Calcium Accumulated by Sickle Cell Anemia Red Cells Does Not Affect Their Potassium \(^{86}\text{Rb}^+\) Flux Components

By Olga E. Ortiz, Virgilio L. Lew, and Robert M. Bookchin

We investigate here the hypothesis that the high Ca content of sickle cell anemia (SS) red cells may produce a sustained activation of the Ca\(^{2+}\)-dependent K\(^+\) permeability (Gardos effect) and that the particularly high Ca levels in the dense SS cell fraction rich in irreversibly sickled cells (ISCs) might account for the Na pump inhibition observed in these cells. We measured active and passive \(^{86}\text{Rb}^+\) influx (as a marker for K\(^+\)) in density-fractionated SS cells before and after extraction of their excess Ca by exposure to the Ca
ionophore (A23187) and ethylene glycol tetra-acetic acid and with or without adenosine triphosphate depletion or addition of guanine. None of these maneuvers revealed any evidence of a Ca\(^{2+}\)-dependent K leak in SS discytes or dense cells. Na pump inhibition in the dense SS cells was associated with normal activation by external K\(^+\) and a low K\(^+\)-channel activity. The results confirmed Na pump inhibition in the ISC fraction and showed evidence of an increase in nonsaturable ouabain-resistant \(^{86}\text{Rb}^+\) influx in the same cells. But there was no evidence of endogenous Ca effects on either pump or leak in any SS cell fraction.

MATERIALS AND METHODS

Heparinized venous blood was obtained with informed consent from normal donors and from patients with SS whose genotype has been established by electrophoretic and family studies at our Hereditary Clinic. The blood was kept refrigerated at 4 °C, and all experiments were performed within 24 hours of venesection. The plasma and buffy coat were removed by aspiration after centrifugation, and the RBCs were routinely washed four times (unless stated otherwise) in the buffer appropriate to each experiment.

Density gradient separation of RBCs. Density fractionation of the RBCs followed the method of Corash et al.\(^6\) with minor modifications. The RBCs were washed and suspended at a hematocrit (HCT) value in buffered saline. The RBCs followed the method of St Regis Paper Co, Libby, Mont) was centrifuged for several hours each at 4 °C with AG50I-X8 (D) 20-50-mesh mixed-bed resin (Bio-Rad Laboratories, Richmond, Calif) until the pH had fallen to about 3.0 and the osmolarity to 100 mosm or less. After the last filtration to remove the resin, the Stractan solution was centrifuged at 20,000 g for 45 minutes at 4 °C. This stock solution, about 40% (wt/wt) Stractan, density 1.163 to 1.170, was diluted with BSKG to obtain four additional densities, 1.087, 1.095, 1.105, and 1.118, as measured on a refractometer (Model 33-45-58, Bausch and Lomb, Austin, Tex), centrifuged again for 30 minutes, passed through a 0.45-μm filter (type S, Nalgene, Rochester, NY), and stored frozen. A discontinuous gradient was made by layering 2.5 mL of each of these four density solutions onto a cushion of 0.5 mL of the stock solution in 12-mL plastic centrifuge tubes; 1 mL of the 20% RBC suspension was layered onto each gradient tube, and they were centrifuged for 45 minutes at 17,000 rpm in an SS24 angle rotor of a Sorvall RC2B centrifuge (Ivan Sorvall, Newtown, Conn.) at 4 °C. The RBC bands were harvested with a Pasteur pipette and, after washing in the appropriate buffer, were sampled into buffered formalin for morphologic evaluation using a Zeiss microscope.

From the Department of Medicine, Albert Einstein College of Medicine, Bronx, NY; and the Physiological Laboratory, University of Cambridge, UK.

Submitted July 29, 1985; accepted Sept 27, 1985.

Supported by Grants HL 28018 and HL 2I0I6 from the National Institutes of Health and Grants from the Wellcome Trust and Medical Research Council of Great Britain.

