Stabilization of the Shape of Sickled Cells by Calcium and A23187

By Margaret R. Clark, Alfred C. Greenquist, and Stephen B. Shohet

Evaluation of the role of calcium in irreversible sickling has been approached by treating sickled cells with calcium and the ionophore A23187. A calcium-dependent stabilization of the sickled cell shape was observed after reoxygenation of cells in the presence of ionophore. At low calcium concentrations, this retention of sickled shape was maintained for periods up to 1 hr. However, the morphology of the oxygen-stable sickled cells was like that of de-oxygenated sickle cells and significantly different from the characteristic morphology of native irreversibly sickled cells (ISCs). Because the stabilized cells did not fulfill the morphological criterion for ISCs, the shape-stabilizing effect of calcium in this system did not provide additional support for the hypothesis that calcium accumulation was the determining factor in ISC generation.

Although the fundamental defect in sickle cell anemia is known to be abnormal genetic coding for the hemoglobin molecule, the mechanism by which the molecular defect is ultimately expressed at the clinical level is not well understood. The principal clinical problems are anemia and painful vasocclusive crises. Serjeant et al.1 have demonstrated that the shortened red cell lifetime responsible for the anemia bears a direct correlation to the proportion of irreversibly sickled cells (ISCs) in the blood of a given patient. There has been no consistent evidence, on the other hand, linking the number of ISCs to the frequency or severity of painful crises. However, as Messer2 has pointed out, because of their high cell hemoglobin concentration, ISCs become rigid much more rapidly than disc-shaped reversible sickle cells (RSCs) upon deoxygenation. In addition, they regain deformability much more slowly than RSCs when reoxygenated. Thus ISCs might occlude areas in the microcirculation during the blood’s rapid passage through the regions of low oxygen tension.

Many people have recognized the possible importance of the ISC in the pathophysiology of sickle cell anemia, and there has been much interest in defining the mechanism by which they are generated from RSCs. Recently, there have been several observations suggesting that calcium accumulation might play an important role in the formation of ISCs. Sickle cells accumulate calcium during deoxygenation,3,4 and ISCs appear to contain excessive amounts of calcium.
of calcium associated with their membranes. In addition, many of the properties of ISCs, such as abnormally low potassium and moderately elevated sodium concentrations, cellular dehydration, and decreased cell deformability are produced in normal cells by calcium accumulation. We have previously used a model system, the hybrid erythrocyte, to investigate the effects of the introduction of calcium into the cell upon the generation of ISCs. In these experiments, hybrids containing EGTA, an efficient calcium-chelating agent, sickle irreversibly to the same extent as do hybrids containing 1 mM calcium. This result has suggested that factors other than calcium accumulation might be important in the generation of ISCs. However, because the hybrid model might not have reproduced the physiologic situation accurately, we have chosen to examine the effect of calcium introduction into intact sickle cells as well.

We have used the calcium ionophore A23187 to increase the internal calcium in sickled cells without severe metabolic depletion. We found that this treatment caused some of the sickled cells to retain their deoxygenated shape after they were reoxygenated, in accord with the results of similar experiments recently reported by Eaton et al. However, the stabilization of the sickled form by calcium and ionophore did not reproduce the characteristic morphology of native ISCs, again suggesting that some factors other than calcium accumulation may influence the genesis of ISCs in vivo.

MATERIALS AND METHODS

Blood was drawn from homozygous sickle cell patients into ACD or EDTA anticoagulant. The cells were washed in buffered saline with glucose and potassium* (BSKG), and were then centrifuged on gradients of Stractan II to remove the dense native ISCs. We followed the method of Corash et al. for the separation of normal cells on the basis of density, with two modifications. Deionization of the Stractan was accomplished by mixing the resin directly into Stractan solutions, followed by suction filtration on Whatman No. 1 paper to remove the resin. In addition, the densities of the Stractan solutions were increased to a range extending from 1.096 to 1.115 g/ml. After removal of ISCs, the disc-shaped sickle cells were washed free of Stractan by centrifugation in BSKG. Prior to deoxygenation, the cells were suspended at approximately 4% hematocrit in BSKG, sometimes with added MgCl2 at 0.25 or 1 mM concentration. Final concentrations of either 1 mM EGTA, or 1, 10, or 100 μM CaCl2 were added to duplicate samples, retaining a pair of samples with addition of equal volumes of saline as controls. With the exception of one experiment, the incubations were performed in protein-free medium because of the fact that A23187 is bound effectively by albumin, so much so that albumin can be used for rapid removal of the ionophore from red cells. The cells were incubated in a humidified nitrogen atmosphere at 37°C until a majority of the cells had sickled, and then A23187 was added to a final concentration of 20 μM to facilitate calcium permeation across the membrane. Deoxygenation was continued for 10 or 30 min, at which time portions of the cell suspensions were fixed in 3% ice-cold glutaraldehyde for determination of the total numbers of sickled cells. At the same time, portions were removed and reoxygenated in air for 30 min to determine if any of the cells had sickled irreversibly under the influence of Ca2+ and ionophore. In some experiments, reoxygenated cells were fixed in glutaraldehyde after various periods of reoxygenation to determine if the stabilization of sickled forms was temporary.

