bath at 37°. The incubation time usually was 15 min, but this was changed in a few experiments as indicated below. After incubation the reaction was stopped by the addition of 4 mL of ice-cold 5 mM EDTA–0.15 M NaCl (pH 7.4), and the cells were washed a further three times in ice-cold 0.155 M NaCl, haemolysed, and separated into cytosolic and stromal fractions by centrifugation as described previously [2]. Radioactivity was counted in these fractions. All of the experiments were performed at least three times, with results similar to those described below.

Analytical Methods

The reticulocyte count was determined by staining with new methylene blue and the packed cell volume by the microhaematocrit method. Haem was extracted by the method of Thunell [11]. Radioactivity was measured in a 3-channel γ-scintillation counter (LKB-Wallac 1282 Compu-gamma). Thin-layer chromatography of nifedipine and its photodegradation products was performed on Merck silica gel 60 F_{254} HPTLC aluminum sheets (*art.* 55600) using petroleum ether:chloroform:acetone (50:30:20), and on a preparative scale by flash chromatography on silica gel using petrol:ethyl acetate (80:20). The active product of nifedipine photodegradation was isolated by the latter technique and examined by mass spectroscopy and NMR spectroscopy. The electron impact mass spectrum was recorded on a Hewlett–Packard model 5986 GC/MC system at 70 eV. The proton NMR spectrum was measured at 200 MHz with $CDCl_3$ as the solvent on a Varian Gemini 200 NMR spectrometer.

RESULTS

The effects of the calcium antagonists on iron uptake were determined using three incubation media that were designed to study uptake of transferrin-bound iron by receptor-mediated endocytosis and of Fe(II) by the high and low affinity transport mechanisms. These were NaCl (pH 7.4), sucrose (pH 6.5), and KCl (pH 7.0), respectively. Reticulocytes were examined using all three media and erythrocytes with the third medium only, since these cells take up negligible amounts of iron in the other two media [2, 3]. With one exception the calcium antagonists inhibited iron uptake in all three incubation systems (Table 1). However, the sensitivity to inhibition was relatively low, marked inhibition occurring only at drug concentrations of 10–100 μM. Also, there was little difference in the sensitivity of the different systems to the inhibitors. A calcium channel agonist, Bay K 8644 (ICN Pharmaceuticals, Costa Mesa, CA, U.S.A.), was also found to have no significant effect on iron uptake at concentrations of 0.1–10 μM, but to produce 25–60% inhibition at 100 μM (data not shown).

The exception to the above pattern of results was obtained with nifedipine. This reagent had little effect on iron uptake from transferrin but markedly stimulated iron uptake in the other incubation systems, even at a concentration as low as 1 μM. This was an unexpected result that was investigated in a series of experiments aimed at determining the mechanism responsible for the increase in iron uptake. The effects of the other antagonists were considered to be of less interest and probably due to non-specific effects on the cell membranes, since they were apparent only at the higher concentrations of the drugs and did not differentiate among the three uptake mechanisms.

Investigation of the effects of nifedipine on iron uptake was performed using reticulocytes or erythrocytes incubated with Fe(II). First, the effects of the pH of the incubation solution were examined using cells incubated with 20 μM

TABLE 1. Effects of Ca^{2+} antagonists on iron uptake into the cytosol of reticulocytes and erythrocytes

Antagonist	Concentration (μmol/L)	Iron uptake (% of control)			
		Reticulocytes			Erythrocytes
		Tf-Fe	Fe(II)–sucrose	Fe(II)–KCl	Fe(II)-KCl
Nifedipine	1	102 ± 2.4	201 ± 37	129 ± 3.1	231 ± 31
	10	105 ± 1.9	260 ± 24	210 ± 4.1	424 ± 57
	100	89 ± 3.3	182 ± 21	287 ± 20	477 ± 33
Nicardipine	1	99 ± 1.5	101 ± 2.6	97 ± 3.2	98 ± 3.6
	10	88 ± 2.3	86 ± 2.1	74 ± 5.8	89 ± 3.2
	100	43 ± 2.2	36 ± 2.7	24 ± 6.9	29 ± 2.9
Diltiazem	1	100 ± 1.5	97 ± 2.1	105 ± 3.7	96 ± 3.9
	10	100 ± 1.7	98 ± 3.0	101 ± 4.5	95 ± 2.6
	100	95 ± 1.7	92 ± 4.9	84 ± 4.0	93 ± 3.2
Verapamil	1	97 ± 1.9	105 ± 4.5	101 ± 2.5	97 ± 2.9
	10	96 ± 2.5	90 ± 8.9	90 ± 4.0	96 ± 3.1
	100	77 ± 1.5	69 ± 5.7	40 ± 3.4	81 ± 2.9
Cinnazarine	1	99 ± 2.1	95 ± 6.1	94 ± 1.5	85 ± 4.1
	10	87 ± 4.1	62 ± 5.1	48 ± 2.5	39 ± 2.6
	100	13 ± 1.5	30 ± 2.3	24 ± 3.9	29 ± 4.5

Cells were incubated at 37° for 15 min with (1) 1 μM transferrin-bound Fe (Tf-Fe), in NaCl, pH 7.4, (2) 1 μM Fe(II) in sucrose, pH 7.0 (Fe(II)–sucrose), and (3) 20 μM Fe(II) in KCl, pH 7.0 (Fe(II)–KCl). The results are expressed as percentages of the values obtained in the absence of the antagonists (percent control) and are means ± SEM of 3–4 determinations. The control values for iron uptake were (1) 0.96 ± 0.12, (2) 1.30 ± 0.22, (3) 12.7 ± 0.57 (reticulocytes), and 9.3 ± 5.6 (erythrocytes) nmol/mL cells/min.