Address reprint requests to Dr Robert M. Bookchin, Albert Einstein College of Medicine, 1300 Morris Park Ave, Bronx, NY 10461.

© 1986 by Grune & Stratton, Inc.

0006-4971/86/6703-0024$03.00/0.

equipped with Nomarsky optics. Cells were designated ISCs if their length exceeded their width by a factor of two or more. Small crumpled RBCs seen in the dense fractions, which did not meet the criteria for ISCs, were designated deformed. Cells with a density between 1.095 and 1.105 were 94% to 95% discocytes, with less than 1% reticulocytes seen on films stained with new methylene blue, and were used as the discocyte fraction; cells with a density between 1.118 and 1.163 were 68% to 73% ISCs, 18% to 19% deformed, and 11% to 14% discocytes and were used as the ISC-rich fraction.

Removal of endogenous RBC calcium. Cells were washed in buffer A containing (in mmol/L) NaCl, 75; KCl, 75; EGTA, 0.1; and Na-HEPES, 20, pH 7.6 (20 °C), 291 mosm. They were then suspended at a 30% HCT value in the same medium containing, in addition, 1 mmol/L EGTA, 0.2 mmol/L MgCl₂, and 10 μmol/L of the calcium ionophore A23187 (Sigma Chemical Co, St Louis) and incubated at 37 °C for 15 minutes. The concentrations of Na⁺, K⁺, and Mg²⁺ in the medium were chosen to maintain cell was chilled on ice and

Mass). The Hb content of an aliquot of each lysate was measured by atomic absorption spectrophotometry as described previously. For determination of cell [Na] and [K], the cells were washed four times in 20 vol of ice-cold buffer containing (in mmol/L) MgCl₂, 108, and Tris-Cl, 10, pH 7.4 (20 °C), 291 mosm, and lysed in a 10 M mol/L LiCl solution, and [Na] and [K] were measured by flame photometry (Model 143, Instrumentation Laboratories, Lexington, Mass). The Hb content of an aliquot of each lysate was measured and the results expressed as mmol/340 g Hb.

RESULTS

Features of experimental design. In attempting to detect a Ca²⁺-dependent component of the passive K⁺ (*⁴⁶Rb⁺*) influx in SS cells, three experimental maneuvers were made. The basic maneuver was to expose an aliquot of each red cell sample to the ionophore A23187 in the presence of EGTA in the medium, which was shown to extract nearly all the excess Ca content of SS cells, and to compare the *⁴⁶Rb⁺* influx in the Ca-containing and Ca-extracted cells. Most of the experiments we report here were performed on fractions of SS cells separated on Straitan density gradients. Since the dense, ISC-rich SS fraction was previously found to lack the Ca²⁺-dependent component of the K⁺ (*⁴⁶Rb⁺*) influx, its use would eliminate these complications. The remaining basic maneuvers consisted of (1) the removal of Ca²⁺ from the medium by the addition of 1 mmol/L EGTA, 0.1; 10 mmol/L, respectively. When K⁺ was present in the medium, the Na⁺ concentration was (150 - K⁺) mmol/L. *⁴⁶Rb⁺* was added as *⁴⁶RbCl, 10 mCi/mg, conditions. Plots were then made of time vs. *⁴⁶Rb⁺* uptake for at least 40 minutes. At a [K⁺]₀ higher than 2 mmol/L, on the other hand, the ouabain-resistant influx was high at 0 °C and leveled off with time at 37 °C, becoming linear between 20 and 60 minutes. Slop were drawn by eye over the linear portions of these curves and plotted as a function of [K⁺]₀. The apparent affinity and V₅₀ of the ouabain-sensitive influx were computed from a fit to Hill's equation with n = 2. Specific variations in the suspension media and in sampling times for the experiments shown in Fig 2 and Tables 1 and 2 are described in their legends.

