In this study, we used a recently developed nuclear magnetic resonance (NMR) technique to measure ionized calcium in sickle erythrocytes. The NMR technique, which involves $^{18}$F NMR studies of a fluorinated calcium chelator quinMF, [2-(2-amino-4-methyl-5-fluorophenoxy)methyl-8-aminoquinoline-$N,N',N'',$N'$$'$-tetraacetic acid] provides a novel approach to the study of ionized calcium in erythrocytes since the presence of hemoglobin precludes the use of fluorescent calcium indicators. The mean value for ionized calcium in oxygenated sickle erythrocytes was $18 \pm 2$ nmol/L (SE). Experiments with normal RBCs gave a mean value of $21 \pm 2$ nmol/L (SE). After 1 hour of deoxygenation, mean values for ionized calcium in sickle erythrocytes did not increase as compared with values obtained under oxygen. To investigate whether deoxygenation stimulated endocytosis, sickle erythrocytes were deoxygenated for 1 hour in the presence of impermeant FABP TA (1.2 bis-(2-amino-5-fluorophenoxy) ethane $N,N',N'',$N'$$'$-tetraacetic acid). Cells were then separated from the extracellular medium and assayed for the presence of FABP TA; they had incorporated significant quantities of the extracellular FABP TA. This incorporation was not observed with normal erythrocytes. These data are consistent with at least a portion of the elevation in total cell calcium in sickle erythrocytes arising as a consequence of an endocytotic process in which extracellular calcium ions are incorporated into vesicles. Additional experiments show that these intracellular vesicles accumulate Ca$^{2+}$ on further deoxygenation, consistent with a transient increase in ionized cell calcium. These studies represent the first use of NMR spectroscopy to evaluate endocytotic processes.

Cytosolic Free Calcium Levels in Sickle Red Blood Cells
By Elizabeth Murphy, Lee R. Berkowitz, Eugene Orringer, Louis Levy, Scott A. Gabel, and Robert E. London

There is a five- to tenfold elevation of total calcium in sickle erythrocytes as compared with normal RBCs, and isotopic calcium influx is increased in deoxygenated sickle erythrocytes, an effect that does not occur when normal RBCs are deoxygenated. Elevation of erythrocyte total calcium, if it is not sequestered, should contribute to the pathogenesis of sickling by activating K+ specific channels in the cell membrane. Activation of these channels would cause loss of K+ and water, which in turn would enhance sickling because cell dehydration promotes the gelation of hemoglobin S. Lew and colleagues recently demonstrated by electron-microprobe analysis that sickle erythrocytes have intracellular vesicles containing mmolar levels of calcium.

Although these studies show that Ca$^{2+}$ is sequestered in sickle erythrocytes, they do not determine whether an increase in cytosolic free calcium (CaF+$^{2+}$) occurs in sickle erythrocytes either under basal conditions or during deoxygenation. Indeed, recent studies of Bookchin and co-workers reported indirect evidence for a transient increase in calcium-induced K-channel in these cells. An indirect, nonkinetic method used to calculate Ca$^{2+}$ levels in erythrocytes suggests that under basal conditions Ca$^{2+}$ levels in sickle and in normal erythrocytes do not differ significantly. Ca$^{2+}$ levels were not, however, measured during deoxygenation, a condition that stimulates Ca$^{2+}$ uptake in sickle erythrocytes. Furthermore, although Lew and colleagues demonstrated the presence of calcium-containing vesicles in sickle erythrocytes, little is known about the formation of these vesicles and about the origin of the calcium contained within them.

In the present study, using a nuclear magnetic resonance (NMR) technique that allows direct measurement of CaF+$^{2+}$, we determined a value of $18 \pm 2$ nmol/L (SE) for ionized calcium in well-oxygenated sickle RBCs. This value does not differ significantly from values obtained with normal human erythrocytes. No increase in ionized calcium is found in sickle erythrocytes on deoxygenation. Moreover, deoxygenation of sickle erythrocytes leads to incorporation of an extracellular impermeant chelator of calcium, suggesting that deoxygenation stimulates endocytosis. In addition, by deoxygenating sickle erythrocytes that had previously incorporated FABP TA, we observed an increase in the calcium level in these vesicles, demonstrating dynamic Ca$^{2+}$ uptake by the vesicles on deoxygenation.

