Calcium Transport and Ultrastructure of Red Cells in β-Thalassemia Intermedia

By Robert M. Bookchin, Olga E. Ortiz, Oded Shalev, Shmuel Tsurel, Eliezer A. Rachmilewitz, Austin Hockaday, and Virgilio L. Lew

Reported findings of elevated total calcium (Ca) contents in erythrocytes (RBCs) from patients with β-thalassemia intermedia (β-TI) prompted the question of whether the state and transport of Ca in these RBCs are similar to those in sickle cell anemia (SS) RBCs where the increased Ca is compartmentalized in endocytic inside-out vesicles and extracted by exposure of the cells to the Ca ionophore A23187 and a Ca chelator (ethylene glycol tetraacetic acid) and the levels of cytoplasmic free ionized Ca ([Ca\(^{2+}\)]\(_{i}\)) are normal. We confirmed a high total Ca content of 51 ± 13 \(\mu\)mol/L RBCs in splenectomized (SPX) β-TI and 24 ± 1 \(\mu\)mol/L RBCs in non-SPX β-TI. Unlike SS RBCs, however, most of the increased Ca was in the lighter, presumably younger β-TI RBCs, and about half the Ca was not iono-

A RECENT REPORT by Shalev et al\(^1\) described an increased total Ca content in RBCs from patients with β-thalassemia intermedia (β-TI), particularly after splenectomy (SPX). Unlike sickle cell anemia (SS) RBCs, however, which also have a high Ca content, β-TI RBCs have not been found to become dense and dehydrated.

With SS RBCs, although the accumulated Ca was shown to be compartmentalized in endocytic inside-out vesicles (EIOVs)\(^3\) and the oxygenated SS cells have normal levels of cytoplasmic ionized Ca ([Ca\(^{2+}\)]\(_{i}\)),\(^5\) recent evidence indicates that transient elevations of [Ca\(^{2+}\)]\(_{i}\), that are associated with periods of increased Ca permeability during deoxygenation-induced sickling\(^4\) activate the Ca\(^{2+}\)-sensitive K channels and promote their dehydra-

To explore these possibilities, we undertook an ultrastructural study of β-TI RBCs combined with a functional study of the state and transport of Ca in these cells.

MATERIALS AND METHODS

Heparinized venous blood was obtained at the Hadassah University Hospital, Kiryat Hadassah, Jerusalem, with informed consent, on several occasions from SPX and non-SPX patients with β-TI and from normal controls. The primary criterion for the diagnosis of β-thalassemia “intermedia” in these patients was the clinical expres-

Measurement of total Ca content of RBCs. The plasma was removed from blood samples after centrifugation with the buffy coat left intact to avoid removing normoblasts. The packed RBCs were passed through columns of microcrystalline cellulose and α-cellulose\(^13\) to remove leukocytes. The effluent RBCs were washed once in ice-cold buffer A containing (in mmol/L) NaCl, 145; KCl, 5; ethylene glycol tetraacetic acid (EGTA), 0.05; and Na-HEPES, 10 (pH 7.4 at 20°C). They were suspended at a 20% hematocrit in the

© 1988 by Grune & Stratton, Inc.

From the Albert Einstein College of Medicine, Bronx, NY; Hadassah University Hospital, Jerusalem; and The Physiological Laboratory, Cambridge, UK.

Submitted March 19, 1987; accepted June 29, 1988.

Supported by research grants from the National Institutes of Health (HL28018 and HL21016), the Wellcome Trust and Medical Research Council (UK), and by a travel award from the

© 1988 by Grune & Stratton, Inc.