Measurement of RBC cation contents. The total RBC Ca was measured by atomic absorption spectrophotometry as described previously.
tionated SS cells. RBCs were suspended at a 5% HCT value in media containing (in mmol/L) NaCl, 145; KCl, 5; Tris-CI, 10, pH 7.7 in Mmol/L pCi/mL of 'Rb. The ionophore A23187 was added from a 0.2
When present, the concentration of ouabain was 0.1 mmol/L and of quinine, 1 mmol/L.

5.8 mol/34O g Hb, respectively; this demonstrated that the Ca sensitivity of the channel might be greater in the dense, ISCs. In this group of experiments, the cells were suspended in a low-K+ (5 mmol/L) medium in which any activation of the Ca2+-dependent K+ channel would increase the membrane potential, resulting in a proportionately larger influx of 86Rb+ by the countertransport mechanism.17

Figure 2 shows an experiment comparing the 86Rb+ influx in suspensions of SS discocytes, ISCs, and unfractionated SS cells. The addition of ionophore A23187 (with excess EGTA in the medium) to a parallel aliquot of each suspension caused a sharp transient increase in the 86Rb+ influx that was barely detectable in the discocytes and maximal in the ISCs. When this response to ionophore exposure was described earlier with unfractionated SS cells,4 it was found to be inhibited by quinine; it was therefore interpreted as an ionophore-mediated increase in the apparent affinity of the K channel for endogenous Ca, either by a direct action of the ionophore on the Ca affinity of the activation site or by its facilitating the access of Ca to the site. The new detail seen here is that the magnitude of the ionophore effect is highest in ISCs, which are also known to contain the highest Ca content.14

Table 1 compares the passive 86Rb+ influx in density-fractionated SS RBCs. Subsequent experiments were performed with density-fractionated SS cells, comparing the reticulocyte-free discocytes and the dense, ISC-rich fractions. In this group of experiments, the cells were suspended in a low-K+ (5 mmol/L) medium in which any activation of the Ca2+-dependent K+ channel would increase the membrane potential, resulting in a proportionately larger influx of 86Rb+ by the countertransport mechanism.

To have the highest Ca content,5,6 the total Ca in the dense SS cell fraction from two donors was measured by atomic absorption spectroscopy before and after Ca extraction. In these samples, the total Ca contents in the dense cells were 89.0 and 71.0 µmol/340 g Hb, and after Ca extraction, 13.2 and 8.8 µmol/340 g Hb, respectively; this demonstrated that most of the excess Ca content in the ISC-rich fraction of cells was ionophore mobilizable, as observed previously in the unseparated SS cells.

Earlier analyses of the conditions for activation of the Ca2+-dependent K+ channel had suggested the possibility that the Ca sensitivity of the channel might be greater in ATP-depleted red cells than in fresh, substrate-fed cells.16 Therefore, the 86Rb+ influx was measured in aliquots of ATP-depleted, Ca-containing and Ca-extracted red cells. Finally, as an additional condition for the ATP-depleted cells, we tested the effect of quinine, a known inhibitor of the Ca2+-sensitive K+ channel.11 Since the combined addition of quinine and ouabain have been observed to produce complex effects on ion transport,9 ouabain was omitted in cell suspensions receiving quinine additions and in the corresponding control suspension.

**Passive 86Rb influx in unfractionated normal and SS RBCs.** Table 1 compares the passive 86Rb+ influx in unfractionated normal and SS cells incubated in a high-K+ (75 mmol/L) medium. For each condition, the 86Rb+ influx in the SS cells was higher than that in normal red cells (generally by about twice). But neither Ca extraction alone nor the additional procedures of ATP depletion or quinine addition revealed any indication of a Ca2+-activated K+ leak.