MATERIALS AND METHODS

Cell preparation. Samples were obtained from normal controls or patients with homozygous sickle cell anemia who had not been transfused for a minimum of 6 months. The samples were drawn into heparinized-vacuum tubes and subsequently diluted in a modified saline solution containing (in mmolar): NaCl 132, MgSO$_4$·7H$_2$O 0.8, KCl 5.4, CaCl$_2$ 1.25, Hepes 5.0, adjusted to pH 7.4 with Tris base. The cells were then centrifuged at 1,000 g for 7 minutes, and the supernatant and buffy coat were removed. For measurement of Ca$^{2+}$, cells were loaded with the fluorinated calcium chelator quinMF, by incubating them at a hematocrit of 3% to 4% in the modified saline, with 50 izmol/L of the acetoxymethyl ester of quinMF (quinMF-AM) for 20 minutes at 37°C. The cells were then centrifuged at 1,000 g for 7 minutes and, after resuspension in the modified saline, were incubated for an additional 60 minutes to allow the cells to recover from the loading procedure. For the NMR studies, the cells were resuspended at a 40% hematocrit in modified saline.

A byproduct of loading erythrocytes with quinMF-AM is formaldehyde, which can inhibit the glycolytic enzyme glyceraldehyde.

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Submitted August 12, 1986; accepted December 19, 1986.

Supported by grants No. R23 HL30467 (L.R.B.) and R01 HL28601 (E.O.) from the National Institutes of Health, Bethesda, MD.

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1469
3-phosphate dehydrogenase as a consequence of an increased NADH/NAD ratio. This can lead in turn to ATP depletion. To ameliorate the effect of formaldehyde, Na pyruvate, 5 mmol/L, was added to the modified saline solution. Na pyruvate generates NAD through the enzyme lactate dehydrogenase so that the cells maintain normal ATP levels despite the presence of formaldehyde. We previously showed that, in the presence of pyruvate, loading RBCs with a similar calcium chelator (FBAPTA) caused only a slight (<20%) fall in ATP.

Measurement of $C_{a}$

Cytosolic free Ca\(^{2+}\) ($C_{a}^{2+}$) was measured by $^{19}$F NMR spectroscopy\(^{10}\) using a newly described fluorinated calcium chelator, quinMF. QuinMF has a much lower dissociation constant than any of the fluorinated chelators originally developed by Smith and co-workers, and it is therefore more useful for measuring $C_{a}$ in erythrocytes. The erythrocytes were suspended in a 20-mm NMR tube fitted with an air-driven stirrer to keep the cells in suspension and to facilitate oxygenation and deoxygenation. The cells were gassed with either 100% O\(_2\) or 100% N\(_2\), resulting in no pH change with the Hepes-Tris buffer. Once inside the cell, the quinMF is in slow chemical exchange with Ca\(^{2+}\). This gives two peaks in the NMR spectrum, one arising from uncomplexed quinMF and another from Ca\(^{2+}\)-complexed quinMF ($Ca$-quinMF). Ca\(^{2+}\) can be calculated using the following equation: 