*Buffered saline with potassium and glucose was prepared as follows: NaCl, 7.808 g; KCl, 0.373 g; Na2HPO4·7H2O, 2.302 g; NaH2PO4·H2O, 0.194 g; glucose, 2.0 g; made up to 1 liter and adjusted to 290–295 mOsm/kg and pH 7.4 if necessary.
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In other experiments, the cells were incubated for various periods of time before addition of the ionophore. These cells were deoxygenated in BSKG with and without glucose, in the presence of 10 \( \mu M \) EGTA to control trace amounts of calcium in the buffer. After 2 and 9 hr of incubation, either 20 \( \mu M \) Ca or 1 \( mM \) EGTA was added, followed immediately by the addition of A23187 to 20 \( \mu M \) concentration. Deoxygenated and reoxygenated cells (30 min equilibration with air) were fixed in glutaraldehyde for morphological evaluation.

Additional experiments involved prolonged deoxygenation of cells in the absence of ionophore. The cells were incubated in the presence of either 1 \( mM \) EGTA or 1 \( mM \) calcium, with other additions as noted in the Results.

Quantitative evaluation of samples for the presence of irreversible sickling was performed by counting glutaraldehyde-fixed cells under phase microscopy. For each sample, we performed four to six counts of 200 cells each. The extent of total sickling was determined from counts on cells fixed prior to reoxygenation. Samples of cells fixed after 30 min reoxygenation in air (except where noted otherwise) were used for determination of the proportion of cells that remained permanently distorted after reoxygenation. Cell morphology was evaluated by phase contrast microscopy and, in some instances, by scanning electron microscopy.

To see whether the introduction of calcium had produced any detectible alterations in membrane proteins, we prepared ghosts from some of the cells after reoxygenation. The ghosts were prepared by the Dodge procedure, except that 20 \( mM \) Tris-HCl buffer at pH 7.4 was used for hemolysis rather than phosphate buffer. Membranes were dissolved in 10\% SDS, 50 \( mM \) dithiothreitol solution, and were electrophoresed on 5\% polyacrylamide gels containing 0.1\% SDS according to the method of Weber and Osborn.

Table 1. Stabilization of the Sickled Form by Ca and A23187

<table>
<thead>
<tr>
<th>Ca (( \mu M ))</th>
<th>Total Sickness</th>
<th>Sickled Cells after 30 min Reoxygenation (%)</th>
<th>Stabilization of Sickled Forms by Ca (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. With N(_2) (%)</td>
<td>0.25 ( mM ) Mg</td>
<td>1 ( mM ) Mg</td>
</tr>
<tr>
<td>1 ( \mu M ) Ca</td>
<td>a 81 ( \pm 3 )*</td>
<td>12 ( \pm 6 )</td>
<td>15*</td>
</tr>
<tr>
<td></td>
<td>b 85 ( \pm 1 )</td>
<td>20 ( \pm 1 )</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>c 45 ( \pm 3 )</td>
<td>34 ( \pm 1 )</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>d 66 ( \pm 3 )</td>
<td>0,44 ( \pm 8 )</td>
<td>2 ( \pm 1 )</td>
</tr>
<tr>
<td>10 ( \mu M ) Ca</td>
<td>c 45 ( \pm 3 )</td>
<td>37 ( \pm 3 )</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>e 52 ( \pm 4 )</td>
<td>26 ( \pm 0 )</td>
<td>50*</td>
</tr>
<tr>
<td></td>
<td>f 55 ( \pm 4 )</td>
<td>37 ( \pm 5 )</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>d 66 ( \pm 4 )</td>
<td>43 ( \pm 6 )</td>
<td>2 ( \pm 1 )</td>
</tr>
<tr>
<td></td>
<td>g 63 ( \pm 5 )</td>
<td>44 ( \pm 6 )</td>
<td>9 ( \pm 0 )</td>
</tr>
<tr>
<td>50 ( \mu M ) Ca</td>
<td>g 63 ( \pm 5 )</td>
<td>29 ( \pm 2 )</td>
<td>30 ( \pm 7 )</td>
</tr>
<tr>
<td>100 ( \mu M ) Ca</td>
<td>c 45 ( \pm 3 )</td>
<td>12 ( \pm 1 )</td>
<td>29 ( \pm 3 )</td>
</tr>
<tr>
<td></td>
<td>h 45 ( \pm 5 )</td>
<td>5 ( \pm 2 )</td>
<td>11 ( \pm 2 )</td>
</tr>
<tr>
<td></td>
<td>d 66 ( \pm 4 )</td>
<td>25 ( \pm 6 )</td>
<td>27 ( \pm 2 )</td>
</tr>
<tr>
<td></td>
<td>g 63 ( \pm 5 )</td>
<td>21 ( \pm 2 )</td>
<td>25 ( \pm 4 )</td>
</tr>
</tbody>
</table>

