Calcium Distribution Within Human Erythrocytes During Endocytosis

By Stanley L. Schrier, Muriel Johnson, Irene Junga, and Judy Krueger

In order to study \(^{40}\)Ca movements within erythrocytes, a method was devised that had minimal deleterious effect on the treated erythrocytes. Agents that induce endocytosis in intact erythrocytes (primaquine, vinblastine, and chlorpromazine) caused a prompt movement of \(^{40}\)Ca from cytosol to membrane-associated sites. This drug-induced movement of \(^{40}\)Ca to membrane sites was blocked by depleting erythrocytes of adenosine triphosphate (ATP) or by incubating them with known inhibitors of endocytosis, NaF or N-ethylmaleimide (NEM). It appears that endocytosis in intact human erythrocytes involves the movement and redistribution of \(^{40}\)Ca from cytosol to membrane-associated sites. Therefore, in the erythrocyte, as in perhaps other cells, movement of Ca from one site to another may modulate important cellular biologic functions.

The phenomenon of drug-induced endocytosis in intact human erythrocytes offers an opportunity for studying plasma membrane properties such as invagination and fusion. Several classes of drugs can cause endocytosis. Agents like primaquine have an absolute requirement for adenosine triphosphate (ATP), while vinblastine and chlorpromazine can induce some endocytosis even in erythrocytes severely depleted of ATP. Our studies on endocytosis in resealed red human erythrocyte ghosts showed that there was a requirement for calcium (Ca) in addition to Mg-ATP. When these studies were extended to intact erythrocytes, it was noted that the Ca ionophore-mediated entry of \(^{45}\)Ca into erythrocytes potentiated primaquine-induced endocytosis. Therefore, Ca distribution within erythrocytes was studied because an understanding of Ca metabolism seemed potentially important in understanding the general phenomenon of endocytosis and because small shifts in cellular Ca have impressive biologic effects.

Accordingly, a method of introducing \(^{45}\)Ca into erythrocytes was designed so that intracellular distribution of \(^{45}\)Ca could be followed when the \(^{45}\)Ca-containing erythrocytes were incubated with endocytosis-inducing and -inhibiting agents. The method depends on the occurrence of an exchange between Ca within erythrocytes and \(^{45}\)Ca in the suspending media induced by exposure of erythrocytes to solutions of butanol, isotonic sucrose, and \(^{45}\)Ca. Specific monitoring indicated that this method produced only minimal perturbation of several modalities of erythrocytic function.

The results of our studies indicated that the amphipathic cations capable of producing endocytosis in intact erythrocytes caused either a net accumulation of \(^{45}\)Ca at membrane-associated sites or resulted in accelerated exchange of newly introduced \(^{45}\)Ca with "cold" Ca already present at membrane sites. This "movement" of \(^{45}\)Ca in erythrocytes was blocked by adding the known inhibitors of endocytosis. Therefore, it is reasonable to propose that the distribution of Ca within erythrocytes is of importance to the phenomenon of erythrocytic endocytosis.

**MATERIALS AND METHODS**

**Materials**

The endocytosis-inducing agents, primaquine, viablastle, and chlorpromazine, as well as the inhibitors, NaF and NEM (N-ethyl maleimide) were obtained from previously described sources. \(^{45}\)Ca was purchased from New England Nuclear, and additions were made to solutions of CaCl\(_2\) so that the specific activity was 2000-20,000 cpm/nmole Ca. Baker's reagents grade butanol was used.

**Methods**

Freshly drawn heparinized venous blood samples were obtained from volunteer donors according to procedures and protocols approved by the Stanford University Committee on Human Experimentation.

The method of induction of \(^{45}\)Ca entry used is described in our other article in this issue.