$$C_{a}^{2+} = K_D [Ca\text{-quinMF}]/[quinMF].$$

Under conditions of nonsaturation or of equal saturation of both $^{19}$F resonances, the concentrations of Ca-quinMF and quinMF are proportional to the area under their respective peaks. Peak area was determined by cutting and weighing. The previously determined $K_D$ of 63 nmol/L was used.\(^{11}\) The level of loading with quinMF was assessed by comparing the area of the quinMF and Ca-quinMF peaks to the area of a known concentration of the standard 6-fluorotryptophan. These studies were performed on a Nicolet (Fremont, CA) NT-360 spectrometer using a 20-mm broad-band Nicolet probe. Observation was made through the decoupler coil, which was set to 539.7 MHz for the $^{19}$F studies. The sample was shimmmed on H\(_2\)O, and we routinely obtained a (nonspinning) line width at half height of ~0.1 ppm. A 40° pulse angle, a 500-μs delay and a 127-ms acquisition time were used for these $^{19}$F studies. EGTA was added to correct for any extracellular calcium indicator (either FBAPTA or quinMF). Addition of excess EGTA will bind extracellular Ca\(^{2+}\); therefore, any extracellular chelator that is complexed will shift to the uncomplexed form.

Estimation of cell lysis

The percentage of cell lysis was calculated using the hemoglobin (Hb) concentration in the supernatant and comparing this with the total cell Hb concentration. Hb concentration was measured spectrophotometrically at 540 nm.\(^{11}\)

Materials

The methyl ester of quinMF was synthesized as described, and Molecular Probes (Junction City, OR) effected the conversion to the aceytoxymethyl ester of quinMF. The potassium salt of FBAPTA was purchased from Molecular Probes. All other chemicals were obtained from Sigma.

RESULTS

We were initially interested in determining whether Ca\(^{2+}\) levels increased in sickle erythrocytes particularly during deoxygenation. FBAPTA has been used previously\(^{8,11,15}\) to measure Ca\(^{2+}\) in erythrocytes. The accuracy of the Ca\(^{2+}\) determination in erythrocytes is limited, however, due to the mismatch between the basal level of Ca\(^{2+}\) and the binding constant of FBAPTA for Ca\(^{2+}\), thus, even for a 1 mmol/L loading of chelator, the signal of Ca-FBAPTA corresponds to <0.1 mmol/L. This leads to the further complication that at such low fractional levels of complexation, a small amount of cell lysis can lead to a large error in the calculated value of Ca\(^{2+}\) due to erroneous inclusion of a contribution from the extracellular chelator complexed with extracellular Ca\(^{2+}\). For these reasons, in this study we used a new fluorinated calcium chelator, quinMF, which has a lower dissociation constant for calcium ($K_D = 63$ nmol/L).

Figure 1, spectrum A (lower trace), shows a representative fluorine NMR spectrum of quinMF-loaded, oxygenated sickle erythrocytes. Based on data from eight similar experiments (corrected for extracellular quinMF) from seven different donors, cytosolic free Ca\(^{2+}\) ($C_{a}^{2+}$) values averaged 18 ± 2 nmol/L, a level not significantly different from a control value of 21 ± 2 nmol/L obtained using quinMF to measure Ca\(^{2+}\) in normal erythrocytes. Spectrum B shows the $^{19}$F spectrum following 30 minutes of deoxygenation. Figure 1B shows an increase in the linewidth of the resonances, which is expected based on the paramagnetism of deoxy- genated hemoglobin. The increase in the ratio of Ca-quinMF to quinMF can reflect either an actual increase in the Ca\(^{2+}\) level or the presence of extracellular calcium complexed quinMF secondary to cell lysis. The latter interpretation is suggested by the fact that the resonance corre-
responding to Ca-quinMF in Fig 1B appears as a relatively sharp resonance superimposed on a broader resonance, potentially reflecting contributions from extracellular and intracellular Ca-quinMF, respectively. To distinguish further between these two possibilities, EGTA was added to the cell suspension and, as shown in spectrum 2C, the addition of EGTA slightly reduced the Ca-quinMF peak. Thus, the increase in the Ca-quinMF peak was due to an increase in extracellular quinMF. In eight experiments with EGTA used to correct for extracellular FBAPTA, Ca$_{\text{e}}$ averaged $21 \pm 2$ nmol/L after deoxygenation. Similar experiments performed on FBAPTA-loaded sickle erythrocytes gave qualitatively similar results, consistent with the suggestion that deoxygenation does not significantly increase Ca$_{\text{e}}$. The levels of Ca$_{\text{e}}$ under both basal conditions and deoxygenation are higher in FBAPTA-loaded cells than in quinMF-loaded cells, however. Reasons for this difference are addressed in the Discussion section.

