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\(^{2+}\)]) are normal. We confirmed a high total Ca content of 51 ± 13 μmol/L RBCs in splenectomized (SPX) β-TI and 24 ± 1 μmol/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 ionophore mobilizable but apparently firmly bound, possibly to remnants of organelles in nucleated and other young RBCs. In the denser RBCs from non-SPX β-TI, total and extractable Ca amounts were normal. β-TI RBCs loaded with the Ca chelator Benz 2 showed an initial influx of Ca in the normal range, which indicated normal Ca permeability, and near–steady-state levels of [Ca\(^{2+}\)] that were normal (22 ± 7 nmol/L RBCs in non-SPX β-TI) or minimally increased (40 ± 19 nmol/L RBCs in SPX β-TI). Serial-section electron microscopy of β-TI ghosts from the denser cell fractions showed more fully enclosed vesicles in non-SPX ghosts than were seen in normal ghosts and many large vesicles and structured, electron-dense material in SPX ghosts. A delayed extrusion of ionophore-preloaded 4Ca only by the SPX β-TI RBCs together with normal [Ca\(^{2+}\)] suggested compartmentalization of the loaded Ca in these RBCs, perhaps in endocytic inside-out vesicles, and normal Ca pumps. Since β-TI RBCs show essentially normal levels of [Ca\(^{2+}\)] and normal Ca influx, their high total Ca content should not be associated with any of the deleterious effects observed in vitro with increased levels of [Ca\(^{2+}\)].

A recent report by Shalev et al. 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) and the oxygenated SS cells have normal levels of cytoplasmic ionized Ca ([Ca\(^{2+}\)]), recent evidence indicates that transient elevations of [Ca\(^{2+}\)], that are associated with periods of increased Ca permeability during deoxygenation-induced sickling, activate the Ca\(^{2+}\)-sensitive K channels and promote their dehydration. Other transport properties of SS cells may also contribute to their dehydration such as the Na pumps acting on a balanced, sickling-induced increase in Na and K leaks or the KCl cotransport, activated by low cell pH or cell swelling. Nevertheless, the absence of many dense RBCs in β-TI suggests that their increased Ca content may not reflect such intermediate states of increased Ca\(^{2+}\). On the other hand, the higher levels of total RBC Ca found in the SPX β-TI patients suggest that the increased Ca might be related to an abnormal abundance of residual Ca-containing organelles and/or increased numbers of EIOVs, which would not be pitted out in the absence of the spleen.

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, Kiyrat 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 expression of the disease, particularly the minimal transfusion requirements: the majority of the patients required no transfusions, and the remainder received transfusions only occasionally, mainly in association with infections or other complications. None of the patients had received blood transfusions for at least 3 months before these studies. Routine blood counts were obtained with a Coulter Counter (Coulter Electronics, Hialeah, FL) and the proportions of reticulocytes and nucleated red cells were determined by standard methods. For those experiments in which the blood was not processed experimentally in Jerusalem on the same day, it was kept on ice and hand carried by one of the investigators to New York or to Cambridge, UK, where experiments were performed within 24 hours of venipuncture.

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 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 same medium containing, in addition, 10 mmol/L inosine and 3 mmol/L adenosine and incubated at 37°C for 30 minutes to replete cellular levels of adenosine triphosphate (ATP) and allow the Ca pumps to extrude any excess cytoplasmic Ca gained during the

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previous one-day period of cold storage, when the whole blood was being carried from Jerusalem to New York. (Although this maneuver could conceivably lower the RBC Ca from their original levels, if that Ca was available to the Ca pumps, our measurements (see the next section) showing normal levels of [Ca $^+^+$], in the beta-TI RBCs not 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 $4^\circ C$, and the remaining RBCs were separated on discontinuous gradients of Stratcan (arabinogalactan, St Regis Paper Co, Libby, MT), as described previously,$^{14}$ by using three densities of Stratcan solutions, 1.087, 1.091, and 1.095, with a cushion density of 1.148. The top fraction ($\delta > 1.087$), containing most of the reticulocytes and any residual leukocytes, was discarded; fraction 2, the next lightest cells (1.087 $< \delta < 1.091$), and fraction 4, the densest cells ($\delta > 1.095$), 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.) 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$^{15}$ 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 $37^\circ C$ in buffer A containing, in addition, 10 $\mu$mol L-Mg, A23187 (alkaloid), 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 $\times 10 = g$ Hb/L RBCs). No measurements of MCHC 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 true 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 [Ca $^+^+$], in physiologic conditions. Unfractionated RBCs from each of three SPX and non-SPX beta-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 the Buffy coat), loaded with the Ca chelator Benz 2, and the uptake of $^{45}$Ca monitored over a 90-minute incubation period as described previously$^{16}$ with the following modifications: the RBCs were suspended at a 20% Hct in a buffer containing (in mmol/L) NaCl, 140; KCl, 5; MgCl$_2$, 0.2; glucose, 10; Na pyruvate, 5; $^{45}$CaCl$_2$, 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, $kd = 50$ nmol/L, the levels of [Ca$^+^+$], (in mmol/L RBC water) in the near steady state were obtained.$^{17}$ Since the RBC Ca pumps become Ca$^2+$ depleted in the presence of a large excess of intracellular chelator, the initial uptake of $^{45}$Ca from the media with plasmalike Ca$^2+$ 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 beta-TI RBCs, the Ca influx rates were expressed as a function of cell number (10$^9$ RBCs).