**Passive 86Rb influx in density-fractionated SS RBCs.** Subsequent experiments were performed with density-fractionated SS cells, comparing the reticulocyte-free discocytes and the dense, ISC-rich fractions. In this group of experiments, the cells were suspended in a low-K+ (5 mmol/L) medium in which any activation of the Ca2+-dependent K+ channel would increase the membrane potential, resulting in a proportionately larger influx of 86Rb+ by the countertransport mechanism.17

Figure 2 shows an experiment comparing the 86Rb+ influx in suspensions of SS discocytes, ISCs, and unfractionated SS cells. The addition of ionophore A23187 (with excess EGTA in the medium) to a parallel aliquot of each suspension caused a sharp transient increase in the 86Rb+ influx that was barely detectable in the discocytes and maximal in the ISCs. When this response to ionophore exposure was described earlier with unfractionated SS cells,4 it was found to be inhibited by quinine; it was therefore interpreted as an ionophore-mediated increase in the apparent affinity of the K channel for endogenous Ca, either by a direct action of the ionophore on the Ca affinity of the activation site or by its facilitating the access of Ca to the site. The new detail seen here is that the magnitude of the ionophore effect is highest in ISCs, which are also known to contain the highest Ca content.14

Following this transient initial effect, however, Ca extraction had only a minimal inhibitory effect on the two-hour 86Rb+ influx. When similar experiments were performed comparing discocytes and ISCs, experiments in which the endogenous Ca was either left in the cells or was extracted with ionophore and EGTA, washing away the ionophore before the incubation, Ca extraction showed no effect on the 86Rb+ influx (Table 2).

Additional results in Table 2 show the passive 86Rb+ influx, with SS discocytes and ISCs suspended in low-K+ media, under each of the conditions used to try to reveal a possible Ca2+-dependent K+ influx. Although some of the maneuvers were associated with small changes in the 86Rb+ influx, the changes bore no consistent relation to Ca extraction procedures. It is particularly noteworthy that in the dense, ISC-rich SS cell fraction, known to have the highest Ca content, neither Ca extraction nor quinine addition produced a significant decrease in 86Rb+ influx, and the

![Fig 2. Effects of the removal of the endogenous cell Ca on the ouabain-resistant 86Rb+ influx in SS discocytes, ISCs, and unfractionated SS cells. RBCs were suspended at a 5% HCT value in media containing (in mmol/L) NaCl, 145; KCl, 5; Tris-Cl, 10, pH 7.7 (37°C); Tris-EGTA, 1; MgCl2, 0.2; glucose, 10; ouabain, 0.1; and 10 µCi/mL of 86Rb. The ionophore A23187 was added from a 0.2 mmol/L solution in ethanol to give a final concentration of 1 µmol/L in the cell suspension. The percentage of lysis, determined at the end of the two-hour incubation by centrifugation and measurement of Hb concentration in the supernatant as compared with the total suspension Hb, was 3.4% and 3.3% for the unseparated cells in the absence and presence of ionophore, respectively: 1.8% and 2.8% for the discocytes; and 6.2% and 7.5% for the ISCs.

Table 1. Effects of Ca Extraction, ATP Depletion, and Quinine on Passive 86Rb+ Influx in Red Cells Incubated in High-K+ Media

<table>
<thead>
<tr>
<th>RBCs</th>
<th>Fed</th>
<th>ATP-Depleted</th>
<th>Fed</th>
<th>ATP-Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA Red Cells</td>
<td>Ouabain</td>
<td>Ouabain</td>
<td>Ouabain</td>
<td>Ouabain</td>
</tr>
<tr>
<td>SS Red Cells</td>
<td>Ouabain</td>
<td>Ouabain</td>
<td>Ouabain</td>
<td>Ouabain</td>
</tr>
<tr>
<td>Ca present</td>
<td>4.2</td>
<td>3.8</td>
<td>8.8</td>
<td>5.8</td>
</tr>
<tr>
<td>Ca extracted</td>
<td>4.4</td>
<td>4.8</td>
<td>10.0</td>
<td>7.2</td>
</tr>
</tbody>
</table>