During deoxygenation, a sample of cells was taken and fixed with a 0.2% solution of glutaraldehyde. Microscopic examination of 500 erythrocytes showed that >80% were in the sickled conformation. Using $^3$P NMR, we also confirmed that deoxygenation of sickled cells caused no decrease in cell ATP (data not shown). Identical deoxygenation experiments were performed with quinMF-loaded normal erythrocytes. After deoxygenation, Ca$_{\text{e}}$ of normal erythrocytes averaged $17 \pm 4$ nmol/L ($n = 3$), a value not significantly different from either oxygenated normal or deoxygenated quinMF-loaded sickle erythrocytes.

Fig 2. Endocytosis of 1,2 bis-(2-amino-5-fluorophenoxy)ethaneN,N,N',N'-tetraacetic acid (FBAPTA) by sickle erythrocytes. Spectrum (A) shows sickle erythrocytes incubated with impermeant FBAPTA (10 mmol/L) plus sufficient extracellular calcium to give a ratio of CaFBAPTA/FBAPTA of 6/4. The suspension was deoxygenated for 1 hour and then washed twice in modified saline. The cells were resuspended in modified saline containing 1.25 mmol/L of CaCl$_2$ and the spectrum (B) was accumulated. Excess EGTA (20 mmol/L) was then added and another spectrum (C) was accumulated. All spectra were 5,000 acquisitions. CaB, CaFBAPTA; B, FBAPTA.

FBAPTA was intracellular in the form of Ca-FBAPTA. The ratio of the resonance intensity of the FBAPTA peak in the spectrum shown in Fig 2B to that in spectrum A, indicates that 0.4% of the CaFBAPTA was sequestered intracellularly. Similarly, the ratio of the CaFBAPTA peak of the spectrum shown in Fig 2C to that of spectrum A was 0.6%. Similar results were obtained after three repetitions of this study.

In Fig 3, using normal erythrocytes, spectra were obtained under conditions similar to those described for Fig 2. The cells were initially suspended in media containing 10 mmol/L of FBAPTA with 8 mmol/L of CaCl$_2$ to give a ratio of CaFBAPTA/FBAPTA of 4/1 (Fig 3A). Subsequent to a 1-hour period of deoxygenation and two washes, the cells
Fig 3. Endocytosis of 1,2-bis(2-amino-5-fluorophenoxy)ethane N,N,N',N'-tetracetic acid (FBAPTA) by normal erythrocytes. (A) Spectrum shows normal erythrocytes incubated with impermeant FBAPTA (10 mmol/L) plus sufficient extracellular calcium to give a ratio of CaFBAPTA/FBAPTA of 8/2. The RBCs were deoxygenated for 1 hour and then washed twice in modified saline. Spectra (B) and (C) were then accumulated with excess calcium and excess EGTA, respectively. All spectra were 5,000 acquisitions. Ca-B, CaFBAPTA; B, FBAPTA.

were resuspended in modified saline containing 1.25 mmol/L CaCl₂ (Fig 3B). No resonance corresponding to free FBAPTA was observed. The addition of excess EGTA resulted in elimination of the CaFBAPTA resonance and the simultaneous appearance of the free FBAPTA resonance, consistent with the interpretation that all the FBAPTA was extracellular. Similarly, if oxygenated sickle erythrocytes are incubated with FBAPTA, incorporation of FBAPTA does not occur.