**Endocytosis in Erythrocytes**

The washed, packed \(^{45}\)Ca-loaded erythrocytes were added to 1 volume of autologous plasma and 1 volume of a solution consisting of 0.154 M NaCl, 5 mM imidazole glycylglycine buffer, pH 7.4, and 2 mg/ml of glucose. Endocytosis-inducing drugs and inhibitors of endocytosis were added to the suspending medium at the onset of the 37°C incubation. The suspension was then incubated at 37°C, and 1.5 ml samples were removed at 0, 15, 30, 60, and 120 min and added to an equal volume of 0.154 M NaCl and centrifuged for 1 min. The supernatant was removed, and aliquots were digested for...
Measurement of $^{45}$Ca in Erythrocytes and Membrane and Cytosol Fractions of Erythrocytes

Following removal of the supernatant solution, the $^{45}$Ca radioactivity of the erythrocyte pellet was determined as well as the packed cell volume (PCV), cell count, and hemoglobin content. The intact erythrocytes were then disrupted into a cytosol and membrane fraction by either freezing and thawing or sonication. Since the results were comparable, only sonication data are presented. The cytosol and membrane fractions were separated and their $^{45}$Ca contents were determined. The results are reported as cpm $^{45}$Ca/ml RBC or as percent of erythrocytic $^{45}$Ca recovered in the membrane or cytosol fraction.

ATP Depletion and Repletion

ATP depletion was produced by shaking sterile heparinized whole blood with a PCV of 40 for 16 hr at 37°C in a horizontally aligned tube. Repletion of ATP was achieved by incubating the depleted erythrocytes for 1 hr at 37°C in the phosphate, inosine, glucose, pyruvate, adenine (PIGPA) medium of Valeri. Morphological Studies

Selected samples fixed in 1.0% glutaraldehyde were studied by phase and Nomarski optics using the Zeiss III photomicroscope at 1260 magnification (Fig. 1).

RESULTS

The Effect of Endocytosis-Inducing Drugs on $^{45}$Ca Distribution Within Erythrocytes

The purpose of these experiments was to determine if endocytosis-inducing agents altered $^{45}$Ca loss or the intracellular distribution of $^{45}$Ca. In the single experiment shown as an example in Table 1, samples containing vinblastine and primaquine were incubated in parallel with control samples. Even in the "0" time sample, which required approximately 2.5 min at room temperature for red cell separation, both agents had produced a distinct shift of $^{45}$Ca to membrane-associated sites. This altered distribution persisted through 120 min of incubation, and prior experiments had established that intracellular-specific activity of $^{45}$Ca became stable after 10 min of incubation. Similar results were obtained whether membrane-associated sites were operationally defined by sonication or by freeze–thawing (data obtained by freezing and thawing are not shown). A summary of the effect of endocytosis-inducing agents is shown in Table 2, where it can be seen that the three classes of agents tested all produced a redistribution of $^{45}$Ca to membrane-associated sites.

The Role of Erythrocytic ATP in Drug-Induced $^{45}$Ca Distribution

Since primaquine endocytosis is an ATP-requiring step and ATP depletion reduces the amount of endocytosis that can be induced by a given concentration of chlorpromazine and vinblastine, experiments...
were performed to determine if ATP depletion interfered with the ability of endocytosis-inducing agents to shift \(^{45}\text{Ca}\) to membrane sites. Erythrocytes from normal donors were incubated overnight (see Materials and Methods) to deplete ATP levels. Aliquots of these ATP-depleted erythrocytes were then incubated with the PIGPA medium for 1 hr to restore ATP levels. The depleted and restored blood samples were then compared with freshly drawn blood from the same normal donor. Either ATP depletion per se or the consequences of severe ATP depletion (i.e., echinocytic shape changes or alterations in properties of membrane-associated proteins) caused a distinct but reversible block in the capacity of endocytosis-inducing drugs to shift \(^{45}\text{Ca}\) to membrane-associated sites.

Five experiments gave comparable results, but only one is shown in Table 3. Endocytosis-inducing agents caused a 2–3-fold increase in percent membrane-associated \(^{45}\text{Ca}\) only in ATP-containing erythrocytes but not in ATP-depleted erythrocytes. Since ATP depletion can increase the amount of membrane-associated protein, the data were recalculated as percent membrane-associated \(^{45}\text{Ca}\) per milligram of membrane protein, and these results were not appreciably different (data not shown) from those shown.