Fe(II) in isotonic sucrose, KCl, or NaCl. As shown in Fig. 1, iron uptake by reticulocytes in the absence of nifedipine was much greater in KCl than in NaCl, and was intermediate between these extremes in the sucrose medium. In KCl, the optimum pH was 7.0. In the presence of nifedipine, Fe(II) uptake from all three media was stimulated markedly and was maximal at about pH 8.0, although in KCl there was relatively little difference between pH 7.5 and 8.0. Also, at pH 8.0, uptake from KCl and NaCl solutions was almost the same. Similar results were obtained with erythrocytes (not shown). These results suggested that the effects of nifedipine were not due to stimulation of known mechanisms of Fe(II) transport since these mechanisms are maximal at pH 6.5 to 7.0 and are inhibited by NaCl [2, 3]. To test this conclusion, we determined the effects of various concentrations of nifedipine on Fe(II) uptake from isotonic KCl, pH 7.0, in the absence or the presence of amiloride and valinomycin which are potent inhibitors of Fe(II) transport in these incubation conditions ([3] and unpublished observations). These reagents inhibited Fe(II) uptake in the absence of nifedipine (Fig. 2, zero nifedipine concentration), but when nifedipine was present the uptake increased above the level seen in the absence of nifedipine to as great a degree when the inhibitors were present as when they were absent (Fig. 2). The results in Table 1 and Fig. 2 also illustrate an effect of nifedipine that was observed in all experiments: the stimulation of Fe(II) uptake was greater with erythrocytes than with reticulocytes.

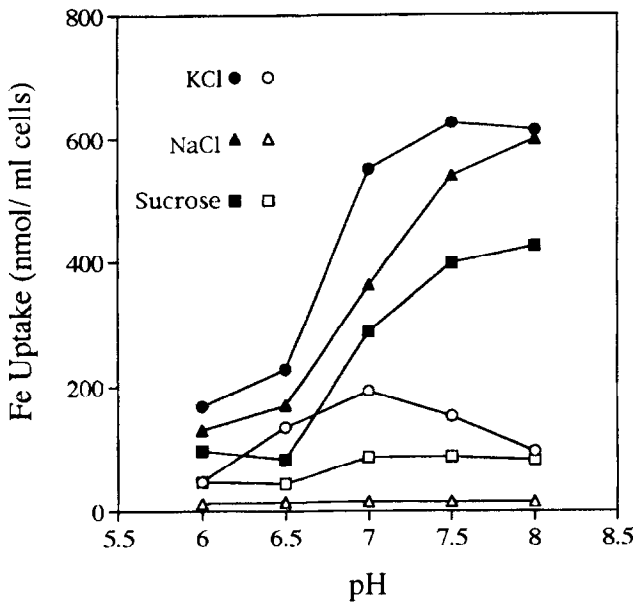


FIG. 1. Effect of pH of the incubation medium on iron uptake into the cytosol of reticulocytes in the absence (open symbols) or presence (closed symbols) of nifedipine. The cells were incubated with 20 μM Fe(II) for 15 min at 37° in isotonic KCl, NaCl, or sucrose buffered at the indicated pH values with 4 mM HEPES. Similar results were obtained with erythrocytes. This experiment is representative of experiments performed three times.

The experiments described above were performed in a laboratory well-lit by daylight and fluorescent light, and no attempt was made to protect the nifedipine from light. However, nifedipine is photodegradable [12, 13]. Therefore, we tested whether the observed effects of nifedipine were the result of photodegradation of the drug. Incubations were performed using the old sample of nifedipine and a freshly prepared solution that was protected from light dur-

