Ca$^{2+}$ Permeability in Deoxygenated Sickle Cells

By M.D. Rhoda, M. Apovo, Y. Beuzard, and F. Giraud

Deoxygenation of sickle cells is known to increase cation permeabilities (Na$^+$, K$^+$, and Ca$^{2+}$). The possible mechanisms involved in the increased uptake of Ca$^{2+}$ were investigated: activation of Ca$^{2+}$ channels, involvement of the anion channel, and the formation of endocytic vacuoles. The Ca$^{2+}$ channel blocker nifedipine reduced the deoxy-stimulated Ca$^{2+}$ uptake by about 30% to 40%. The anion channel inhibitor DIDS (4,4'-diisothiocyanate stilbene 2,2' disulfonate) inhibited the deoxy-stimulated Ca$^{2+}$ uptake by approximately 50%. Maximal possible endocytic uptake, measured by using an impermeant marker ([3H] inulin), accounted for 6% to 9% of the total Ca$^{2+}$ uptake. These data indicate that the deoxygenation-induced increase in Ca$^{2+}$ permeability could result from both the activation of a Ca$^{2+}$ channel and of a transport system for cations involving interactions between polymerized hemoglobin S, band 3 and other membrane components. Endocytosis appears to play only a minor role in the Ca$^{2+}$ uptake of deoxygenated sickle cells.

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DEOXYGENATION OF sickle cells induces the polymerization of hemoglobin S, sickling, and a variety of functional abnormalities of the red blood cell membrane, particularly an elevated permeability to Na$^+$, K$^+$, and Ca$^{2+}$. While reoxygenation restores the normal shape and membrane properties of most cells (reversibly sickled cells), prolonged deoxygenation or cycles of deoxygenation-reoxygenation results in the presence of a subpopulation of cells that becomes permanently deformed, the so-called irreversibly sickled cells (ISC). These ISC exhibit a loss of cell volume associated with an increased monovalent cation permeability resulting in a K$^+$ loss exceeding the Na$^+$ gain. In addition, Ca$^{2+}$ accumulates in intracellular inside-out vesicles, increasing the total cellular calcium content (40 to 50 μmol/L instead of 5 to 10 μmol/L in normal erythrocytes). However, the concentration of the free cytosolic ionized Ca$^{2+}$ ([Ca$^{2+}$]i) remains normal when determined in the oxygenated state (20 to 40 nmol/L). The increased Ca$^{2+}$ permeability upon deoxygenation of sickle cells could contribute to cell dehydration by activation of a Ca$^{2+}$-dependent K$^+$ channel during cycles of deoxygenation-reoxygenation. The apparent Ca$^{2+}$-dependent formation of dense cells during such cycles, and the inhibition of this process by some Ca$^{2+}$ channel blockers, led us to examine, using nifedipine, the contribution of a putative Ca$^{2+}$ channel to the deoxygenation-induced Ca$^{2+}$ uptake. Additionally the effect of DIDS (4,4'-diisothiocyanate stilbene 2,2' disulfonate), an anion channel inhibitor, on the Ca$^{2+}$ influx was also investigated, since (1) DIDS has been shown to inhibit the K$^+$ loss in deoxygenated sickle cells, and (2) Ca$^{2+}$ could enter the cell through the anion channel, as a monovalent anion complex with chloride, as already reported for Zn$^{2+}$. Finally, the extent of Ca$^{2+}$ uptake due to the formation of endocytic vacuoles was evaluated using an impermeant marker, [3H] inulin.

MATERIAL AND METHODS

Heparinized blood samples were obtained from healthy, nontransfused adult patients homozygous for sickle cell hemoglobin (SS cells) and from normal adults (AA cells).

Preparation of erythrocytes. Freshly drawn erythrocytes were washed three times in the following buffer: 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl$_2$, 5 mmol/L glucose, 1 mmol/L Na$_2$HPO$_4$/NaH$_2$PO$_4$, 10 mmol/L HEPES (pH 7.4) (buffer A). These cells were either immediately used for chelator loading and Ca$^{2+}$ flux measurements or kept overnight at 4°C and subsequently incubated in an adenosine triphosphate (ATP) regenerating system (2 mmol/L adenosine, 10 mmol/L inosine in buffer A, 45 minutes at 37°C, hematocrit 15%) before chelator loading.