subjected to this treatment suggested that the maneuver would improve rather than reduce the fidelity of our measurements of total RBC Ca.) After three further washes in buffer A, an aliquot of each RBC sample was set aside at 4°C, and the remaining RBCs were separated on discontinuous gradients of Stractan (arabinogalactan, St Regis Paper Co, Libby, MT), as described previously, by using three densities of Stractan solutions, 1.087, 1.091, and 1.095, with a cushion density of 1.148. The top fraction (Δ < 1.087), containing most of the reticulocytes and any residual leukocytes, was discarded; fraction 2, the next lightest cells (1.087 < Δ < 1.091), and fraction 4, the densest cells (Δ > 1.095), were harvested. Examination of stained smears of these fractions showed considerably more normoblasts in fraction 2 than in fraction 4 RBCs from SPX patients. For example, with one such RBC sample, which originally contained nine normoblasts per 1,000 RBCs, fraction 2 contained six, and fraction 4 contained 3 normoblasts per 1,000 RBCs.) With each RBC sample, the total Ca content was measured in these two fractions and in the unseparated RBCs by atomic absorption spectroscopy by methods described previously that include RBC washes in EGTA-containing buffers. The total Ca content was also measured in aliquots of each RBC sample and fraction after incubation at a 10% Hct for ten minutes at 37°C in buffer A containing, in addition, 10 μmol ionophore (A23187)/L RBCs and 2 mmol/L EGTA, to extract any mobilizable Ca. The hemoglobin (Hb) content of each sample processed for Ca content was measured by the cyanmethemoglobin method, and the total Ca content was expressed per liter of RBCs as adjusted by the mean corpuscular Hb content (MCHC) of each whole blood sample (MCHC × 10 = g Hb/L RBCs). No measurements of MCHCs were made on the density fractions of RBCs, and their total Ca levels were expressed with reference to the whole blood MCHCs. Since the MCHCs of the lighter cells must be lower than those of the denser fractions, the total Ca values per liter of RBCs should be slightly lower in the light fractions and slightly higher in the dense fractions than those referred to the whole blood MCHCs.

Measurement of the Ca influx and [Ca2+]i, in physiologic conditions. Unfractionated RBCs from each of three SPX and non-SPX β-TI patients and from one normal control (R.B.; blood drawn at the same time) were carried on ice to Cambridge where, within 24 hours from venipuncture, they were washed (with repeated removal of theuffy coat), loaded with the Ca chelator Benz 2, and the uptake of 45Ca monitored over a 90-minute incubation period as described previously with the following modifications: the RBCs were suspended at a 20% Hct in a buffer containing (in mmol/L) NaCl, 140; KCl, 5; MgCl2, 0.2; glucose, 10; Na pyruvate, 5; CaCl2, 1.2; and Tris-HCl, 10 (pH 7.7). The chelator content of an aliquot of each RBC sample was determined and, with knowledge of the in situ dissociation constant of Benz 2 for Ca, = 50 nmol/L, the levels of [Ca2+], (in mmol/L RBC water) in the near steady state were obtained. Since the RBC Ca pumps become depleted in the presence of a large excess of intracellular chelator, the initial uptake of 45Ca from the media with plasmalike Ca2+ concentrations can be taken as a measure of Ca influx under physiologic conditions. In each case, this value was derived from the uptake over the first five minutes, which is the initial (and maximal) slope of the uptake curve (see Fig 1). Since the mean cell volumes of the patients’ RBCs varied considerably and there is no reliable information about the membrane surface area of β-TI RBCs, the Ca influx rates were expressed as a function of cell number (1012 RBCs). Measurement of Ca extrusion from RBCs. Ca extrusion from β-TI and normal RBCs preloaded with 45Ca was measured by methods described previously with minor modifications. Briefly, fresh, washed RBCs were suspended at a 10% Hct in buffer containing (in mmol/L) KCl, 80; NaCl, 70; HEPES, 10 (pH 7.6), inosine, 10; MgCl2, 0.2; 4CaCl2, 0.2; and the ionophore A23187, 6 mmol/L suspension (60 μmol/L RBC) at 37°C for two minutes. After chilling and washing with ice-cold buffer (containing 1% albumin in the first two washes to extract all ionophore), zero-time samples were taken, the suspension was incubated on a shaking water bath at 37°C, and timed aliquots were taken for one hour and rapidly processed for 45Ca as described.