Measurement of Ca extrusion from RBCs. Ca extrusion from beta-TI and normal RBCs preloaded with $^{45}$Ca was measured by methods described previously$^{17}$ 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; MgCl$_2$, 0.2; $^{45}$CaCl$_2$, 0.2; and the ionophore A23187, 6 $\mu$mol/L suspension (60 $\mu$mol/L RBC) at 37$\circ C$ 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$\circ C$, and timed aliquots were taken for one hour and rapidly processed for $^{45}$Ca as described.$^{18}$

Electron microscopy of beta-TI ghosts. Aliquots (20 $\mu$L) of the dense fraction of RBCs from an SPX and non-SPX beta-TI patient were lysed in 1 mL of hypotonic buffer (L-Mg) containing (in mmol/L) K-HEPES, 1.5 (pH 7.5), and MgCl$_2$, 1.0, conditions known to prevent spontaneous formation of inside-out vesicles.$^{17}$ 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 packed in ice and carried to the Physiological Laboratory in Cambridge, UK, where they were processed by the same investigators who previously described these procedures by the same methods$^2$ 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 Shalve et al.$^1$ of a high total Ca content in beta-TI RBCs, here amounting to 51 ± 13 and 24 ± 1 $\mu$mol/L RBCs in samples from SPX and non-SPX patients, respectively. Unlike SS cells,$^{19}$ however, a substantial fraction of the increased Ca in the beta-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 beta-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 [Ca$^+^+$]. The RBC Ca permeability and near-steady-state [Ca$^+^+$]$_i$, (the Ca influx and fraction from the media with plasmalike Ca$^2+$ concentrations) were measured by the Ca uptake in unfractionated, Benz 2-loaded RBCs, as illustrated for one beta-TI sample in Fig 1. The initial Ca 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 obtained in populations of normal RBCs,$^1$ and this probably reflects the morphologic heterogeneity observed with beta-TI cells. The values of the Ca content from which the [Ca$^+^+$]$_i$ 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 [Ca$^+^+$].

The results obtained for each sample are summarized in...
fl-TI patients whole blood were 5.6\% ± Lew'.

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 ± content of each RBC sample after incubation in media containing the ionophore A23 1 87 and excess EGTA (see Materials and Methods and Bookchin and Table 2. In both the normal control and non-SPX /3-TI patients. The RBCs from SPX /3-TI patients, however, showed a markedly delayed extrusion pattern, quite similar to the pattern observed previously with 55 RBCs,'5 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 /3-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 /3-TI RBCs showed a distinct increase in the number of small vesicles. Ghosts from SPX /3-TI RBCs also showed an increased number of vesicles, which were considerably larger than those seen in the ghosts from /3-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 /3-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 /3-thalassemia major.18 The use of serial sections for the present electron microscopic studies establishes the presence of vesicles fully enclosed in the cytoplasm of /3-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 /3-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

Table 1. Total and Ionophore-Mobilizable Ca in /3-TI RBCs

<table>
<thead>
<tr>
<th>Sample</th>
<th>RBC Ca Content (μmol/L RBC)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Unfractionated RBCs</td>
</tr>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Normal</td>
<td>7.2</td>
</tr>
<tr>
<td>/β-TI, spleen intact</td>
<td></td>
</tr>
<tr>
<td>A.Z.</td>
<td>21.5</td>
</tr>
<tr>
<td>T.A.</td>
<td>23.3</td>
</tr>
<tr>
<td>M.A.</td>
<td>25.2</td>
</tr>
<tr>
<td>L.A.</td>
<td>26.7</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>24.2 ± 1.1</td>
</tr>
<tr>
<td>/β-TI, SPX</td>
<td></td>
</tr>
<tr>
<td>S.H.</td>
<td>35.2</td>
</tr>
<tr>
<td>A.W.</td>
<td>40.9</td>
</tr>
<tr>
<td>G.J.</td>
<td>75.8</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>50.6 ± 12.7</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 Lew's). 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 × 109/L in the Spx patients' blood.

![Fig 1. Uptake of 45Ca in Ca chelator (Benz 2)-loaded RBCs from an SPX /β-TI patient (D.S.). Maximum uptake, during the first five minutes (18.9 μmol/1012 RBC/h), was considered to reflect Ca permeability. [Ca2+]i calculated at 90 minutes' incubation (20 nmol/L RBC water), was taken as the near-steady-state level of [Ca2+]i. These values are included in Table 2.](image-url)
spleen is intact. Among the 12 SPX /3-TI samples, each row of values was obtained from a different donor.