*Data given as the mean of four to six determinations, \( \pm \) SEM.

\( * \) In these experiments, samples were taken only 10 min after addition of A23187. In all others, samples were taken 30 min after A23187 addition.

Cells were incubated in buffered saline at 37\(^\circ\)C in a N\(_2\) atmosphere. Ca and Mg were added at the beginning of deoxygenation. After 1 hr deoxygenation, A23187 was added and allowed to equilibrate (with continued deoxygenation) for 10 or 30 min. Samples of cells were then fixed in 3\% glutaraldehyde for determination of total sickling with N\(_2\). At the same time, paired samples were reoxygenated for 30 min and then fixed in glutaraldehyde for determination of the sickled forms present after reoxygenation. Percent stabilization of sickled forms was calculated from the ratio of sickled cell counts after and before deoxygenation. With the exception of experiments a, b, and e, controls in which 1 \( mM \) EGTA was included showed 2\% or fewer sickled cells after reoxygenation. Blood used in experiments a, b, and e contained 5\%, 13\%, and 6\% residual ISCs; additional stabilization of sickled cells was not observed in the EGTA controls.
Table 2. Effect of Reoxygenation Time on Sickled Shape Retention

<table>
<thead>
<tr>
<th>Ca²⁺ Concentration</th>
<th>Time After Initial Reoxygenation (min)</th>
<th>Sickled Cells in N₂ (%)</th>
<th>Sickled Cells after O₂ (%)</th>
<th>Stabilization of Sickled Form (%)</th>
</tr>
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<tr>
<td>10 µM</td>
<td>5</td>
<td>45 ± 3*</td>
<td>43 ± 5*</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>45 ± 3</td>
<td>37 ± 5</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>45 ± 3</td>
<td>37 ± 3</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>45 ± 3</td>
<td>32 ± 3</td>
<td>71</td>
</tr>
<tr>
<td>100 µM</td>
<td>5</td>
<td>43 ± 1</td>
<td>18 ± 5</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>43 ± 1</td>
<td>13 ± 3</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>43 ± 1</td>
<td>12 ± 1</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>43 ± 1</td>
<td>7 ± 2</td>
<td>16</td>
</tr>
</tbody>
</table>

*Cells were incubated under the same conditions described for Table 1. Thirty minutes after addition of A23187, samples were fixed for determination of total sickled cells, and additional samples were reoxygenated. At the indicated times, portions of the reoxygenated samples were fixed for determination of stabilized sickled cells. Percent stabilization was calculated from the ratio of sickle cells after and before reoxygenation.

*Data given as the mean of four to six determinations ± SEM.

RESULTS

The addition of A23187 to sickled cells in a calcium-containing medium promotes the retention of the sickled form. As the data in Table 1 demonstrate,* this effect is dependent upon the presence of calcium and does not occur when the ionophore is added to suspensions containing EGTA. In these initial experiments, in which we counted fixed sickle forms after 30 min of reoxygenation, calcium appears to have had its maximum effect near 10 µM concentration. Calcium at 1 µM concentration gave a variable effect and may represent the minimum borderline concentration at which calcium causes retention of the sickled shape. Calcium at 50 and 100 µM concentrations also stabilizes the sickled cells, but appears to be less effective under these conditions.