Electron microscopy of β-TI ghosts. Aliquots (20 μL) of the dense fraction of RBCs from an SPX and non-SPX β-TI patient were lysed in 1 mL of hypotonic buffer (L-Mg) containing (in mmol/L) K-HEPES, 1.5 (pH 7.5), and MgCl2, 1.0, conditions known to prevent spontaneous formation of inside-out vesicles. After spinning 15 seconds in an Eppendorf centrifuge, the packed ghosts were fixed in 1 mL of 2.5% glutaraldehyde in L-Mg (pH 7.2) for 15 minutes at room temperature and washed twice with 1 mL L-Mg (pH 7.2). Solutions used in this processing were brought (sterile) from Cambridge to maintain consistency for comparison with other data. The fixed samples were processed by the same investigators who previously described these procedures by the same methods for 0.15-μm serial-section electron micrographs.

RESULTS

Ca content of RBCs. Measurements of RBC Ca by atomic absorption spectroscopy, shown in Table 1, confirmed the findings of Shalev et al of a high total Ca content in β-TI RBCs, here amounting to 51 ± 13 and 24 ± 1 μmol/L RBCs in samples from SPX and non-SPX patients, respectively. Unlike SS cells, however, a substantial fraction of the increased Ca in the β-TI RBCs was not extracted by exposure of the cells to the ionophore (A23187) and EGTA, and the Ca content in both the total population and in each fraction was higher in the lighter than in the denser cells.

In the RBCs from SPX patients, the lighter cell fraction, which contained a much higher Ca content than did the denser cells, also contained most of the normoblasts. Although the mean Ca content was moderately increased in RBCs from β-TI patients with intact spleens, the increase was primarily in the lighter, presumably younger RBCs; in the dense fraction of these RBCs, both the total and extractable Ca amounts were only slightly increased. Ca influx and [Ca2+]i. The RBC Ca permeability and near–steady-state [Ca2+]i, levels were estimated from measurements of 45Ca uptake in unfractionated, Benz 2–loaded RBCs, as illustrated for one β-TI sample in Fig 1. The initial 4Ca influx was calculated from the first five-minute uptake, which is the steepest portion of the curve (see Materials and Methods). The uptake curve was not as smooth as that observed in populations of normal RBCs, and this probably reflects the morphologic heterogeneity observed with β-TI cells. The values of the 4Ca content from which the [Ca2+]i, was derived were taken at 90 minutes when all influx components were leveling off and represent approximate population mean values for the near–steady-state levels of [Ca2+]i.
fl-TI patients whole blood were 5.6% ± Lew'5). The difference, if any, of the total Ca less this residual Ca represents the ionophore-mobilizable component. The mean reticulocyte counts in the spleens and numbered 20.0 ±

<table>
<thead>
<tr>
<th>Sample</th>
<th>Unfractionated RBCs</th>
<th>Fraction 2 (Light RBCs)</th>
<th>Fraction 4 (Dense RBCs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (μmol/L RBC)</td>
<td>I + EGTA (μmol/L RBC)</td>
<td>Total (μmol/L RBC)</td>
</tr>
<tr>
<td>Normal</td>
<td>7.2</td>
<td>4.5</td>
<td>32.2</td>
</tr>
<tr>
<td>β-TI, spleen intact</td>
<td>21.5</td>
<td>13.3</td>
<td>50.1</td>
</tr>
<tr>
<td>A.Z.</td>
<td>25.2</td>
<td>13.5</td>
<td>82.0</td>
</tr>
<tr>
<td>T.A.</td>
<td>26.7</td>
<td>4.2</td>
<td>58.9</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>24.2 ± 1.1</td>
<td>9.9 ± 2.2</td>
<td>55.8 ± 10.4</td>
</tr>
<tr>
<td>β-TI, SPX</td>
<td>35.2</td>
<td>14.5</td>
<td>59.6</td>
</tr>
<tr>
<td>S.H.</td>
<td>40.9</td>
<td>20.6</td>
<td>80.4</td>
</tr>
<tr>
<td>A.W.</td>
<td>75.8</td>
<td>30.4</td>
<td>81.6</td>
</tr>
<tr>
<td>G.J.</td>
<td>50.6 ± 12.7</td>
<td>21.8 ± 4.6</td>
<td>73.9 ± 7.1</td>
</tr>
</tbody>
</table>