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>MCV (fl)</th>
<th>Initial 45Ca Influx (mmol/10^12 RBC x h)</th>
<th>Steady State [Ca2+] (nmol/L RBC Water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous normals</td>
<td></td>
<td>19.6 ± 2.6</td>
<td>28.4 ± 4.7</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td></td>
<td>19.6 ± 2.6</td>
<td>21.7 ± 5.4</td>
</tr>
<tr>
<td>Normal control</td>
<td></td>
<td>29.9 ± 11</td>
<td>35.8 ± 28</td>
</tr>
<tr>
<td>R.B.</td>
<td>90.6</td>
<td>16.5 ± 3</td>
<td>19 ± 4</td>
</tr>
<tr>
<td>/3-TI spleen intact</td>
<td></td>
<td>5.7 ± 1.0</td>
<td>10.7 ± 2</td>
</tr>
<tr>
<td>A.Z.</td>
<td>57.9</td>
<td>10.6 ± 1.7</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>K.D.</td>
<td>74.1</td>
<td>13.6 ± 1.7</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>T.A.</td>
<td>70.9</td>
<td>10.2 ± 1.7</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td></td>
<td>11.5 ± 1.1</td>
<td>21.7 ± 5.0</td>
</tr>
<tr>
<td>/3-TI Spx</td>
<td></td>
<td>29.9 ± 11</td>
<td>35.8 ± 28</td>
</tr>
<tr>
<td>S.H.</td>
<td>92.5</td>
<td>43.7 ± 6.5</td>
<td>66 ± 11</td>
</tr>
<tr>
<td>A.W.</td>
<td>72.1</td>
<td>28.9 ± 4.7</td>
<td>35 ± 5</td>
</tr>
<tr>
<td>D.S.</td>
<td>65.9</td>
<td>18.9 ± 3.5</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td></td>
<td>30.5 ± 7.2</td>
<td>40.3 ± 13.5</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 Benzo 2 incorporated into the cells ranged in these experiments from 0.9 to 2.5 mmol/kg dry weight; in cell, so that during the first five minutes the [Ca2+] levels should always have been below those that activate the Ca pumps and the 45Ca uptake should have been maximal in that time. Steady state [Ca2+] represents the near-steady-state [Ca2+] levels calculated at about 90 minutes' incubation, when the 45Ca influx was leveling off. "Previous normal" values were taken from Bookchin et al 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.

Findings of a markedly delayed pattern of extrusion of ionophore-loaded Ca by the SPX /3-TI RBCs together with a normal or near-normal [Ca2+], (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 /3-TI patients. The normal Ca extrusion pattern by the non-SPX /3-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 /3-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 suggestion 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 disease are EIOVs, the reported findings that such RBCs have normal Ca contents 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 /3-TI RBCs.

![Fig 2. Extrusion of 45Ca from normal and /3-TI RBCs preloaded with 45Ca in the presence of ionophore A23187. Initial 45Ca contents (representing 100% values at the start of incubation at 37°C) expressed as micromoles per 10^12 RBC, were as follows: for 12 normal samples, 5.3 ± 0.9 (mean ± SEM) (range, 0.9 to 10.8); for 12 SPX /3-TI samples, 4.8 ± 1.5 (range, 1.8 to 11.5); and for five non-SPX /3-TI samples, 5.7 ± 1.8 (range, 2.5 to 10.3). Plotted at each time point are the mean ± SD percent remaining 45Ca for each group. Among the 12 SPX /3-TI samples, which showed a high mean 45Ca retention after 60 minutes' incubation, there was no consistent relation between the initial (zero time) 45Ca content and the amount retained.](http://www.bloodjournal.org by guest on September 24, 2017. For personal use only.)
obtained independently about the Ca content, influx, and $[Ca^{2+}]$, in SPX and non-SPX β-TI RBCs.25

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ADDENDUM

After submission of this manuscript and publication of the results in preliminary (abstract) form,24 similar results were obtained independently about the Ca content, influx, and $[Ca^{2+}]$, in SPX and non-SPX β-TI RBCs.25

Fig 3. Transmission electron micrographs of β-TI RBC ghosts. Each micrograph is typical of a group of 0.15-μm serial sections of ghosts made from (A) non-SPX and (B) SPX patients. The arrows point to vesicles that by examination of several adjacent serial sections were found to be fully enclosed in the cytoplasmin (Original magnification ×11,200).

The present findings could result if the residual organelles of the very young RBCs found in β-TI patients contain increased amounts of both bound and extractable Ca.

In contrast to the present findings with β-TI RBCs, an increased Ca permeability has been described in RBCs from patients with β-thalassemia major; the reason for the difference is unknown but might be related to the greater degree of either membrane immaturity or acquired membrane damage in the latter cells. Similar causes may underlie the observed increase in K permeability in these RBCs.22

Overall, our results indicate that since the $[Ca^{2+}]$ levels in β-TI RBCs are normal the elevated total Ca content in these cells, whether tightly bound or contained in EIOVs or other organelles, may not be expected to have any of the deleterious effects observed in vitro with increased levels of $[Ca^{2+}]$, such as ATP depletion, membrane protein cross-linking, sodium pump inhibition, or RBC dehydration due to K-channel activation.21

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