Ca$^{2+}$ flux measurements. Ca$^{2+}$ fluxes were measured after incorporation of a Ca$^{2+}$ chelator, quin2, in order to increase the exchangeable intracellular Ca$^{2+}$ pool. Chelator loading can be carried out either in the absence or in the presence of Ca$^{2+}$ in the medium. In the first case, during the chelator loading, the endogenous Ca$^{2+}$ pool was depleted, and upon Ca$^{2+}$ addition, a unidirectional Ca$^{2+}$ influx was measured. In the second case, the endogenous pool was increased up to an equilibrium during the chelator loading, and upon Ca$^{2+}$ addition, bidirectional Ca$^{2+}$ fluxes were observed. Either one or the other procedure was used, as indicated in the text.

Cells were incubated in buffer A containing either 1 mmol/L CaCl$_2$ or no CaCl$_2$ (37°C, hematocrit 30%) and quin2-AM (Lancaster Synthesis, Morecamb, UK; 200 μmol/L of cells). After 45 to 60 minutes, the cells were washed three times in buffer A, with or without 1 mmol/L CaCl$_2$. The concentration of intracellular free quin2, determined as previously described, was found to be 100 to 140 μmol/L of cells. The concentration of free cytoplasmic Ca$^{2+}$ ([Ca$^{2+}$]i) was calculated as previously described.

Chelator-loaded cells in the presence of CaCl$_2$ and resuspended in buffer A (hematocrit 10%) containing 1 mmol/L CaCl$_2$ were incubated at 37°C with 45Ca (CEA, Oris, France; 370 kBq/pmol). After 60 minutes' incubation with 45Ca under air, deoxygenation experiments were done on a part of the cell suspension by flushing it with humidified N$_2$. The 45Ca content of the cells was measured from time zero corresponding to the addition of 45Ca up to 120 minutes, as described.

When required, the cells were preincubated at 37°C for 10 minutes with or without nifedipine (N 7634; Sigma, St Louis, MO) before the addition of 45Ca. The effect of DIDS (D3514; Sigma) was determined in cells preincubated for 20 minutes at 37°C with DIDS and then washed and loaded with quin2-AM. The contribution of endocytosis to the 45Ca uptake was determined by

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suspension of the cells in buffer A containing [3H] inulin (2.6 MBq/mL of cells), an impermeant marker, and 45CaCl2 (1 mmol/L).

Chelator-loaded erythrocytes in the absence of CaCl2 were suspended in buffer A (hematocrit 10%) and preincubated at 37°C for 10 minutes with or without nifedipine before the addition of 0.1 or 1 mmol/L 4CaCl2 (3.7 or 370 kBq/μmol). In some experiments, cells were deoxygenated at 37°C, 30 minutes before the addition of 45Ca. The 45Ca content of the cells was measured at different times up to 20 minutes.

The ATP content, measured after chelator loading (UV test combination, Boehringer-Mannheim, West Germany), was reduced by 40% in AA cells, but only by 30% in SS cells, as previously reported. The percentage of cell lysis was calculated by measuring the hemoglobin concentration in the supernatant and in the total cell suspension spectrophotometrically at 540 nm.

RESULTS

Stimulation of Ca2+ uptake in deoxygenated SS cells and inhibition by nifedipine. In the experiments shown in Fig 1, quin2 loading and Ca2+ uptake were done in the presence of 1 mmol/L CaCl2. Ca2+ uptake in SS cells slightly exceeded that in AA cells under oxygenated conditions, but was strongly enhanced upon deoxygenation (Fig 1A and B). Deoxygenation had no effect on the Ca2+ uptake in AA cells (Fig 1A). Ca2+ influx, under oxygenated conditions, was calculated from the plot of Log (45Ca0 - 45Ca(t)) = f(t), as previously described. Ca2+ influx in SS cells due to deoxygenation was estimated by the measurement of the initial rate of the 45Ca uptake. As reported in previous studies, the rate of influx in deoxygenated SS cells was found to be about five times that of oxygenated SS cells (Table 1).