Table 1 also contains the results of experiments in which 1 mM magnesium was included in the incubation medium. These data show that 1 mM magnesium virtually eliminates the stabilizing effect of calcium up to 10 µM concentration, but that it does not inhibit the stabilization of sickled form by 50 or 100 µM calcium. Magnesium (0.25 mM) has no effect on the stabilization phenomenon.

We investigated the permanence of the calcium stabilization of the sickled form by fixing cells at various periods following reoxygenation. These experiments showed a sharp reduction in the percentage of sickled cells with increasing periods of reoxygenation (Table 2). At 10 µM calcium, some reduction of sickling with increased reoxygenation time also occurred, but the effect was much less pronounced.

Accompanying the variations in the proportion of oxygen-stable sickled cells, an expected increase in echinocytes with increasing calcium concentration also

*Note the relatively low percentage of sickling with N₂ in most of the samples (45%-66%). The blood for these experiments was obtained from patients who had received transfusions of normal blood within the recent past. The degree of sickling was consistent with the amount of hemoglobin A determined by electrophoresis (from 40%, Hb S 3 days prior to blood withdrawal to 70%, Hb S the day before blood withdrawal).
occurred.\textsuperscript{11,21} The echinocytic transformation was not the result of incubation in protein-free medium, since it occurred only in the samples containing calcium, and only after addition of the ionophore permitted entry of calcium to the cell interior. The degree of echinocytosis was not uniform; i.e., there were earlier- and later-stage echinocytic forms in all the calcium-containing samples. However, there was a distinct advance in the proportion of late-stage echinocytes and spheroechinocytes as the calcium concentration was increased from 1 to 100 \( \mu M \). Most of the cells that did not remain sickled in the presence of 100 \( \mu M \) calcium became spheroechinocytes upon addition of the A23187.

In several experiments the ionophore was added to the cells, together with 100 \( \mu M \) calcium or 1 mM EGTA, just before deoxygenation was begun. The presence of calcium in these experiments greatly reduced the extent of total sickling during deoxygenation. In a typical experiment, 80\% of the cells with EGTA plus A23187 sickled after 1\( \frac{1}{2} \) hr deoxygenation, whereas only 20\% of the cells with 100 \( \mu M \) calcium plus the ionophore were sickled at the same time. Under these conditions, there was little stabilization of the few sickled forms in the presence of calcium.

Fig. 1. Isolated membranes dissolved in 1\% SDS and electrophoresed on 5\% polyacrylamide gels containing 0.1\% SDS. Sample on left is from cells incubated with EGTA and A23187, and on right cells incubated with Ca\(^{2+}\) and A23187.
The amount of A23187 relative to the number of cells was important. When the ionophore to cell ratio was decreased from 0.5 zmole/ml cells by increasing the hematocrit from 4% to 24%, there was no stabilization of sickled cells. This observation was true at 1, 10, and 100 mM Ca concentrations.

To determine if irreversible modification in membrane polypeptides occurred as a consequence of the calcium-ionophore-induced stabilization of the deoxy sickle cell configuration, the polypeptides of isolated membranes treated with ionophore were examined on SDS-PAGE, as shown in Fig. 1. In this representative example, membranes were examined from cells described in Table I, experiment c. Membrane polypeptides from samples exposed to ionophore and EGTA (on left) showed no marked differences from membranes exposed to ionophore plus calcium (on right). Quantitative densitometry of the stained gels confirmed the absence of pronounced differences in polypeptide patterns.

Although the presence of calcium plus A23187 stabilized some cells in sickled conformation, the morphology of the stabilized cells after reoxygenation was...
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identical to the morphology of deoxygenated cells and, significantly, not equivalent to the form of native ISCs. Figure 2 presents scanning electron micrographs of sickle cells treated with 10 \( \mu M \) calcium and 20 \( \mu M \) A23187 in deoxygenated and reoxygenated states. Representative native ISCs separated from the blood of the same patient are also shown. Note the presence of distinct spicules of the cells prepared in vitro (Figs. 2A-2D). In contrast, such spicules are totally absent in native ISCs (Fig. 2E).

To test the possibility that the spiculated morphology of the “fixed” sickle cells was merely the result of incubation in protein-free buffer, we added calcium and ionophore to sickled cells in the presence of 0.3 g/100 ml bovine serum albumin. Although the effective concentration of ionophore was not well defined under these conditions, there was sufficient drug available to the cells to stabilize the sickled form. The stabilized cells were identical to those observed in the absence of protein, characterized by multiple, sharp spikes.