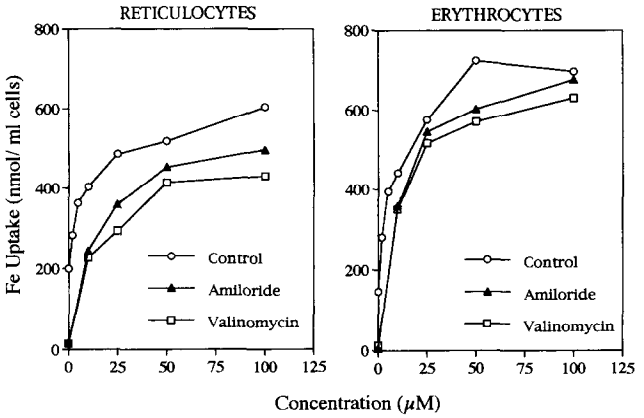


FIG. 2. Effects of amiloride and valinomycin on nifedipine-mediated iron uptake into the cytosol of reticulocytes and erythrocytes. The cells were incubated with 20 μM Fe(II) at 37° for 15 min in isotonic KCl, pH 7.0, in the presence of various concentrations of nifedipine without (control) or with the addition of 500 μM amiloride or 0.5 μM valinomycin. Note that these two reagents virtually abolished iron uptake in the absence of nifedipine. This experiment is representative of experiments performed four times.

ing preparation and incubation with the cells. The light-protected sample did not stimulate Fe(II) uptake (Fig. 3). When new solutions of nifedipine were exposed to the light in the laboratory for varying periods of time, activity developed rapidly (Fig. 4). These solutions were analysed by thin-layer chromatography, which showed the disappearance of the original substance and the appearance of one major and several minor products with increasing periods of exposure to light (Fig. 5A). These products were scraped from the plate, extracted with 0.15 M NaCl–0.02 M HEPES (pH 7.4), and tested for their ability to mediate iron uptake by erythrocytes. As shown in Fig. 5B, this activity was localized to the major photodegradation product.

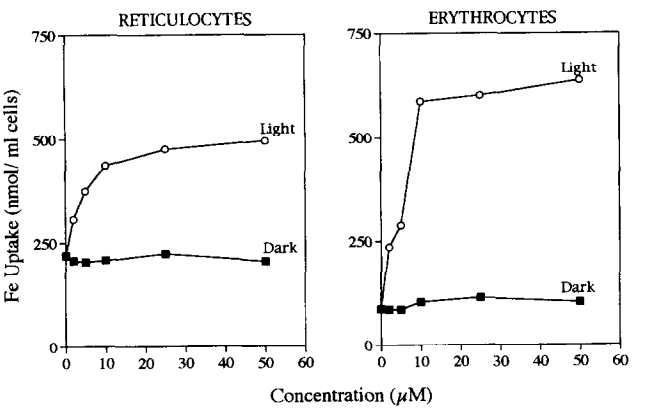


FIG. 3. Iron uptake into the cytosol of reticulocytes in the presence of various nifedipine concentrations that had been exposed to light (Light) or protected from light (Dark). The incubations were performed with 20 μM Fe(II) in KCl, pH 7.0, for 15 min at 37°. The cell incubations with the nifedipine that had been protected from light were performed under light-protected conditions. This experiment is representative of experiments performed six times.

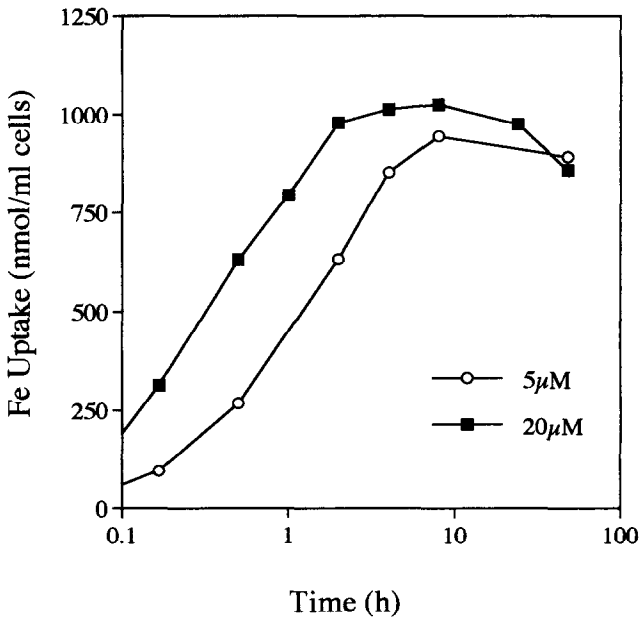
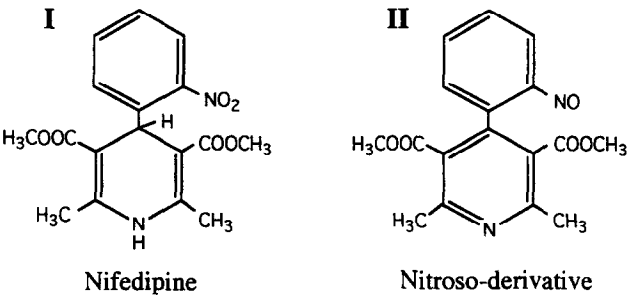


FIG. 4. Effect of duration of exposure of nifedipine to light on nifedipine-mediated iron uptake into the cytosol of reticulocytes. The incubations were performed in the dark with 20 μM Fe(II) in isotonic NaCl, pH 7.4, for 15 min at 37°. Samples of a nifedipine solution (50 mM in chloroform) were exposed to daylight near the laboratory window for the indicated time periods. Two concentrations of nifedipine (5 and 20 μM) were used for the incubations with the cells. This experiment is representative of experiments performed four times.