Nifedipine was used to define the fraction of total Ca2+ influx due to the Ca2+ channel. After preincubation with nifedipine (10 minutes, 50 μmol/L), 45Ca2+ influx was inhibited in oxygenated AA and SS cells, as well as in deoxygenated SS cells (Fig 1A and B). Deoxygenation of SS cells enhanced both the nifedipine-sensitive and the nifedipine-resistant part of the Ca2+ influx (Table 1). However, the nifedipine-sensitive influx was increased by eightfold, whereas the nifedipine-resistant one was increased by only threefold.

The extent of inhibition of Ca2+ uptake by nifedipine in AA cells in our experiments was found to be lower than previously reported data. Previous data were obtained with nitrendipine or verapamil in vanadate-treated erythrocytes, a condition that induces a carrier-mediated Ca2+ uptake.

The same experiments were done with the erythrocytes loaded with quin2 in the presence of Ca2+. This procedure, which causes a decrease in the intracellular Ca2+ concentration, may induce a stimulation of the influx of Ca2+, as previously reported, and may increase the extent of inhibition by the Ca2+ entry blockers. In fact, under these conditions, Ca2+ influx was not stimulated (Table 1), and the extent of inhibition by nifedipine remained low (30% to 40% maximum, Fig 2). Ca2+ uptake in these Ca2+-depleted SS cells, reincubated with 1 mmol/L CaCl2 (Table 1B), was stimulated by deoxygenation to a similar extent than under steady-state conditions of internal Ca2+ (Table 1A). Again, the nifedipine-sensitive fraction of the Ca2+ uptake in SS cells was much more stimulated by deoxygenation than was the nifedipine-resistant one. Ca2+ uptake was also investigated in SS cells that had been loaded with quin2-AM in the presence of 1 mmol/L CaCl2, washed, and reincubated with 1 mmol/L CaCl2. When required, nifedipine (50 μmol/L) was added 10 minutes before 45Ca. After 60 minutes under air, deoxygenation was induced by humidified N2. Data are means of duplicate determinations in a single experiment, and three others gave similar values. In the experiment shown, the ATP content was 0.42 and 0.55 mmol/L of AA and SS cells, respectively, after quin2-AM loading. Intracellular quin2 concentration was 140 and 120 μmol/L of AA and SS cells, respectively. Hemolysis was 4% maximum after 1 hour of deoxygenation. A: AA cells oxy; Δ, deoxy; □, oxy + nifedipine. B: SS cells oxy; △, deoxy; ■, oxy + nifedipine; *, deoxy + nifedipine.
Table 1. Effect of Nifedipine on Ca\(^{2+}\) Influx

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Cells were loaded with quin2-AM in the presence (A) or in the absence (B and C) of CaCl\(_2\), washed and reincubated with either 1 mmol/L (A and B) or 0.1 mmol/L (C) 45Ca under air (oxy) or under humidified N\(_2\) (deoxy). (A) Deoxygenation of SS cells was started after 60 minutes of equilibrium with 45Ca under air, as described in the text (Methods) and in the legend of Fig 1. (B and C) SS cells were deoxygenated for 30 minutes before the addition of 45Ca. Values given are the means ± SE of five experiments or means of two experiments.

*P < .02.

In other experiments (data not shown), maximal inhibition of the Ca\(^{2+}\) uptake (30% to 40%) by oxygenated AA or SS cells was also observed with 10 µmol/L nicardipine or 0.5 µmol/L nifedipine.

Inhibition of deoxy-stimulated Ca\(^{2+}\) uptake by DIDS. Pretreatment with DIDS (done before quin2-AM loading) had no effect on the 45Ca uptake of oxygenated SS cells, but inhibited Ca\(^{2+}\) uptake under deoxygenated conditions (Fig 3). This inhibition was concentration-dependent, and the maximum inhibition occurred at about 15 µmol/L of DIDS (Fig 4). Replacement of NaCl and KCl by NaNO\(_3\) and KNO\(_3\) did not modify the Ca\(^{2+}\) uptake of SS cells under either oxygenated or deoxygenated conditions nor did it modify the extent of inhibition of DIDS of the deoxy-stimulated Ca\(^{2+}\) uptake (data not shown).