Experiments involving prolonged incubation before addition of the drug further emphasized the importance of cell morphology in these experiments. If the ionophore and calcium were added to cells following only 2 hr deoxygenation, all the cells remaining sickled after deoxygenation were of the sharply spiculated type illustrated in Figs. 2C and D. If, on the other hand, the cells were deoxygenated for 9 hr before addition of calcium and A23187, approximately half the cells remaining sickled after reoxygenation had the rounded contours of native ISCs. In addition, the other sickled cells did not retain the sharp spikes of the sickled cells stabilized early during deoxygenation, but they had the appearance of cells in the process of unsickling, with apparent fragmentation occurring at the ends of the spicules.

An additional group of experiments was performed involving prolonged deoxygenation of sickle cells without addition of ionophore. Cells were incubated in the presence of either 1 mM EGTA or 1 mM calcium. Other conditions that were varied included the presence and absence of glucose, magnesium, and bovine serum albumin. A consistent observation was the generation of irreversibly sickled cells in the presence of EGTA following deoxygenation for 18 hr. The percentage of ISCs varied from 10% to 30% in samples containing EGTA. In the corresponding samples containing calcium, the ISCs ranged from 6% to 37% after 18 hr. An additional point of interest in these experiments was that the classic native morphology of ISCs was present only when the cells were incubated with glucose. Although cells were distorted in a manner reminiscent of ISCs in the absence of glucose, they tended to be swollen and echinocytic. The echinocytic spines on these cells were clearly different from the sharply extended spikes on the ionophore-stabilized cells. In none of the experiments involving prolonged deoxygenation did we observe the spiculated “fixed sickle” form characteristic of the ionophore experiments. It should be emphasized that ISCs were generated after 18 hr deoxygenation in the presence of glucose, both with EGTA and with calcium in the medium.

DISCUSSION

These experiments demonstrate that introduction of calcium into a sickled cell by means of A23187 can stabilize the cell in its deoxy configuration.
The retention of the sickled form in calcium-treated cells persisted up to 1 hr at low calcium concentrations. While these experiments were being completed, Eaton et al.\textsuperscript{16} also reported preliminary results on the production of ISCs in the presence of A23187 and calcium. However, they found a complete transformation of sickled cells to spheroechinocytes within a few minutes. Slight differences in method possibly may account for this result.

Our experiments conducted over a range of calcium concentrations indicate that the extracellular concentration of calcium is an important factor in the stabilization of the sickled form. The variable results at 1 \( \mu M \) suggest that this may be the lower limit at which this effect can be observed. The apparent decrease in the ability of calcium to stabilize sickled cells at 100 \( \mu M \) suggests that at this concentration the echinocytogenic effect of calcium predominates over its stabilizing effect upon the membrane.\textsuperscript{21} We have not measured the amount of calcium accumulated by the cells in these experiments. Kirkpatrick et al.\textsuperscript{14} have shown that 10 \( \mu M \) A23187 added to cells at 10% hematocrit results in uptake of calcium from the medium to a level of at least 80\% of the external calcium concentration. Our experiments have used twice this ionophore concentration and only 4\% hematocrit, and we expect that the internal calcium concentration would lie between 80\% and 100\% of the external concentration upon addition of ionophore.

Our results qualitatively confirm the observation of Eaton et al.\textsuperscript{16} that magnesium can inhibit the stabilization of sickled cells by calcium. However, the effect is only observed at low calcium concentrations.

Carraway et al.\textsuperscript{22} have shown that calcium can induce aggregation of ghost membrane polypeptides observed in SDS PAGE. This effect is apparent when ghosts are hemolyzed in the presence of calcium at 500 \( \mu M \), but not 100 \( \mu M \) concentration. We have not observed membrane polypeptide changes after exposure of cells to ionophore and calcium. However, the formation of SDS dissociable aggregates or minor polypeptide modifications that are morphologically significant cannot be dismissed.

Although these experiments demonstrate that calcium can cause sickled cells to remain sickled after reoxygenation, it appears inappropriate to extrapolate from this observation to a causal role for calcium in the generation of ISCs. In order to show that calcium accumulation could be a mechanism for the generation of ISCs, it is necessary to show that the in vitro calcium-mediated model meets criteria established for the identification of physiologic ISCs. At least three criteria need to be satisfied: (1) characteristic changes in intracellular contents, (2) permanence of the shape change, and (3) specific morphology. Calcium introduction into sickled cells does produce cells that contain abnormally low K, high Na, decreased ATP, increased MCHC, and reduced deformability.\textsuperscript{16} Indeed, this change could have been anticipated, since calcium is known to produce the same effects in normal red cells. In fact, the similarities in the physical characteristics of ISCs and normal cells containing abnormally high levels of calcium had provided the original basis for the suggestion that calcium might be important in the generation of ISCs.\textsuperscript{23} Hence, the reproduction of these effects simply by facilitating calcium ingress with the ionophore constitutes insufficient evidence for a primary role of calcium in ISC
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A second criterion is the persistence of permanently altered morphology. Although Eaton’s experiments have not reproduced this feature, our experiments do show that appropriate selection of calcium concentration can produce a persistent change of shape in this system.