Red cell Ca content was measured by atomic absorption spectroscopy. The values under the columns labeled I + EGTA indicate the residual Ca content of each RBC sample after incubation in media containing the ionophore A23187 and excess EGTA (see Materials and Methods and Bookchin and Lew14). The difference, if any, of the total Ca less this residual Ca represents the ionophore-mobilizable component. The mean reticulocyte counts in the β-TI patients' whole blood were 5.6% ± 3.8% (spleen intact) and 9.0% ± 4.3% (SPX); nucleated RBCs were rare in samples from patients with intact spleens and numbered 20.0 ± 13.6 x 10^9/L in the Spx patients' blood.

Table 1. Total and Ionophore-Mobilizable Ca in β-TI RBCs

Table 2. In both the normal control and non-SPX β-TI patients the values for Ca influx were slightly lower than were the previously observed normal values (by the same methods).14 If we compare only within the present set of data, the mean Ca influx and quasi-steady-state [Ca^{2+}], levels are both slightly higher in the RBCs from SPX than in the non-SPX β-TI patients. None of these differences, however, was statistically significant (P > .05 by Student's two-tailed t test).

Ca extrusion from RBCs. As illustrated in Fig 2, Ca preloaded into the cells in the presence of the ionophore A23187 was extruded normally by RBCs from non-SPX β-TI patients. The RBCs from SPX β-TI patients, however, showed a markedly delayed extrusion pattern, quite similar to the pattern observed previously with SS RBCs,15 and even after one hour, they retained a substantial fraction of the 45Ca loaded.

Electron microscopy. Transmission electron micrographs were obtained on 0.15-μm serial sections of ghosts made from the dense RBC fractions from SPX and non-SPX β-TI patients. The dense cells were chosen for examination to minimize the presence of normoblasts and other immature RBCs containing organelles. One typical section of each dense ghost fraction is shown in Fig 3, with arrows indicating vesicles confirmed to be fully enclosed in the cytoplasm by examination of adjacent serial sections. Compared with normal ghosts2 (not shown), the ghosts from non-SPX β-TI RBCs showed a distinct increase in the number of small vesicles. Ghosts from SPX β-TI RBCs also showed an increased number of vesicles, which were considerably larger than those seen in the ghosts from β-TI patients with intact spleens; in addition, the ghosts from the SPX donors contained much electron-dense material, often structured, which must represent residual organelles, as expected in the absence of the spleen.

DISCUSSION

The present results first confirm the initial findings of Shalev et al1 that the total Ca content of β-TI RBCs is significantly increased and is higher in the RBCs from SPX persons. Unlike SS RBCs, however, most of the increased Ca is found in the lighter, presumably younger cell fractions, which include the majority of normoblasts in the blood from SPX patients.

Vesicular structures have been seen previously by transmission electron microscopy in RBCs from patients with β-thalassemia major.16 The use of serial sections for the present electron microscopic studies establishes the presence of vesicles fully enclosed in the cytoplasm of β-TI RBCs.

In SS RBCs, essentially all the increased Ca was found to be readily mobilizable since it was extracted by a few minutes exposure of the RBCs to the ionophore A23187 and EGTA.15 By contrast, about half of the increased Ca in β-TI RBC was not ionophore mobilizable, which indicates that it is firmly bound. The findings that the increase in tightly bound Ca is greatest in the lighter cells and is higher after
splenectomy raises the possibility that it is associated with the remnants of organelles in the normoblasts and other immature RBCs. Further studies would be required to test this possibility.