Ca\(^{2+}\) uptake due to endocytosis. [3H] inulin, an impermeant marker, was used to measure the extent of endocytosis and to calculate the fraction of the Ca\(^{2+}\) uptake due to this process. AA and SS cells, chelator-loaded in the presence of 1 mmol/L CaCl\(_2\), were incubated in the same medium containing 45Ca and [3H] inulin. After 60 minutes under air, a fraction of the suspension was deoxygenated. The uptake of each marker is shown in Fig 5. The volume of external medium incorporated in the cells (expressed as percent of cell volume) was calculated from [3H] radioactivity incorporated in the cells (cpm/µL of cells) and from the specific radioactivity of [3H] inulin in the external medium (cpm/µL of external medium). Under oxygenated conditions, a small incorporation of the [3H] marker was observed in normal AA cells and a slightly higher one in SS cells. In both cases a plateau level was reached after 10 minutes. Deoxygenation of SS cells resulted in a delayed increase in the incorporation of the [3H] marker up to a plateau level reached after 60 minutes. The difference between the oxy and deoxy plateau levels in SS cells could reflect the endovesiculation due to deoxygenation. This was found to be 1% of the cell volume (Fig 5B). Since the extracellular Ca\(^{2+}\) concentration is 1 mmol/L, this incorporation of extracellular medium allowed the uptake of 10 µmol Ca\(^{2+}\)/L of cells. During the same time, the total exchangeable Ca\(^{2+}\) in this experiment was about 110 µmol/L of cells, a value corresponding to the plateau level obtained upon deoxygenation. Thus, the contribution of endocytosis to the Ca\(^{2+}\) uptake would be 10 µmol/L of cells, or 9% of the total Ca\(^{2+}\) uptake. In two additional experiments, endocytosis accounted for 6.5% and 6.8% of the total Ca\(^{2+}\) uptake. In other experiments (data not shown), incorporation of [3H] inulin was observed both in light (1.068 < d < 1.078) and in dense (d > 1.096) SS cell fractions obtained from Strasctan density gradients. In addition, when deoxygenation was done after addition of sufficient EGTA to reduce the external Ca\(^{2+}\) concentration to less than 50 nmol/L, [3H] inulin remained at the same level as that observed under oxygen conditions, indicating that the process was dependent upon the presence of Ca\(^{2+}\) in the medium (data not shown).
**DISCUSSION**

The purpose of the present study was to investigate the possible mechanisms involved in the increased Ca\(^{2+}\) uptake noted in sickle cells upon deoxygenation. Experimental evidence was shown for the presence of a dihydropyridine-sensitive (30% to 40%) and a DIDS-sensitive Ca\(^{2+}\) influx (40% to 50%), as well as the direct endocytosis (6% to 9%) of Ca\(^{2+}\).

All the experiments were done after loading the erythrocytes with the calcium chelator quin2. Moderate concentrations of chelator were incorporated (100 to 140 pmol/L of cells) in order to prevent important ATP depletion.\(^{26}\) The level of ATP, measured after chelator loading, remained within values sufficient to maintain a normal activity of the Ca\(^{2+}\) pump.\(^{27}\)

The \(^{45}\)Ca content of incubated oxygenated SS cells increased gradually up to a pump-leak steady-state of 15 to 25 pmol/L of cells. From this value and that of the concentration of intracellular quin2, [Ca\(^{2+}\)], was calculated (18 to 30 nmol/L). This value was not different from that in AA cells, in agreement with previous reports.\(^{8,10}\) Upon deoxygenation, \(^{45}\)Ca uptake increased up to a new steady-state of about 100 to 120 pmol/L of cells, a value exceeding that expected from the concentration of quin2 incorporated if [Ca\(^{2+}\)], was maintained at its low level, as should be the case in SS cells with normal Ca\(^{2+}\) pump activity. The resealing of some lysed cells during deoxygenation could account for part of the observed Ca\(^{2+}\) uptake. However, assuming that all the uptake of the extracellular marker \(^{[3]H}\) inulin (1% of cell volume) was due to cell damage and resealing, the Ca\(^{2+}\) uptake that can be attributed to this process would be only 10 pmol/L of cells. Since SS cell populations are heterogeneous, it is possible that Ca\(^{2+}\) permeability is increased in some cells that do not contain quin2. In one experiment, in which SS cells were fractionated into light and dense populations (see...
results), sickling induced the same \( \text{Ca}^{2+} \) uptake in both fractions. A more likely explanation of our data is that this excess \( \text{Ca}^{2+} \) entering the cell is rapidly sequestered into an intracellular compartment lacking quin2, such as the endocytic inside-out vesicles previously described in SS cells.\(^7\) They can pump actively cytosolic \( \text{Ca}^{2+} \) but do not contain quin2, since this latter cannot cross the membrane in its free form.