Larger quantities of this product were then isolated by flash chromatography on silica gel and examined by mass spectroscopy and NMR spectroscopy. These studies identified the active product as the fully aromatic nitrosopyridine derivative of nifedipine (II, below). This has been identified previously as the major product of daylight-induced degradation of nifedipine [12–14]. The remainder of the experiments described below were performed using fully photodegraded nifedipine and, for brevity, the active constituent with respect to iron transport will be referred to as the nitroso-derivative. The concentrations given in these experiments were calculated from the quantities of nifedipine that were used to produce the active derivative. Since there were only minor quantities of other products in fully photodegraded nifedipine (Fig. 5A), the values given for the concentrations of the derivative closely approximate the true values.



Nifedipine Nitroso-derivative

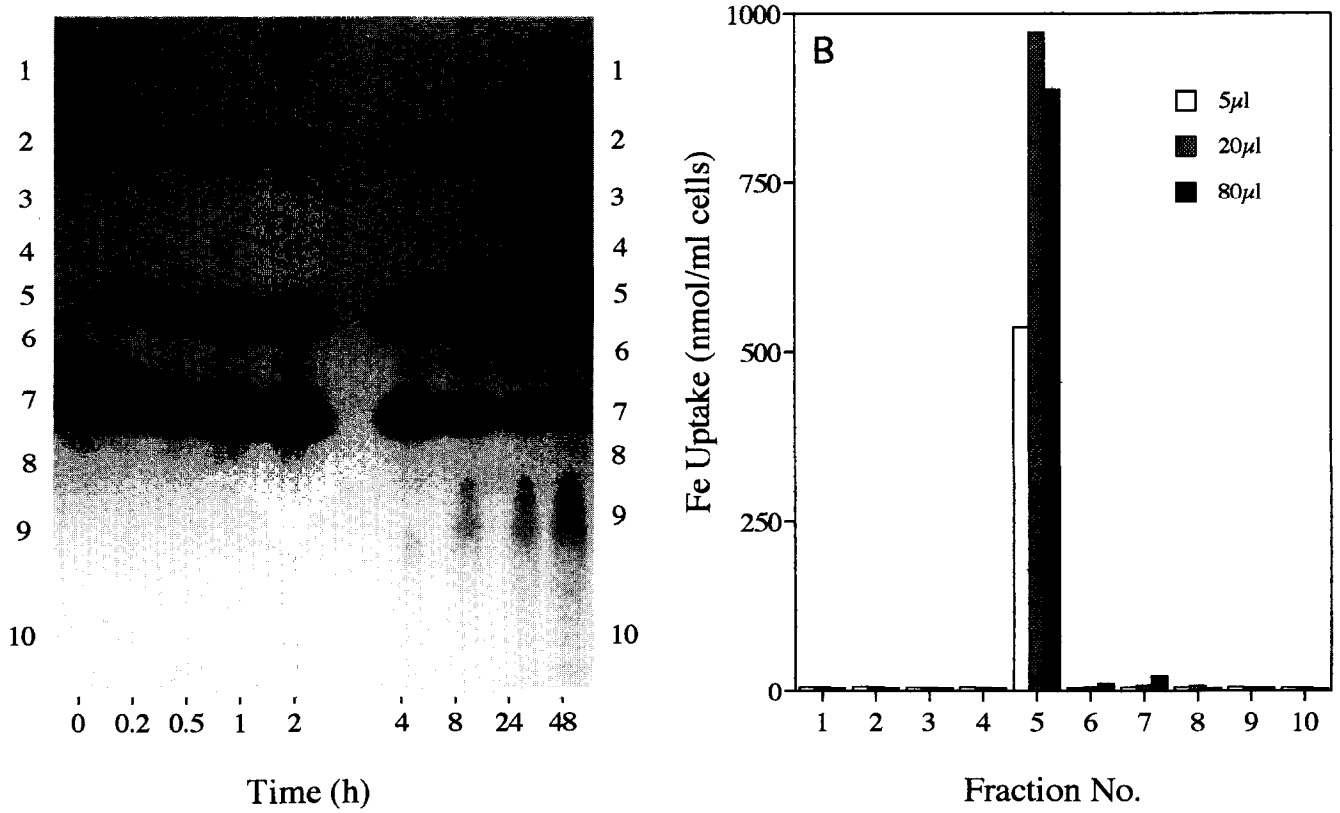


FIG. 5. Effect of duration of exposure to light on the appearance of photodegradation products of nifedipine (A) and the ability of the products to mediate iron uptake into the cytosol of erythrocytes (B). The same nifedipine solutions as in Fig. 4 were examined by thin-layer chromotography on silica gel (Fig. 4A). The sample exposed to light for 8 hr was then run as a band across the silica gel; 10 fractions were scraped from the gel as indicated in Fig. 4A, and eluted with 2 mL HEPES-buffered NaCl (pH 7.4); then 5-, 20- and 80-μL aliquots of the extracts were tested for their ability to mediate iron uptake by erythrocytes [20 μM Fe(II) in NaCl, pH 7.4]. This experiment is representative of experiments performed three times.

