Increased Ca\(^{++}\), Mg\(^{++}\), and Na\(^{+}\) + K\(^{+}\) ATPase Activities in Erythrocytes of Sickle Cell Anemia

By Madan G. Luthra and David A. Sears

RED BLOOD CELLS (RBC) from patients with sickle cell anemia (SS) show significant membrane abnormalities,\(1,2\) and it has been suggested that the accumulation of Ca\(^{++}\) in cells plays a role in producing these abnormalities.\(3,4\) The Ca\(^{++}\) content of SS RBC is at least twice that of normal cells, and the most dense cells, enriched with irreversibly sickled cells (ISC), have Ca\(^{++}\) levels as high as four times that of normal cells.\(5,6\) Increased Ca\(^{++}\) uptake in deoxygenated cells has also been reported by Eaton et al.\(6\) and by Palek.\(7\) Recently, Gopinath and Vincenzi\(9\) and Dixon and Winslow\(10\) reported significantly lower calmodulin-sensitive Ca\(^{++}\) ATPase activity in SS RBC membranes and suggested that defective Ca\(^{++}\) extrusion contributes to the accumulation of Ca\(^{++}\) in these cells.

The level of activity of calmodulin-sensitive Ca\(^{++}\) ATPase expressed in normal RBC depends significantly on the type of hemolytic procedure employed.\(11\) Cells hemolyzed in saponin expose greater activity than isolated membranes or reconstituted systems of membranes plus cytosol. SS RBC hemolysates had greater Ca\(^{++}\) ATPase activity than normal hemolysates; they exhibited higher Mg\(^{++}\) and Na\(^{+}\) + K\(^{+}\) ATPase activities as well. Assays on density (age) fractions of SS and normal red cells demonstrated that all ATPase activities were highest in low density (young) cells, and activities in SS red cells exceeded those in normals in all fractions studied. Thus, when studied under conditions that maximize enzyme activity, Ca\(^{++}\) ATPase activity, like Mg\(^{++}\) and Na\(^{+}\) + K\(^{+}\) ATPase, is actually increased in SS RBC, probably due to the young red cell population present. The elevated Ca\(^{++}\) levels in these cells are more likely due to an increased Ca\(^{++}\) leak or abnormal calcium binding than to defective extrusion by the ATPase pump.

Materials and Methods

Chemicals

1-Histidine, disodium ATP, Trizma base, and EGTA were purchased from Sigma Chemical Company (St. Louis, Mo). Saponin was a product of Calbiochem (La Jolla, Calif.); it was used without further purification. Chloroform and methanol were purchased from Fisher Scientific Company (Pittsburgh, Pa.).

Blood Samples, Counts, Density Separation

Blood was drawn from healthy donors and patients with sickle cell anemia and anticoagulated with heparin. Reticulocyte percentages were determined by counts of at least 500 cells after staining with new methylene blue. Percentages of ISC were determined by counts of at least 500 cells in wet preparations utilizing the criteria of Lux et al.\(11\) Density separation of RBC suspended in autologous plasma was carried out by the method of Murphy\(14\) as outlined previously.\(15\) Five equal fractions were collected from the top to bottom of the centrifuged tube and numbered 1–5, respectively. Fractions 1, 3, and 5 were used for the measurement of ATPase activities.

Preparation of Hemolysates and Membranes

Unfractionated and density fractionated cells were centrifuged, and plasma and buffy coat were removed. The packed cells were washed three times in 0.17 M Tris-HCl buffer, pH 7.6, resuspended in the same buffer, and stored on ice. Saponin hemolysates were prepared by the addition of saponin (0.2 mg/ml) to 10% RBC suspensions. Calmodulin-deficient membranes were isolated from RBC by lysis in 20 milliosmolar Tris-HCl buffer, pH 7.6, as described previously.\(15\) Unless otherwise indicated, the supernatant fluid from the first step of this hypotonic lysis procedure was used as the source of membrane-free hemolysate containing calmodulin. Hemoglobin was measured by the method of Kachmar.\(16\)

From the Department of Medicine, Baylor College of Medicine, Houston, Texas.

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Address reprint requests to Madan G. Luthra, Department of Medicine, Baylor College of Medicine, 1200 Moursund, Houston, Texas 77030.