The calcium channel blocker nifedipine (50 \( \mu \text{mol/L} \)) inhibited partially the \( \text{Ca}^{2+} \) influx in deoxygenated SS cells and in oxygenated SS or AA cells preloaded with quin2-AM either in the absence or presence of \( \text{Ca}^{2+} \) in the medium. The extent of inhibition of \( \text{Ca}^{2+} \) influx by nifedipine was low (20% to 40%). Interestingly, when the external \( \text{Ca}^{2+} \) concentration was 1 \( \text{mmol/L} \), nifedipine was a slightly more potent inhibitor of \( \text{Ca}^{2+} \) uptake in deoxygenated SS cells than in oxygenated SS cells or oxygenated AA cells, suggesting that deoxygenation of SS cells induces an activation of a \( \text{Ca}^{2+} \) channel. Although the concentration dependence of this process on external \( \text{Ca}^{2+} \) was not investigated over a whole range of external \( \text{Ca}^{2+} \) concentrations, the data show that at 0.1 \( \text{mmol/L} \) there was no activation.

Nifedipine (15 \( \mu \text{mol/L} \)) has been reported to inhibit 70% of the \( \text{Ca}^{2+} \) influx in vanadate-treated oxygenated AA cells.\(^{21,22}\) In addition to inhibiting Ca-ATPase, vanadate opens a carrier-mediated transport pathway similar to \( \text{Ca}^{2+} \) channels of excitable tissue.\(^{23}\) An inhibitory effect of nifedipine was also observed by Engelman and Duhm\(^{28}\) in vanadate-treated but not in quin2-loaded erythrocytes. The lack of effect by nifedipine in the latter case was presumably due to the fact that, in their study, the rate of \( \text{Ca}^{2+} \) uptake was determined by atomic absorption spectrophotometry, a method less sensitive than \( \text{Ca}^{45} \) measurements and only between 0 and 15 minutes a period too short for an accurate determination of the small inhibition induced by nifedipine.

The possibility that deoxygenation in SS cells induces a transient increase in [Ca], triggering the activation of the \( \text{Ca}^{2+} \)-dependent K+ channel, resulting in K+ loss and cell dehydration has been suggested previously.\(^{11,12}\) However, the mechanism responsible for the increase in \( \text{Ca}^{2+} \) permeability has not been clearly understood. The fact that nifedipine, nisoldipine, dilthiazem, and other calcium channel blockers have been shown to inhibit K+ loss and sickle cell dehydration, induced by prolonged deoxygenation or cycles of deoxygenation-reoxygenation\(^{11,14}\) of sickle cells in the presence of \( \text{Ca}^{2+} \), could also suggest that these drugs inhibit \( \text{Ca}^{2+} \) entry. The concentrations of nifedipine (20 to 50 \( \mu \text{mol/L} \)) at which \( \text{Ca}^{2+} \) influx was inhibited are of the same order of magnitude as those inhibiting the formation of dehydrated SS cells. However, these concentrations are higher than their usual pharmacologic concentrations (10-6 to 10-4 \( \text{mol/L} \)), and therefore, possible nonspecific effects of sickling-induced cation leaks cannot be excluded.

The concentration of DIDS required to inhibit 50% of the deoxy-stimulated \( \text{Ca}^{2+} \) uptake (15 \( \mu \text{mol/L} \)) was greater than that necessary to block the anion transport (\( \approx 5 \mu \text{mol/L} \)). It is thus likely that the effects of DIDS on anion transport and \( \text{Ca}^{2+} \) influx are not directly related. In addition, chloride replacement with nitrate had no effect on the inhibition of \( \text{Ca}^{2+} \) uptake by DIDS, excluding the possibility of the entry of \( \text{Ca}^{2+} \) in the form of a monovalent anion complex with chloride, as described for \( \text{Zn}^{2+} \).\(^{16}\) Inhibition of the deoxy-stimulated Na+ and K+ fluxes in SS cells by similar doses of DIDS, also amounting to about 50%, have been reported previously,\(^{11,12}\) indicating that the DIDS inhibitory effect is not restricted to \( \text{Ca}^{2+} \). It is also known that the interaction of DIDS with band 3 results in changes in the binding properties of the cytoplasmic domain of band 3 with hemoglobin A.\(^{20}\) It may be assumed that as a consequence of sickling, the interaction of polymerized HbS with band 3 activates a cation transport system responsible for the increased permeability to Na+, K+, and \( \text{Ca}^{2+} \). Modifications of the conformation of band 3 after its interaction with DIDS may result in a diminution in the activation of the putative cation transport system, as previously suggested.\(^{29,30}\)