The data presented suggest that on deoxygenation, endocytotic vesicles are formed that incorporate extracellular medium, thereby increasing total cell calcium. A final experiment was designed to test the possibility that deoxygenation causes small transient increases in intracellular calcium, which is actively pumped into and sequestered by endocytotic vesicles. For this study, sickle erythrocytes were deoxygenated in a medium in which the ratio of CaFBAPTA/FBAPTA was adjusted to 1/15 (15 mmol/L of FBAPTA with 1 mmol/L of CaCl₂). After incorporation of CaFBAPTA/FBAPTA (1:15) into the vesicles, the cells were washed and resuspended in calcium-containing saline. The erythrocytes were then deoxygenated for a second time while ¹⁹F spectra were observed. The spectrum in Fig 4A shows the ratio of extracellular CaFBAPTA/FBAPTA present when the cells were sickled by deoxygenation for 1 hour. The cells were then washed twice and resuspended in saline containing 1.25 mmol/L of CaCl₂. The spectrum shown in Fig 4B, obtained under oxygen, revealed a peak correspond-

Fig 4. Calcium uptake into endocytotic vesicles during deoxygenation. (A) Spectrum shows erythrocytes incubated with 15 mmol/L of impermeant 1,2 bis(2-amino-5-fluorophenoxy)ethane N,N,N',N'-tetracetic acid (FBAPTA) plus calcium to give a ratio of CaFBAPTA/FBAPTA of 1/15. Cells were deoxygenated for 1 hour, washed and resuspended in an oxygenated buffer containing excess extracellular calcium, and then spectrum B was accumulated. (C) Spectrum was accumulated after the cells were deoxygenated for a second time (20 minutes) in the presence of Ca²⁺. Following addition of excess EGTA to the deoxygenated suspension, spectrum shown in (D) was accumulated. Cells were then washed and resuspended in excess Ca²⁺; and spectrum shown in (E) was obtained.
deoxygenation of sickle erythrocytes. Our studies show that a considerable amount of Ca is sequestered in sickle erythrocytes. The experiments of Lew and colleagues elegantly demonstrate that a transient increase in Ca occurs during deoxygenation of sickle erythrocytes. These data suggest that only deoxygenation of sickle erythrocytes stimulates endocytosis. Incorporation of FBAPTA in sickle cells cannot be due to trapping of FBAPTA between the cells, since: (a) endocytosis could not be demonstrated in normal cells, and it has been shown that the trapped extracellular space is the same in comparisons of oxygenated normal and sickle cells; and (b) the cells were washed twice, and a portion of FBAPTA was not shifted by addition of extracellular Ca²⁺ or EGTA.

Data presented also support the idea that intracellular vesicles can actively accumulate calcium. Such accumulation was demonstrated by deoxygenating sickle erythrocytes that had previously incorporated FBAPTA. When we resickle these cells that have incorporated FBAPTA, we observe a sufficient increase in the ratio of CaFBAPTA/FBAPTA to indicate a 600% increase in calcium in these vesicles. These data suggest that deoxygenation stimulates Ca²⁺ uptake into these intracellular vesicles, consistent with a transient increase in Caᵢ.

These studies indicate that despite the high total calcium that has consistently been observed in sickle RBCs the ionized calcium in the cytosol is not different from that of normal cells. Deoxygenation of sickle RBCs does increase calcium permeability and may result in a slight transient increase in Caᵢ, but this excess calcium is rapidly pumped into and sequestrated by intracellular, endocytotic vesicles and is pumped out of the cytoplasm by CaATPase. In this manner, such vesicles may have an important role in protecting the sickle erythrocyte from the adverse effects known to result from an increase in intracellular calcium. These studies leave open the possibility, however, that despite the capacity of the vesicles for calcium accumulation, brief transients may exist in intracellular calcium and activate a K⁺ channel, leading to ion and water loss from the sickle erythrocyte.

ACKNOWLEDGMENT

We thank Drs. C. Tyler Burt and John C. Parker for their helpful suggestions and critical reading of the manuscript. We also thank Robin Sorrell and Amy Lewter for excellent secretarial assistance, and Robert Hall for designing and constructing the air-driven stirrer.
REFERENCES

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