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

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 $^{19}$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 ± 2 nmol/L (SE). Experiments with normal RBCs gave a mean value of 21 ± 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 FABPTA (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 FABPTA; they had incorporated significant quantities of the extracellular FABPTA. 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.

© 1987 by Grune & Stratton, Inc.

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 anduffy 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 solution, with 50 ìmol/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

From the National Institute of Environmental Health Sciences, Laboratory of Molecular Biophysics, Research Triangle Park, NC; and the Department of Medicine, Division of Hematology, The University of North Carolina at Chapel Hill.

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.

Address reprint requests to Dr Elizabeth Murphy, National Institute of Environmental Health Sciences, Laboratory of Molecular Biophysics, PO Box 12233, Research Triangle Park, NC 27709.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1987 by Grune & Stratton, Inc.

0006-4971/87/6905-0031$3.00/0
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 Ca2+. Cytosolic free Ca2+ (Ca2+) was measured by 19F NMR spectroscopy using a newly described fluorinated calcium chelator, quinMF.11 QuinMF has a much lower dissociation constant than any of the fluorinated chelators originally developed by Smith and co-workers,16 and it is therefore more useful for measuring Ca2+ in erythrocytes.13 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% O2 or 100% N2, resulting in no pH change with the Hepes-Tris buffer. Once inside the cell, the quinMF is in slow chemical exchange with Ca2+. This gives two peaks in the NMR spectrum, one arising from uncomplexed quinMF and another from Ca2+-complexed quinMF (Ca-quinMF). Ca2+ can be calculated using the following equation:

$$\text{Ca}^2+ = K_D \left[\text{Ca-quinMF}\right]/\left[\text{quinMF}\right]$$

Under conditions of nonsaturation or of equal saturation of both 19F 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 tuned to 339.7 MHz for the 19F studies. The sample was shimmed on H2O, 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 19F studies. EGTA was added to correct for any extracellular chelator complexed with extracellular Ca2+. This gives two peaks in the NMR spectrum, one arising from uncomplexed quinMF and another from Ca2+-complexed quinMF (Ca-quinMF). Ca2+ can be calculated using the following equation:

$$\text{Ca}^2+ = K_D \left[\text{Ca-quinMF}\right]/\left[\text{quinMF}\right]$$

Estimation of cell lysis. The percentage of cell lysis was calculated by measuring the hemoglobin (Hb) concentration in the supernatant and comparing this with the total cell Hb concentration. Hb concentration was measured spectrophotometrically at 540 nm.

Materials. The methyl ester of quinMF was synthesized as described,14 and Molecular Probes (Junction City, OR) effected the conversion to the acetoxyethyl ester of quinMF. The potassium salt of FBAPTA was purchased from Sigma.

RESULTS

We were initially interested in determining whether Ca2+ levels increased in sickle erythrocytes particularly during deoxygenation. FBAPTA has been used previously9,11,15 to measure Ca2+ in erythrocytes. The accuracy of the Ca2+ determination in erythrocytes is limited, however, due to the mismatch between the basal level of Ca2+ and the binding constant of FBAPTA for Ca2+, 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 Ca2+ due to erroneous inclusion of a contribution from the extracellular chelator complexed with extracellular Ca2+ present at much higher levels. 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 Ca2+ (Ca2+) 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 Ca2+ in normal erythrocytes. Spectrum B shows the 19F 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 deoxygenated hemoglobin.14 The increase in the ratio of Ca-quinMF to quinMF can reflect either an actual increase in the Ca2+ 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-
sponding 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\(^{2+}\) averaged 21 ± 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\(^{2+}\). The levels of Ca\(^{2+}\) 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 \(^{31}\)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\(^{2+}\) of normal erythrocytes averaged 17 ± 4 nmol/L (n = 3), a value not significantly different from either oxygenated normal or deoxygenated quinMF-loaded sickle erythrocytes.