It has been shown previously that oxygenated SS cells contain internal endocytic vesicles enriched in calcium.\(^{7,8}\) Evidence was also provided that these vesicles were formed by endocytosis upon deoxygenation.\(^{10,31}\) In order to quantify this process under our experimental conditions, \([\text{H}]\) inulin, an impermeant extracellular marker, was used to measure the extent of endocytosis induced by deoxygenation in SS cells. The data show that \([\text{H}]\) incorporation into oxygenated AA and SS cells amounted to 0.8% of the total cell volume. In oxygenated cells, the incorporation of \([\text{H}]\) inulin most likely did not indicate the formation of endocytic vesicles but only reflected the presence of leaky cells that then resealed upon reincubation at 37°C. This is further indicated by the very rapid saturation of the process (10 minutes). In contrast, the increase in \([\text{H}]\) incorporation upon deoxygenation observed only in SS cells and only after a lag time could represent endocytosis. Similar data were obtained by Bookchin et al.\(^{8}\) who have used \( ^{40} \text{Co}-\text{EDTA} \) or \( ^{45} \text{Ca}-\text{EGTA} \) as extracellular marker and slightly different experimental conditions: (1) SS cells in which \( \text{Ca}^{2+} \) pump was inhibited and that were deoxygenated for 3 hours in autologous plasma and (2) SS cells subjected to four repeated 20-minute cycles of deoxygenation-reoxygenation. As mentioned by these authors, the uptake of an extracellular marker does not necessarily represent endocytosis, since lysis and resealing of a small fraction of the cells could also cause the same uptake. However, using an impermeant fluorescent marker, Rubin et al.\(^{19}\) have observed the presence of brightly staining vesicles in SS cells that increase in number upon deoxygenation. Although this methodology does not permit quantification of the process in terms of its contribution to \( \text{Ca}^{2+} \) uptake, it provides direct evidence for endocytosis. Omitting \( \text{Ca}^{2+} \) from the medium blocked both the stimulation of \( \text{Ca}^{2+} \) influx and the endocytosis. Ben-Bassat et al.\(^{16}\) have reported that \( \text{Ca}^{2+} \) was not required for endocytosis in erythrocytes. However, in the same laboratory it was shown that, in resealed ghost, internal \( \text{Ca}^{2+} \) at a low concentration (\( < 1 \mu \text{mol/L} \)) stimulated vacuole formation.\(^{13}\) It may be suggested that the deoxy-stimulated endocytosis was caused by the transient increase in [Ca], resulting from the stimulated \( \text{Ca}^{2+} \) influx. The extent of endocytosis induced by deoxygenation in SS cells, assuming that all the marker uptake is due to this process and calculated from the difference between the deoxy and the oxy conditions, amounted to approximately...
0.8% to 1% of the total cell volume. Interestingly, we have observed that the formation of the vesicles occurred in all fractions, light or dense, of SS cells, as also reported by Rubin et al. The contribution of endocytosis to the Ca$^{2+}$ uptake upon deoxygenation was found to account for only 6% to 9% of the total Ca$^{2+}$ uptake.

In conclusion, the increase in Ca$^{2+}$ permeability induced by deoxygenation of sickle cells may be linked to the activation of a Ca$^{2+}$ channel. The inhibitory effects of DIDS on Ca$^{2+}$ uptake in deoxygenated SS cells indicates the presence of an additional pathway that merits further work to determine the mechanism underlying its mode of action. Finally, endocytosis was found to play only a minor role in the Ca$^{2+}$ uptake of deoxygenated sickle cells.

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