The effects of varying both iron and the nitroso-derivative were investigated by measuring Fe(II) uptake into cytosol and stroma of erythrocytes and reticulocytes incubated in NaCl solution, pH 7.4. As shown in Fig. 6 for erythrocytes, the pattern of results was different for cytosol and stroma. Similar results were obtained with reticulocytes. Fe(II) uptake into the cytosol increased rapidly as the concentration of the derivative was raised from 0 to 20 μM but showed very little further increase at higher concentrations (up to 100 μM). By contrast, Fe(II) uptake into the stromal fraction of the cells increased in an approximately linear manner as the concentration of the derivative was raised from 20 to 100 μM. However, even at 100 μM, the uptake of Fe(II) into the stroma was not as great as that into the cytosol, except at the lowest Fe(II) concentration that was used (5 μM). The uptake of Fe(II) mediated by the nitroso-derivative increased as the Fe(II) concentration was raised from 5 to 100 μM.

It was also observed that the stimulation of Fe(II) uptake by the nitroso-derivative was reversible (Fig. 7), that the iron can be used for haem synthesis (Fig. 8), that the derivative was more effective in mediating the uptake of Fe(II) than of Fe(III) (Fig. 9), and that the stimulation of Fe(II) uptake was a temperature-sensitive process (Fig. 10).

In other experiments, the ability of the derivative to mediate the uptake of Mn^{2+} by reticulocytes and erythrocytes was examined by incubating the cells with ^{54}Mn-labelled $MnCl_2$ (20 μM) in the NaCl or KCl incubation medium.

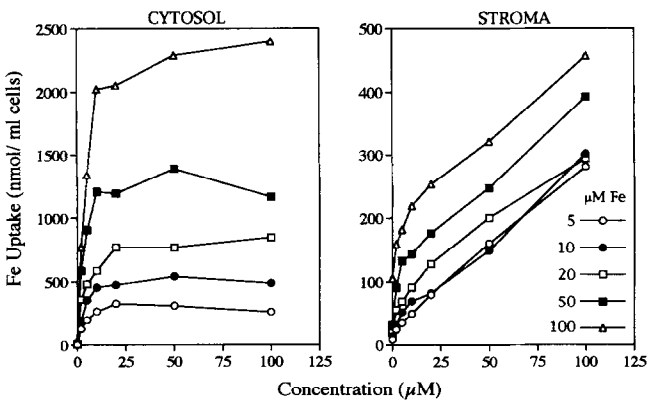


FIG. 6. Effect of various concentrations of the nitroso-derivative of nifedipine on iron uptake into the cytosol and stroma of erythrocytes. The incubations were performed with Fe(II) at 5–100 μM in NaCl, pH 7.4, for 15 min at 37°. Similar results were obtained with reticulocytes. This experiment is representative of experiments performed three times.

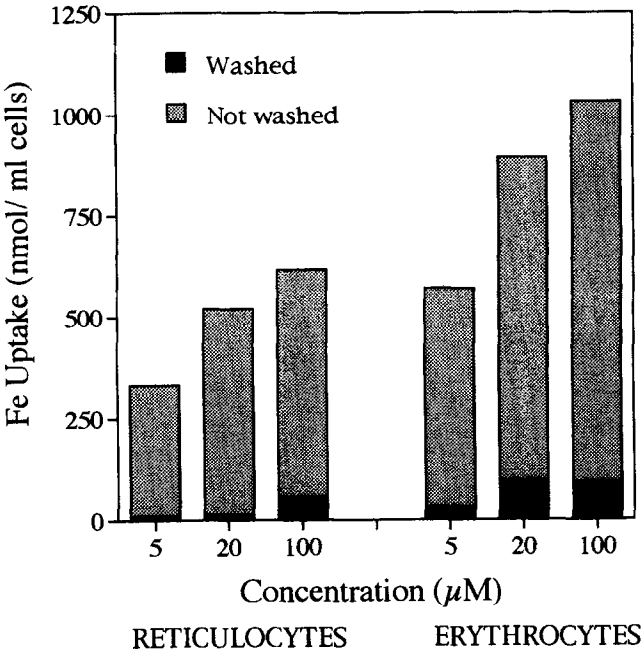


FIG. 7. Reversibility of nitroso-derivative-mediated iron uptake into the cytosol of reticulocytes and erythrocytes. The cells were incubated for 10 min at 37° in NaCl, pH 7.4, with the derivative (5, 20, and 100 μM). Next, samples of the cells were washed three times with ice-cold NaCl or not washed (as indicated) and then incubated at 37° for 15 min in NaCl, pH 7.4, with 20 μM Fe(II). This experiment is representative of experiments performed three times.