RBCs were incubated in media containing (in mmol/L) KCl, 75; NaCl, 75; Tris-phosphate, 10, pH 7.5 (37°C); Tris-EGTA, 0.1; and 10 µCi/mL of 86Rb+. Samples were taken at zero time (at 0°C) and at 2, 4, 6, 10, 15, 20, 25, and 30 minutes' incubation at 37°C and processed as described in Materials and Methods. A high initial (zero time) uptake was seen with the Ca-extracted fed cells and especially with the ATP-depleted, Ca-extracted cells and was attributed to a small number of very leaky cells produced during these manipulations that equilibrated with the external 86Rb+ in the cold. Subsequent 86Rb+ uptake was linear in all samples over the 30-minute incubation period, and influx values were obtained from the slopes of lines drawn by eye through the data points. When present, the concentration of ouabain was 0.1 mmol/L and of quinine, 1 mmol/L.
influx was not increased in the Ca-containing cells by ATP depletion.

Active and passive $^{86}$Rb$^+$ influx as a function of $[K^+]_o$. Measurement of the ouabain-sensitive and ouabain-resistant $^{86}$Rb$^+$ influx in SS discocytes and ISCs as a function of $[K^+]_o$, illustrated in Fig 1, shows that the passive $K^+$ ($K^+$) influx of the discocytes fell within a normal range, whereas the ISC fraction showed an increased passive influx of $^{86}$Rb$^+$ that was nonsaturable over this range of $[K^+]_o$. The saturable component of the ouabain-resistant $^{86}$Rb$^+$ influxes, plotted separately after subtraction of the nonsaturable linear components (not illustrated), was not significantly different for the discocytes and ISCs. In the experiment in Fig 1 and another similar experiment, Na pump activity, as estimated by ouabain-sensitive $^{86}$Rb$^+$ influx (expressed in relation to $[K^+]_o$), appeared normal in the SS discocytes ($K^+$ = 1.02 and 0.98; $V_{max}$ = 2.1 and 2.2 mmol/340 g Hb/hr) but was inhibited in the ISC fraction, which showed normal $K^+$ values of 1.10 and 0.94 but reduced $V_{max}$ values of 1.6 and 1.4 mmol/340 g Hb/hr.

Effect of Ca extraction on active $^{86}$Rb$^+$ influx. In another experiment (not illustrated), ouabain-sensitive $^{86}$Rb$^+$ influx was measured in discocyte and ISC fractions, with an aliquot of each fraction having first been incubated with ionophore A23187 and EGTA to extract the endogenous Ca. With $[K^+]_o$ at 10 mmol/L to fully saturate the pump with $K^+$, the $^{86}$Rb$^+$ influx was 3.4 mmol/340 g Hb/hr for the discocytes and 1.0 mmol/340 g Hb/hr for the ISC fraction. Ca extraction produced no change in either value, indicating that the marked Na pump inhibition found with the ISC fraction was not corrected by removal of their excess Ca content.

### DISCUSSION

The findings described here demonstrate that neither the discocyte SS cells nor the dense ISC-rich fraction, whose total Ca content generally exceeds 100 μmol/L RBCs, show a sustained Ca$^{2+}$-dependent component of the passive $K^+$ ($^{86}$Rb$^+$) flux whether the cells are metabolically replete or ATP depleted.

The various predicted consequences of the established high Ca content of SS red cells, including dehydration resulting from Ca$^{2+}$ activation of the $K^+$ channel, have presumed that much if not all the excess Ca found in these cells is freely distributed in their cytoplasm. If this were so, then since SS cells have been found to show cytoplasmic Ca$^{2+}$ buffering in the normal range with a 20% to 30% ionized fraction, a mean total Ca content of 40 to 60 μmol/L RBCs would indicate a cytoplasmic-free ionized Ca$^{2+}$ content of 8 to 18 μmol/L, and in the ISCs, whose total Ca content may be 100 to 200 μmol/L. Measurements of the $^{86}$Rb$^+$ influx in ATP-depleted normal red cells show maximal activation of the Ca$^{2+}$-sensitive channel above 4 μmol/L [Ca$^{2+}$], with half-maximal activation around 2 μmol/L [Ca$^{2+}$].