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1332
Measurement of ATPase Activities

One-tenth milliliter of saponin hemolysate was assayed for ATPase activities in a total volume of 0.7 ml containing 3.1 mM MgCl₂, 2.1 mM disodium ATP, 68 mM NaCl, 28 mM KCl, 68.5 mM histidine (pH 7.5), and, in appropriate cases, 4.8 μM Ca²⁺, μM EGTA, or 180 μM EGTA and 0.5 mM ouabain. The reaction was begun by the addition of hemolysate, quickly followed by the addition of ATP. The reaction was terminated after incubation for 2 hr at 37°C by the addition of 1.4 ml of chloroform-methanol mixture (2:1, v/v). The inorganic phosphate released from ATP was measured in the aqueous phase as described previously. ATPase activities were expressed as μmoles of inorganic phosphate (Pi) released per gram hemoglobin. In the case of hemoglobin-free membranes, the reference is to the hemoglobin content of the RBC from which the membranes were prepared. The activity obtained in the presence of 180 μM EGTA was considered to be Na⁺ + K⁺ + Mg²⁺ ATPase and was subtracted from the activity obtained in the presence of added calcium (total ATPase) to obtain Ca²⁺ ATPase. The activity obtained in the presence of 180 μM EGTA and 0.5 mM ouabain was defined as Mg⁺⁺ ATPase. The activity of Na⁺ + K⁺ + Mg⁺⁺ ATPase minus that of Mg⁺⁺ ATPase gave Na⁺ + K⁺ ATPase. The definitions of these ATPase activities are summarized in Table 1.

RESULTS

ATPase activities varied with the concentration of saponin and the time of exposure of RBC to the lytic agent (Figs. 1 and 2). Maximum ATPase activities were produced by 0.2 mg/ml concentrations of saponin and 20-min exposure times, so these conditions were chosen for the assays.

Isolated RBC membranes contained less ATPase activity than hemolysates, and the difference was especially marked in the case of Ca²⁺ ATPase (Table 2). Ca²⁺ ATPase activity was increased when the incubation medium was fortified with membrane-free hemolysate containing calmodulin (Fig. 3). Nevertheless, the Ca²⁺ ATPase activity in saponin hemolysates still exceeded that in reconstituted systems of membranes plus membrane-free hemolysate (Fig. 3).

Figure 4 shows Ca²⁺ ATPase, Mg⁺⁺ ATPase, and Na⁺ + K⁺ ATPase activities of normal and SS hemolysates. Ca²⁺ ATPase activities in normal and SS hemolysates were 137 ± 17 (mean ± SD) and 157 ± 31, respectively. Thus Ca²⁺ ATPase activity in SS RBC was slightly but significantly greater than that in normal RBC (t test, p < 0.02). Similarly, the values for Mg⁺⁺ and Na⁺ + K⁺ ATPase activities were greater

<table>
<thead>
<tr>
<th>Table 1. Definition of ATPase Activities</th>
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<td>ATPase Activity</td>
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</tr>
<tr>
<td>(1) Total</td>
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<tr>
<td>(2) Na⁺ + K⁺ + Mg⁺⁺</td>
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<tr>
<td>(3) Mg⁺⁺</td>
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<td>(4) Na⁺ + K⁺</td>
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<td>(5) Ca²⁺</td>
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Fig. 1. Effect of saponin concentrations on ATPase activities of normal and SS hemolysates after 20-min incubation at 22°C. The solid lines show SS hemolysates, the dotted lines normal hemolysates.

Fig. 2. Effect of incubation time on ATPase activities of normal and SS hemolysates in the presence of 0.2 mg saponin/ml at 22°C. The solid lines show SS hemolysates, the dotted lines normal hemolysates.
in SS than normal RBC ($p < 0.001$). All the ATPase activities maintained the observed difference between normal and SS when the data were expressed on the basis of the number of cells (data not shown). The magnitude of the difference in activities was greatest for Mg$^{2+}$ ATPase and least for Ca$^{2+}$ ATPase.

The observed increase in Ca$^{2+}$ ATPase activity in SS RBC could have been due to the elevated levels of Ca$^{2+}$ in these cells. Therefore, we compared Ca$^{2+}$ ATPase activities of normal and SS RBC at various Ca$^{2+}$ concentrations. As shown in Fig. 5, SS RBC displayed higher Ca$^{2+}$ ATPase activity than normal cells at all Ca$^{2+}$ concentrations tested.