There still remains one additional critical problem in the fact that the morphological criterion for ISCs is not met by the cells in these experiments. The stabilized sickled cells produced by the ionophore and calcium are spiculated like deoxy sickled cells. This shape is in striking contrast to the smoothly contoured native ISCs found in vivo. It may be argued that the ISCs formed in vivo originally had spicules, but that movement through the circulation caused their loss and resulted in the rounded contours of the ISCs seen on peripheral smears. However, Shen has shown that ISCs which are generated during prolonged in vitro deoxygenation without metabolic substrate24 lack spicules and are identical in appearance to native ISCs. Shen’s experiments have involved incubation of whole blood, in which the presence of plasma proteins could conceivably have influenced cell morphology. However, we also find that cells with the native morphology of ISCs are generated during prolonged deoxygenation in protein-free buffer solutions. Moreover, the presence of albumin during the stabilization of sickled form by calcium and A23187 has no effect on stabilized cell morphology. (It should be noted that in vitro ISCs generated during anoxic incubation also display the other known characteristics of native ISCs, such as altered cations, MCHC, etc.25, 26)

Further indication that the morphological distinction is important comes from the ionophore experiments involving prolonged deoxygenation. These experiments demonstrate that if ionophore is added to calcium-incubated cells early in the incubation only the spiculated “fixed sickle” morphology is found. If the cells are incubated for prolonged periods, however, addition of ionophore and calcium produces permanently distorted cells without sharp spicules, very similar in appearance to native ISCs. Thus it can be concluded that a “fixation” form is induced by calcium and that it differs from either native ISCs or those generated in vitro during prolonged deoxygenation. It may be relevant that the “irreversibly sickled” erythrocyte hybrids which are formed in the presence of calcium appear to be spiculated “fixed” sickle forms, whereas those generated in EGTA medium look like native ISCs (data not shown here).

The hypothesis that calcium accumulation is the critical event in the generation of ISCs is appealing because of its logical consistency in terms of the known effects of calcium on normal cells. However, there are experimental observations that are not consistent with a primary role for calcium. For example, while Jensen et al.27 have found that addition of EDTA to serum-free buffer solutions eliminates the generation of ISCs during lengthy deoxygenation, in the presence of serum and 5 mM EDTA the formation of ISCs is reduced but not abolished. Using the data of Portzehl et al.28 on binding constants of EDTA for magnesium and calcium, we calculate that the concentration of free calcium in those experiments should have been only $1.5 \times 10^{-8} \, M$. In addition, Glader25 has reported that EDTA reduces but does not prevent irreversible sickling in serum-free medium. Our own experiments reported here, involving prolonged incubation with EGTA, show persistence of irreversible
sickling in the absence of calcium. Furthermore, in the experiments with erythrocyte hybrids, EGTA has had no inhibitory effect upon irreversible sickling.\textsuperscript{13}

All these observations, along with our present results concerning the transformation in morphology of calcium–A23187-stabilized cells to a more native conformation with prolonged incubation, suggest that there are calcium-independent factors, perhaps related to the metabolic state of the cell, which are involved in the generation of ISCs. Thus, while calcium accumulation may in some circumstances accelerate or enhance the formation of ISCs, it may not be the initiating or even a requisite step in the process. While ATP depletion has been implicated in the generation of ISCs, it may also be that this mechanism does not provide sufficient explanation for the process by itself. Such a possibility is suggested by our observations that the native morphology of ISCs is produced during in vitro incubation only in the presence of glucose. It may well be that the generation of ISCs involves the concerted effect of more than one factor, rather than proceeding as the inevitable result of a single critical event. Even apart from this consideration, the present experiments demonstrating stabilization of the sickled form by calcium and A23187 do not provide any new support for the hypothesis that calcium accumulation is the primary factor in ISC generation, because they do not reproduce the natural phenomenon of irreversible sickling.

REFERENCES

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