The Effect of Inhibitors of Drug-Induced Endocytosis on \(^{45}\text{Ca}\) Distribution Within Erythrocytes

Some of the inhibitors of drug-induced endocytosis may work, in part, by causing ATP depletion.12

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Table 1. Effect of Endocytosis-Inducing Drugs on \(^{45}\text{Ca}\) Distribution in Erythrocytes

<table>
<thead>
<tr>
<th>Time of incubation at 37°C</th>
<th>Membrane</th>
<th>Intact ABC (^{45}\text{Ca}) cpm/ml ABC</th>
<th>Cytosol (^{45}\text{Ca}) cpm/ml ABC</th>
<th>Percent ABC (^{45}\text{Ca})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td></td>
<td>47,915</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11,655</td>
<td>10,360</td>
<td>1,140</td>
<td>10%</td>
</tr>
<tr>
<td>Primaquine, 1.5 mM</td>
<td>11,137</td>
<td>1,865</td>
<td>7,718</td>
<td>69%</td>
</tr>
<tr>
<td>Vinblastine, 0.5 mM</td>
<td>11,914</td>
<td>2,590</td>
<td>8,806</td>
<td>74%</td>
</tr>
<tr>
<td>30 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6,475</td>
<td>5,180</td>
<td>622</td>
<td>10%</td>
</tr>
<tr>
<td>Primaquine, 1.5 mM</td>
<td>6,216</td>
<td>1,295</td>
<td>3,108</td>
<td>50%</td>
</tr>
<tr>
<td>Vinblastine, 0.5 mM</td>
<td>5,698</td>
<td>1,140</td>
<td>4,144</td>
<td>73%</td>
</tr>
<tr>
<td>120 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6,734</td>
<td>4,403</td>
<td>518</td>
<td>8%</td>
</tr>
<tr>
<td>Primaquine, 1.5 mM</td>
<td>6,216</td>
<td>1,088</td>
<td>3,108</td>
<td>50%</td>
</tr>
<tr>
<td>Vinblastine, 0.5 mM</td>
<td>6,475</td>
<td>1,295</td>
<td>3,367</td>
<td>52%</td>
</tr>
</tbody>
</table>

Table 2. Effect of Endocytosis-Inducing Drugs on the Intraerythrocytic Distribution of \(^{45}\text{Ca}\)

<table>
<thead>
<tr>
<th>Membrane-Associated (^{45}\text{Ca})</th>
<th>0-min Incubation at 37°C</th>
<th>15-min Incubation at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent ABC (^{45}\text{Ca}) in Membrane Pellet</td>
<td>(p) Value as Compared With Control</td>
</tr>
<tr>
<td>(13)* Control</td>
<td>13.9 ± 1.2†</td>
<td>14.9 ± 1.1</td>
</tr>
<tr>
<td>(4) Primaquine, 2 mM</td>
<td>53.8 ± 11.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(8) Vinblastine, 0.5 mM</td>
<td>49.5 ± 6.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(8) Chlorpromazine, 0.6 mM</td>
<td>44.1 ± 8.2</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*The number of experiments are shown in parentheses.
†Mean ± SEM.

Table 3. Role of ATP in Drug-Induced Intraerythrocytic Distribution of \(^{45}\text{Ca}\)

<table>
<thead>
<tr>
<th>ATP Content* ((\mu) mole/ml Packed RBC)</th>
<th>Membrane-Associated (^{45}\text{Ca}) After 15-min Incubation at 37°C (% of Total RBC (^{45}\text{Ca}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Fresh erythrocytes</td>
<td>0.96</td>
</tr>
<tr>
<td>Depleted erythrocytes</td>
<td>0.13</td>
</tr>
<tr>
<td>Restored erythrocytes†</td>
<td>1.36</td>
</tr>
</tbody>
</table>