We have previously shown in normal subjects that young red cells contain higher Ca$^{2+}$ and other ATPase activities than older cells. Therefore, we compared ATPase activities of density (age) separated normal and sickle cells. Figure 6 shows data on ATPase activities of fractions 1, 3, and 5 (top, middle, and bottom 20% fractions, respectively) of RBC from 8 normal and 8 sickle cell anemia patients. Confirming our previous observations, the lighter cells from normals expressed more ATPase activities than heavier cells. Similar patterns were observed in SS RBC. Moreover, the differences between lightest (young) and heaviest (old) RBC from SS patients were greater than in normal RBC (Fig. 6). All 3 density fractions

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**Table 2. ATPase Activities of Saponin Hemolysates and Isolated Red Cell Membranes in Normal Subjects**

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<tr>
<th>Type of ATPase</th>
<th>Activity (μmole Pi Released/2 hr/g Hb)</th>
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<tr>
<td>Na$^+$ + K$^+$</td>
<td>Hemolysates 17.4 ± 1.8 Membranes 8.2 ± 1.2</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>Hemolysates 14.7 ± 1.8 Membranes 4.8 ± 1.0</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Hemolysates 137.7 ± 17.0 Membranes 13.0 ± 1.5</td>
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The data shown are means and standard deviations of assays in 15 normal subjects.
assayed showed higher ATPase activities in SS than in normal RBC. Differences between SS and normal RBC were greater in Mg$^{2+}$ and Na$^+ + K^+$ ATPase activities than in Ca$^{2+}$ ATPase activity, particularly in the lightest cells.

DISCUSSION

Red cell Ca$^{2+}$ levels have been shown to be elevated in SS, and it has been suggested that this may have pathologic importance. The mechanism by which red cell Ca$^{2+}$ becomes elevated in SS red cells is not clear. Palek and Eaton et al. showed increased Ca$^{2+}$ influx in SS cells, suggesting abnormal red cell Ca$^{2+}$ permeability, and Palek demonstrated normal extrusion of Ca$^{2+}$ from SS RBC compared to reticulocyte-rich controls. On the basis of studies of Ca$^{2+}$ extrusion from resealed RBC membranes, Litosch and Lee postulated increased Ca$^{2+}$ binding by SS RBC membranes at sites unavailable to the pump. They found slightly reduced Ca$^{2+}$ ATPase activity in SS RBC, but the enzyme activity was stimulated by calmodulin. Bookchin and Lew suggested progressive Ca$^{2+}$ pump failure due to the sickling process but did not assay ATPase. On the other hand, Gopinath and Vincenzi and Dixon and Winslow reported that calmodulin-stimulated ATPase in SS RBC is reduced, and therefore suggested that Ca$^{2+}$ extrusion from SS RBC is impaired. Both of the latter groups of investigators employed isolated red cell membranes to study Ca$^{2+}$ ATPase activities of SS cells and showed that isolated membranes, to which calmodulin (the cytoplasmic activator of Ca$^{2+}$ ATPase) was added, displayed substantially lower activities than normal red cell membranes. The decrease in Ca$^{2+}$ ATPase was not a result of reduced calmodulin activity in SS RBC.

The levels of activity of calmodulin-stimulated Ca$^{2+}$ ATPase in normal RBC depend on the type of hemolytic procedure employed. Cells hemolyzed in saponin expose greater activity than isolated membranes or reconstituted systems of isolated membranes and membrane-free cytosol. Therefore, we utilized saponin hemolysates to study the ATPase activities, reasoning that the system that maximizes activity would provide the most valid comparison among different RBC. The concentration of saponin and time of incubation were optimized to expose maximum ATPase activities (Figs. 1 and 2). Under these conditions the Ca$^{2+}$ ATPase activity of SS RBC was slightly but significantly greater than that of normal RBC. Confirming previous observations, we found no evidence of altered calmodulin activity in SS RBC when added to red cell membranes from normal subjects (data not presented).

Other ATPase activities—Mg$^{2+}$ ATPase and Na$^+ + K^+$ ATPase—were also greater in SS than normal RBC. Since all the ATPase activities were higher in SS hemolysate, it seemed likely that the increases reflected the younger red cell population in SS patients. Therefore, we measured ATPase activities of density (age) separated normal and SS RBC. As expected, the lighter (young) cells from normal subjects expressed greater ATPase activities than the more dense (old) cells. Similar patterns were observed in SS RBC. The differences between lightest and heaviest SS RBC were greater than those in normal RBC. We conclude that the higher ATPase activities in SS are related to the increased proportion of young red cells in SS patients. In view of the fact that calmodulin-stimulated Ca$^{2+}$ ATPase is not reduced, as previously reported, but is rather increased when the
assay is carried out in optimum systems, it is more likely that the elevated Ca⁺⁺ levels in SS RBC are due to an increased Ca⁺⁺ leak, abnormal Ca⁺⁺ binding, or some sort of "sequestration" of Ca⁺⁺ from pump sites than to defective Ca⁺⁺ extrusion by the ATPase pump.

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Increased Ca++, Mg++, and Na+ + K+ ATPase activities in erythrocytes of sickle cell anemia

MG Luthra and DA Sears