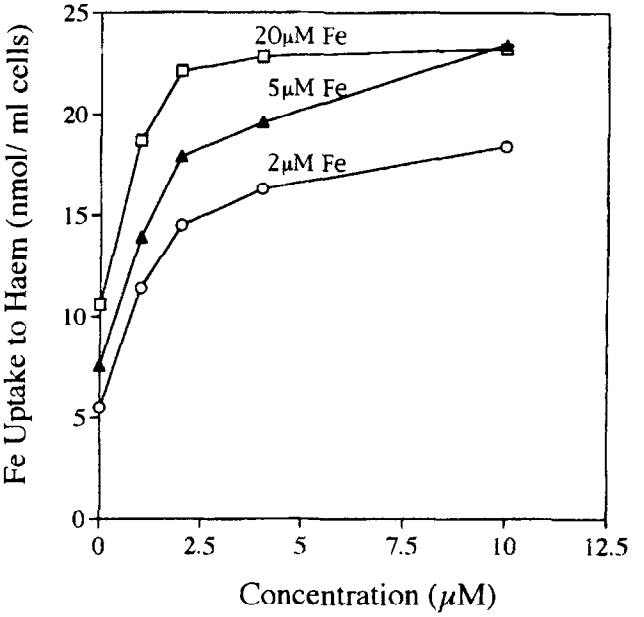


FIG. 8. Incorporation into haem of iron taken up by reticulocytes in the presence of the nitroso-derivative of nifedipine. Cells were incubated for 15 min at 37° in NaCl, pH 7.4, with the indicated concentrations of Fe(II) and 1–10 μM derivative. They were then washed, and haem was extracted and counted for ^{59}Fe as described in the text. This experiment is representative of experiments performed three times.

No increase in uptake was found when 20 μM nitroso-derivative was added to the incubation medium.

The nitroso-derivative was able to mediate iron release from reticulocytes and erythrocytes as well as its uptake. To study this, reticulocytes were labelled with ^{59}Fe by incubation for 30 min at 37° with ^{59}Fe-labelled transferrin-bound iron (1 μM Fe) in NaCl, pH 7.4, or with Fe(II) in sucrose pH 6.5 (1 μM Fe) or in KCl, pH 7.0 (20 μM Fe), and with erythrocytes using 20 μM Fe(II) in KCl, pH 7.0. The cells were then washed four times in ice-cold 0.155 M NaCl and resuspended in NaCl, pH 7.4, with or without the addition of 10 μM derivative and 100 μM desferrioxamine. Then they were reincubated at 37° for periods up to 60 min. At each time point, samples of the cell suspension were centrifuged, the supernatant solution was removed and counted for radioactivity, the cells were haemolysed and recentrifuged, and radioactivity was measured in the cytosolic and stromal fractions. In the case of the reticulocytes incubated with transferrin-iron and 1 μM Fe(II), 1 mM succinyl acetone (an inhibitor of haem synthesis) was included in the initial incubation solution because a large proportion of the iron taken up under these conditions is normally incorporated into haemoglobin [1, 2], and this would prevent the possibility of subsequent release from the cells. This is not a problem with uptake from 20 μM Fe(II) in KCl because the amount of iron taken up greatly exceeds that which is incorporated into haemoglobin [3].

In all of the experiments, very little ^{59}Fe was released from the cells when desferrioxamine was not included in the reincubation solution, and little ^{59}Fe was released when no nitroso-derivative was present with or without desferrioxamine. This is illustrated in Fig. 11 for erythrocytes that had been labelled by incubation with 20 μM Fe(II) in KCl solution, but similar results were observed with reticulocytes and the other preincubation conditions. However, when derivative and desferrioxamine were present in the reincubation, a large proportion of the ^{59}Fe originally taken up by erythrocytes from 20 μM Fe(II) was released, the released ^{59}Fe being largely derived from the cytosolic fraction of the cells (Fig. 11). In a similar manner, nitroso-derivative was able to mediate iron release from reticulocytes that had been labelled with ^{59}Fe by all of the three incubation procedures described above. With the cells that had been labelled with ^{59}Fe(II), the effluxed ^{59}Fe was derived mainly from the cytosol. In the case of reticulocytes preincubated with transferrin-Fe, the majority of the ^{59}Fe was in the stromal fraction of the cells and this was the source of most of the effluxed ^{59}Fe (Fig. 12). It has been shown previously that reticulocytes incubated with transferrin-Fe in the presence of inhibitors of haem synthesis continue to take up the iron at a normal rate, and the iron is deposited in mitochondria [15] which form part of the stromal fraction of reticulocytes.