The moderately increased extractable Ca might also be located in the residual organelles and/or in the vesicles seen in the β-TI RBCs. Since these RBCs show a normal Ca influx, however, EIOVs would not be expected to accumulate Ca to the levels found in the vesicles of dense SS cells (which averaged 35 mmol/kg dry weight).2

Findings of a markedly delayed pattern of extrusion of ionophore-loaded Ca by the SPX β-TI RBCs together with a normal or near-normal [Ca2+]i, (indicative of essentially normal Ca pumps) suggests that during Ca loading a substantial portion of the Ca entering these RBCs is compartmentalized. The observed extrusion pattern could be accounted for by Ca accumulation by intracellular IOVs (with inward-directed Ca pumps) to millimolar levels during Ca-ionophore exposure, with a subsequent slow net passive efflux from the IOVs to the cytoplasm after removal of the ionophore. This, in turn, is consistent with the relatively high total and extractable Ca in these RBCs and with the electronmicroscopic observations of increased numbers of vesicles in the cells from SPX β-TI patients. The normal Ca extrusion pattern by the non-SPX β-TI RBCs, on the other hand, is consistent with their much smaller total (and extractable) Ca contents and the smaller number and size of observed intracellular vesicles.

If many of the increased numbers of vesicles seen in the dense fraction of RBCs from β-TI patients with intact spleens are indeed EIOVs, they could provide the capacity to record any periods of increased Ca permeability by accumulating increased amounts of extractable Ca. In that case, the finding that both the total and ionophore-mobilizable Ca levels in this cell fraction are normal would argue against the suggestion19 that physiologic shear stress in the circulation results in a large increase in RBC Ca permeability. Similarly, if the vesicular structures described in the RBCs from SPX persons without hematologic disease29 are EIOVs, the reported findings that such RBCs have normal Ca contents1 would point to the same conclusion and would also suggest that an increased number and size of EIOVs containing plasma concentrations of Ca may not in themselves account for the observed increase in extractable Ca in β-TI RBCs.

### Table 2. Calcium Permeability and Steady-State [Ca2+]i Levels in Red Cells Loaded With an Intracellular Ca Chelator

<table>
<thead>
<tr>
<th>Sample</th>
<th>MCV (fl)</th>
<th>Initial 45Ca Influx (μmol/10^11 RBC × h)</th>
<th>Steady State [Ca2+]i (nmol/L RBC Water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous normals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal control</td>
<td>90.6</td>
<td>16.5</td>
<td>19</td>
</tr>
<tr>
<td>β-TI spleen intact</td>
<td></td>
<td></td>
<td>19</td>
</tr>
<tr>
<td>A.Z.</td>
<td>57.9</td>
<td>10.6</td>
<td>31</td>
</tr>
<tr>
<td>K.D.</td>
<td>74.1</td>
<td>13.6</td>
<td>20</td>
</tr>
<tr>
<td>T.A.</td>
<td>70.9</td>
<td>10.2</td>
<td>14</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td></td>
<td>11.5 ± 1.1</td>
<td>21.7 ± 5.0</td>
</tr>
</tbody>
</table>

Values for initial 45Ca influx represent the maximum influx, which was always in the first five minutes of incubation (eg, see Fig 1). The concentration of the Ca chelator Benz 2 incorporated into the cells ranged in these experiments from 20.9 to 41.9 μmol/10^11 RBCs, so that during the first five minutes the [Ca2+]i levels should always have been below those that activate the Ca pumps and the 45Ca uptake should have been maximal at that time. Steady state [Ca2+]i represents the near-steady-state [Ca2+]i levels calculated at about 90 minutes' incubation, when the 45Ca influx was leveling off. "Previous normal" values were taken from Bookchin et al2 and from other experiments in our laboratories, and the same methods were used. As with the other samples, each row of values was obtained from a different donor.
obtained independently about the Ca content, influx, and [Ca\(^{2+}\)], in SPX and non-SPX \(\beta\)-TI RBCs.\(^{23}\)

REFERENCES


14. Ortiz OE, Lew VL, Bookchin RM: Calcium accumulated by sickle cell anemia red cells does not affect their potassium (\(^{86}\)Rb) flux components. Blood 67:710, 1986


ADDENDUM

After submission of this manuscript and publication of the results in preliminary (abstract) form,\(^{24}\) similar results were
Calcium transport and ultrastructure of red cells in beta-thalassemia intermedia

RM Bookchin, OE Ortiz, O Shalev, S Tsurel, EA Rachmilewitz, A Hockaday and VL Lew