The present findings are consistent with the compartmentalization of SS cell Ca in EIOVs. Immediately following exposure of the cells to ionophore while the Ca is being mobilized out of the vesicles and through the cytoplasm, there is transient activation of the Ca$^{2+}$-sensitive $K^+$ channel, as seen in Fig 2.

Partial inhibition of the Na pump in ISCs, as first observed by Clark et al., is shown here to be characterized by normal sensitivity to $[K^+]_o$ but a low $V_{max}$, which is particularly abnormal in view of the elevated Na$^+$ concentrations in ISCs.
ISCs as well as normal Na\(^{-}\)-K\(^{-}\) ATPase activities in their membranes, and it seemed possible that the Na pump inhibition observed in the intact ISCs might result from their high Ca content. We have shown here that the reduction in the \(V_{\text{max}}\) of the \(^{86}\text{Rb}\) influx of the ISCs persists after extraction of their excess Ca with ionophore and EGTA. Brown and Lew\(^{2}\) have found that a persistence of Na pump inhibition in red cells exposed to ionophore and Ca\(^{2+}\) is associated with an irreversible reduction in the ATP content of those cells. In view of the normal ATP concentrations found in ISCs, the persistence of Na pump inhibition after Ca extraction suggests that the increased cell Ca is not responsible for the inhibition. This interpretation is also consistent with compartmentalization of the SS Ca. If Ca had been involved in Na pump inhibition, its effects must have been irreversible and not mediated by ATP depletion.

Although the sickle cells showed no evidence of an ongoing Ca\(^{2+}\)-sensitive K\(^{+}\) leak, the ouabain-resistant component of the \(^{86}\text{Rb}\) influx, although of normal magnitude in the SS discocytes, was clearly increased in the dense, ISC-rich fraction of SS RBCs. This increase was shown to be limited to the unsaturable electrodiffusional component of the passive \(^{86}\text{Rb}\) influx since it increased linearly with \([\text{K}^{+}]_0\) over the range observed. With the high outward K\(^{+}\) gradient in RBCs, this increased Ca\(^{2+}\)-independent passive K\(^{+}\) leak could be substantial and, if uncompensated, could contribute to the cell dehydration observed with ISCs.

This finding of an increase only in the nonsaturable component of the \(^{86}\text{Rb}\) influx of ISCs may help explain the apparent discrepancy in the results of Clark et al., who described a normal passive \(^{86}\text{Rb}\) influx in the ISCs despite an increase in the passive K\(^{+}\) efflux. This increase in the K\(^{+}\) efflux was similarly high in the middle and bottom SS cell density fractions when expressed as a function of the mean cell [K\(^{+}\)] (as a constant rate). Their passive \(^{86}\text{Rb}\) influx measurements were made at [K\(^{+}\)]\(_0\) concentrations of 5 mmol/L, conditions under which we found little or no differences between discocytes, ISCs, and normal reported values. Our findings of an increased nonsaturable passive \(^{86}\text{Rb}\) influx in the ISCs, which was greater than that in the discocytes, is also compatible with the observation of Clark et al of similar rate constants of a passive K\(^{+}\) efflux for middle and bottom SS cell fractions since a greater leakiness in the dense cells would be counterbalanced by the lower cell [K\(^{+}\)] in that fraction.

The absence of evidence of Ca\(^{2+}\)-sensitive K\(^{+}\) channel activity in oxygenated SS cells does not exclude the possibility that transient small increases in cytoplasmic-free Ca\(^{2+}\), which might occur during periods of increased Ca permeability associated with deoxygenation-induced Hb S polymerization and RBC sickling, could briefly activate K\(^{+}\) channels in some cells, thereby contributing to their dehydration. The findings of Ohnishi\(^{26}\) of an apparent Ca\(^{2+}\) dependence of the formation of dense cells during cycles of deoxygenation and oxygenation of SS cells in vitro provides some indirect evidence for such a mechanism.

REFERENCES

Calcium accumulated by sickle cell anemia red cells does not affect their potassium (86Rb+) flux components

OE Ortiz, VL Lew and RM Bookchin