DISCUSSION

The results of these experiments show that nifedipine had different effects on iron uptake by erythroid cells than did

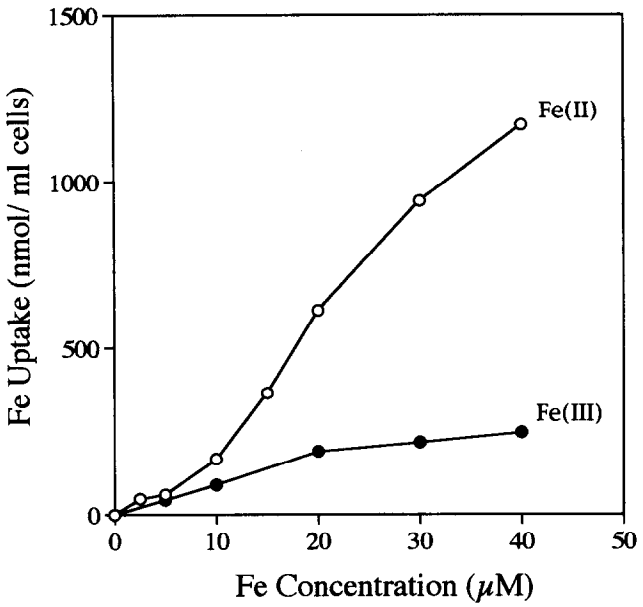


FIG. 9. Effect of the nitroso-derivative of nifedipine on the uptake into the cytosol of Fe(II) and Fe(III) by reticulocytes. The cells were incubated for 15 min at 37° in NaCl, pH 7.4, with the indicated concentrations of Fe(II) or Fe(III) in the presence of 50 μM derivative. The Fe(III) was in the form of Fe–citrate (Fe:citrate = 1:100), but similar results were obtained if the Fe(III) was added as $FeCl_3$. This experiment is representative of experiments performed three times.

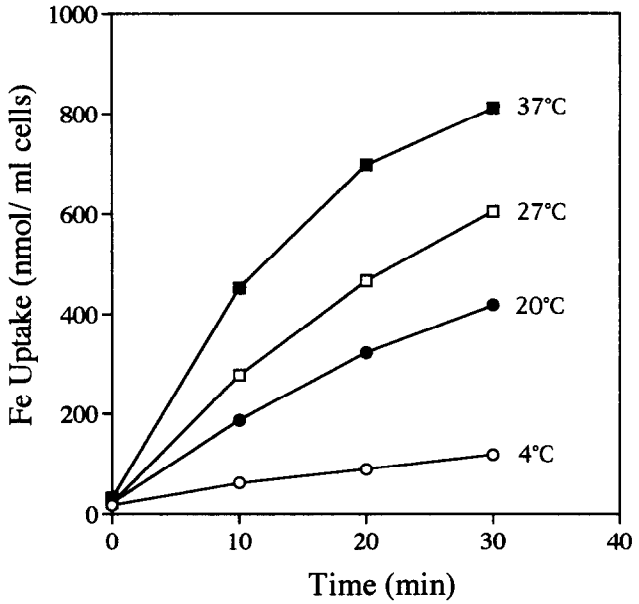


FIG. 10. Effect of incubation temperature on iron uptake into the cytosol of reticulocytes. The incubations were performed with 20 μM Fe(II) and 50 μM nitroso-derivative of nifedipine in NaCl, pH 7.4. This experiment is representative of experiments performed three times.

the other Ca^{2+} antagonists tested, and that the unique properties of nifedipine appeared only after it had been subjected to photodegradation. The results obtained with the other antagonists were unremarkable in that they were all inhibitory, appeared only at relatively high concentrations of the drugs, and did not distinguish between the different iron uptake mechanisms that were studied. Hence, they were not investigated further. However, the effects of nifedipine were so striking that they demanded further investigation.

The observation that the nitroso-derivative of nifedipine stimulated the uptake of non-transferrin-bound iron but not that of transferrin-Fe suggested that it may be acting on one of the pathways involved in the uptake of transferrin-free iron. However, this is unlikely to be the case since stimulated uptake occurred in the presence of potent inhibitors of Fe(II) uptake: NaCl, valinomycin, and amiloride. It therefore appears that the derivative is opening up a new pathway for iron transport across the membrane or is acting as a mobile iron ionophore (an "ironophore"). It is not surprising that the derivative did not stimulate iron uptake from transferrin since the rate-limiting step in this iron transport process is the rate of endocytosis of transferrin [16] and, with reticulocytes, each molecule of transferrin donates both iron atoms to the cell during its endocytic cycle [16]. Hence, the derivative or any other drug can accelerate the rate of iron uptake from transferrin into reticulocytes only by increasing the rate of transferrin endocytosis, not by stimulating iron transfer across cell membranes.