We were also interested in investigating whether deoxygenation stimulated formation of the intracellular vesicles observed by Lew and colleagues using electron-probe microanalysis. To test this hypothesis, cells (that had not been loaded with the acetoxymethyl ester of FBAPTA) were incubated with the ionized form of FBAPTA, which cannot permeate the membrane. If deoxygenation stimulates endocytosis, FBAPTA, although impermeant, should be detected in the sickle erythrocytes. Results are shown in Fig 2. The initial spectrum, (Fig 2A), was taken with 10 mmol/L of FBAPTA and 6.0 mmol/L of CaCl\(_2\) in the incubating medium, so that the ratio of CaFBAPTA/FBAPTA was 1.5/1.0. The high ratio of signal to noise reflects the high concentration of FBAPTA and the large extracellular volume. The cells were then deoxygenated for 1 hour at 37°C, washed twice in the modified saline solution containing 1.25 mmol/L of CaCl\(_2\) (room air). The \(^{19}\)F NMR spectrum (Fig 2B) was then obtained. This spectrum, both CaFBAPTA and FBAPTA peaks were present, although the ratio of the CaFBAPTA resonance to FBAPTA was larger than in the spectrum shown in Fig 2A. Because excess Ca was present extracellularly, the free FBAPTA peak must have been intracellular. To demonstrate further the presence of endocytosis as opposed to residual extracellular chelator, excess EGTA (20 mmol/L) was added to the incubation medium (spectrum shown in Fig 2C). EGTA shifted some but not all of the resonance intensity from the Ca-FBAPTA position to the free FBAPTA position, confirming that a fraction of the
Fig 3. Endocytosis of 1,2-bis(2-amino-5-fluorophenoxy)ethane N,N,N',N'-tetraacetic acid (FBAPTA) by normal erythrocyes. (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, endocytic 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 endocytic 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 ³¹P 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 endocytic 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'-tetraacetic 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.
ing to uncomplexed FBAPTA despite the addition of 1.25 mmol/L of CaCl2 to the suspending medium. This finding indicates that FBAPTA was incorporated into the endocytic vesicle. The spectrum shown in Fig 4C, taken during a second deoxygenation period, shows that resickling caused an increase in the ratio of CaFBAPTA/FBAPTA to 1/1.5. In this second deoxygenation, we deoxygenated until we saw an increase in the CaFBAPTA peak; this time period (20 to 40 minutes) varied from donor to donor. To confirm that this increase was due to Ca uptake into the vesicles and not to cell lysis and subsequent leakage of FBAPTA into the extracellular high Ca medium, we added excess EGTA. In the spectrum shown in Fig 4D, even with the FBAPTA concentration overestimated due to a pool of extracellular FBAPTA (in excess EGTA, extracellular FBAPTA would be uncomplexed), the ratio of CaFBAPTA/FBAPTA increased. This was confirmed in the spectrum in Fig 4E, which shows the intravesicular ratio of FBAPTA/CaFBAPTA following washing and resuspension of the cells in modified saline.

**DISCUSSION**

NMR studies using fluorinated calcium chelators provide a novel approach to the direct measurement of ionized calcium in human erythrocytes. The technique, originally described by Smith and co-workers and used to measure Ca2+ in thymocytes, is based on the detection of two 19F resonances corresponding to free and calcium-complexed intracellular chelator. Ionized Ca values, calculated using the area under the 19F resonance peaks and the Kd of FBAPTA, range from 30 to 70 nmol/L for normal erythrocytes. The principal limitation encountered arises from a small amount of cell lysis (1% to 3%), which results in FBAPTA in the extracellular medium. With CaCl2 present in the incubating media, any extracellular FBAPTA will be entirely in the CaFBAPTA form, leading to an overestimation of the CaFBAPTA peak and thereby to an overestimation of the calculated level of Ca2+. Because FBAPTA has a Kd more than tenfold above basal Ca2+ levels, the CaFBAPTA peak is less than one-tenth of the FBAPTA peak; therefore, small amounts of fluorinated chelator leakage produce a large percentage of increase in the CaFBAPTA peak. Because of these difficulties, we developed new fluorinated calcium chelators with tighter calcium binding. QuinMF has a Kd of 63 nmol/L and, when loaded into normal erythrocytes, the calculated level of Ca2+ ranges from 15 to 30 nmol/L. The Ca2+ values calculated using FBAPTA are probably overestimated due to slight errors in the estimation of extracellular FBAPTA.

Our experiments have used this NMR technique to investigate calcium homeostasis in sickle erythrocytes. Total calcium measurements in sickle erythrocytes are substantially elevated as compared with those of normal erythrocytes. The experiments of Lew and colleagues elegantly demonstrate that a considerable amount of Ca is sequestered in sickle erythrocytes. These data, however, do not provide information on whether an increase in Ca2+ occurs during deoxygenation of sickle erythrocytes. Our studies show that Ca2+ in well-oxygenated sickle erythrocytes is not significantly different from values of Ca2+ measured in normal erythrocytes. Thirty minutes of deoxygenation give a slight but not significant increase of Ca2+ in sickle erythrocytes, although small transient elevations of Ca2+ during deoxygenation could be missed, since we are signal-averaging for 10 minutes.

It is of interest to study the stimulus for the formation of these vesicles as well as to investigate the means by which these vesicles sequester Ca2+. Data presented in this article showing cellular incorporation of impermeant FBAPTA on deoxygenation suggest that these calcium-containing vesicles are formed by endocytosis of the cell membrane and that during endocytosis they envelop extracellular Ca2+. Similar incorporation could not be demonstrated with either oxygenated sickle erythrocytes or deoxygenated RBCs from normal control subjects, suggesting 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 or deoxygenated 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 Ca2+.

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 Ca2+, but this excess calcium is rapidly pumped into and sequestered 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

Cytosolic free calcium levels in sickle red blood cells

E Murphy, LR Berkowitz, E Orringer, L Levy, SA Gabel and RE London