The nitroso-derivative of nifedipine has the ability to mediate iron transfer in both directions across the cell between extracellular medium and cytoplasm. This is shown by the changes in the ^{59}Fe content of the cytosolic fraction of the cells in the uptake and efflux experiments and the

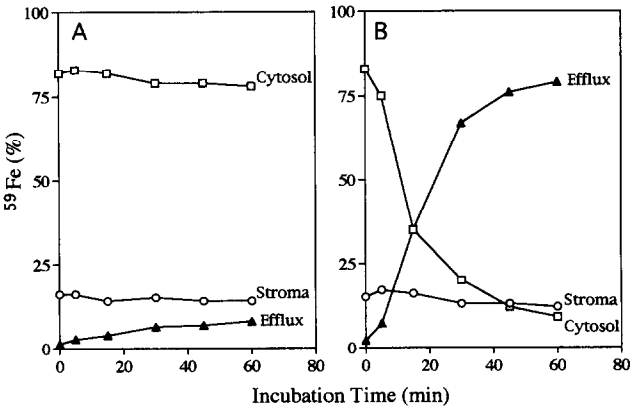


FIG. 11. Iron efflux from erythrocytes in the absence (A) or presence (B) of nitroso-derivative after uptake of Fe(II). The cells were labelled with ^{59}Fe by incubation for 30 min with 20 μM Fe(II) in KCl, pH 7.0, washed three times with ice-cold NaCl, and reincubated at 37° with 100 μM desferrioxamine in NaCl, pH 7.4, in the absence (A) or presence (B) of 10 μM derivative. The figure shows the ^{59}Fe that was released from the cells (efflux) and that present in the cytosolic and stromal fractions expressed as percent of the total ^{59}Fe in the cells. This experiment is representative of experiments performed five times.

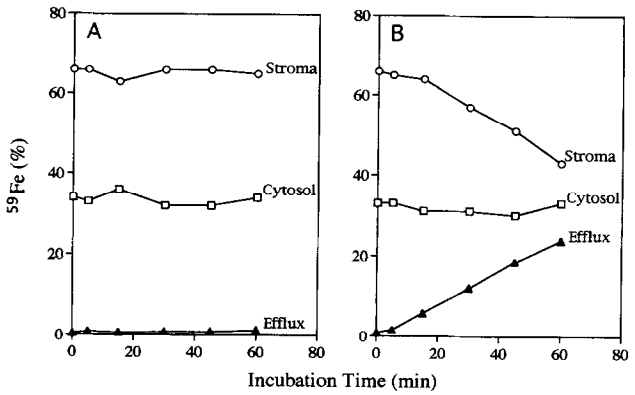


FIG. 12. Iron efflux from reticulocytes in the absence (A) or presence (B) of nitroso-derivative after uptake of transferrin-bound iron. The cells were labelled with ^{59}Fe by incubation for 30 min with 1 μM transferrin-bound Fe in the presence of 1 mM succinyl acetone. They were then washed and reincubated as described for Fig. 11. This experiment is representative of experiments performed three times.

incorporation of some of the Fe(II) taken up under the influence of derivative into haem. Although the amount of Fe(II) incorporated into haem was low compared with the total stimulated Fe(II) uptake, it increased in the presence of the derivative to reach a plateau level, which was probably determined by the maximum capacity of the cells for haem synthesis.

These experiments fail to provide a definitive answer to the question of how the nitroso-derivative mediates iron exchange across erythroid cell membranes. However, they provide some clues and should stimulate further investigations into the mechanisms involved. The exchange of Fe(II) was mediated much more effectively than that of Fe(III) or Mn^{2+}. Also, the effect was reversed by simply washing the cells in ice-cold NaCl solution, indicating that the rapid Fe(II) exchange was unlikely to be due to structural damage to the cell membrane, such as that caused by free radical reactants. Indeed, haemolysis, which is a useful indicator of membrane damage in erythroid cells, was not observed in any of the experiments in this investigation. The experiments in which the concentrations of Fe(II) and the derivative were varied also raise questions which, if answered, should aid in understanding the processes involved. Thus, Fe(II) transfer to the cytosol increased with Fe(II) concentration up to 100 μM, but with respect to derivative concentration, only up to 20 μM. However, Fe(II) incorporation into the stroma increased as the derivative concentration was raised up to the maximum tested (100 μM). The maximal or near maximal rate of Fe(II) transfer to the cytosol for each Fe(II) concentration was reached at a derivative concentration of 20 μM, whereas Fe(II) uptake to the stroma continued to increase with higher concentrations, although the effect of concentration was curvilinear with the change from curved to linear occurring at about a 20-μM concentration of the derivative. Possibly a change in the interaction between molecules of the derivative and/or the components of the cell membrane occurs when the concentration in the extracellular medium is raised above 20 μM.

Although any conclusion regarding the mode of action of the nitroso-derivative of nifedipine on Fe(II) transport across erythroid cell membranes must be tentative at the present time, a useful working hypothesis is that the drug acts as an ionophore, with the capability of transferring Fe(II) but not Mn^{2+} across the membranes. If this activity is found to be specific for iron, it may provide a valuable adjunct in chelation therapy for iron overload. Since the photodegradation products of nifedipine lack Ca^{2+} channel blocking properties [17, 18], there should be no risk of side-effects due to actions on these channels. The product, in combination with desferrioxamine, was able to mediate iron release from erythroid cells at a far greater rate than was observed with desferrioxamine alone. If this action also occurs with other types of cells, and *in vivo*, it could provide a means of accentuating the excretion of excess body iron by desferrioxamine, the only iron chelator that has achieved widespread clinical acceptance for this purpose.

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