*At the beginning of the incubation.
†Depleted RBC incubated with PIGPA to restore ATP levels.
Certainly inhibition of primaquine-induced endocytosis by NaF is paralleled by the fall in RBC ATP levels. However, inhibitors of endocytosis could interfere directly with 45Ca distribution within erythrocytes. Therefore, two classes of endocytosis-inhibiting agents, NaF and the penetrable sulfhydryl inhibitor NEM, were studied for their possible effect on 45Ca distribution within erythrocytes. These experiments were performed in duplicate and the endocytosis-inducing and -inhibiting agents were added simultaneously at “0” time, just before erythrocyte addition. The effect on 45Ca distribution is recorded in Tables 4 and 5. The associated erythrocytic shape changes using primaquine as endocytosis inducer are shown in Fig. 1. Six experiments using vinblastine and inhibitors were performed giving similar results and one is shown in Table 4. Four chlorpromazine and two primaquine and inhibitor experiments were performed and a combined experiment is shown in Table 5. The ATP values at the end of the incubation (15 or 30 min) are included in Tables 4 and 5, and it can be seen that with this short incubation period NaF produced modest ATP depletion. Neither NEM nor NaF acting alone had any effect on the distribution of 45Ca. However, movement of 45Ca to membrane-associated sites, which is regularly produced by the three classes of endocytosis-inducing drugs, was blocked by NEM and NaF (Tables 4 and 5). In this short incubation period some echinocytosis occurred, but only in samples incubated with 20 mM NaF (Fig. 1). Therefore, the ability of the inhibitors to block the 45Ca shifts produced by the endocytosis-inducing agents is probably not dependent on the formation of echinocytosis.

**DISCUSSION**

**Effect of Endocytosis-Inducing Drugs on 45Ca Distribution**

Drug-induced endocytosis is a complex phenomenon that can be thought of as occurring in several steps. In intact erythrocytes, the process probably begins with the amphipathic cationic drug inducing conversion of erythrocytes to stomatocytes. Then, invaginations appear on the stomatocytic face that deepen while the neck begins to constrict. Finally the neck of the vacuole fuses, and the endocytic vacuole is separated from the bulk of the membrane. Because Ca plays a role in red ghost endocytosis and in primaquine endocytosis in intact erythrocytes, intraerythrocytic Ca distribution was studied in intact erythrocytes during incubation with the endocytosis-inducing agents. Three classes of endocytosis-inducing agents were studied because there are significant differences in the mechanisms by which they induce endocytosis. For example, if Ca is introduced into erythrocytes, primaquine endocytosis is enhanced, while vinblastine endocytosis is inhibited and chlorpromazine endocytosis is not regularly affected. Each of these three agents caused a prompt and persistent increase in 45Ca at membrane-associated sites (Tables 1 and 2). This drug-induced increase in membrane-associated 45Ca is consistent with either an increased net accumulation of Ca at the membrane and/or an enhanced exchange of 45Ca with “cold” Ca previously present in membrane-associated pools. Several attempts have been made to resolve these alternatives by making net calcium measurements using atomic absorption spectrometry. However, because of interfering substances in the large sample size required, we have been unable to accurately detect net shifts of calcium that are calculated to be in the order of 0.25–2 n mole/ml of RBC.

All three endocytosis-inducing drugs produce stomatocytosis and increase in 45Ca membrane asso-
cation. Perhaps the drug-induced increase in 45Ca membrane association may be a consequence or an antecedent of stomatocytosis and may only secondarily be related to the subsequent endocytosis. Alternatively, the drug-induced shift of 45Ca to membrane sites may be a very early step in the endocytosis process.

We can only speculate on the actual mechanisms by which these drugs cause an increase in membrane-associated 45Ca. Chlorpromazine displaces Ca from ghost membrane-binding sites and conceivably could have the same effect in intact erythrocytes. Calcium-binding sites have been identified on the cytosol face of erythrocyte membranes. If after displacing cold Ca, the chlorpromazine, for unknown reasons, moved away from the membrane Ca-binding site, there would be an opportunity for newly introduced 45Ca to take the vacated site, thereby resulting in an enhanced exchange of 45Ca from cytosol to membrane.

The Effect of ATP Depletion on Endocytosis-Related 45Ca Redistribution

When erythrocytes were depleted of ATP, endocytosis was inhibited to varying degrees. Therefore, in order to test the hypothesis that drug-induced 45Ca shifts are important in endocytosis, we studied the effect of ATP depletion of drug-induced 45Ca shifts. When erythrocytes were depleted of ATP, the ability of the three endocytosis-inducing agents to shift 45Ca to membrane-associated sites was blocked (Table 3). Restoration of ATP was paralleled by restoration of the ability of endocytosis-inducing drugs to increase 45Ca membrane association. Perhaps ATP is required for 45Ca redistribution from cytosol to membrane, via mechanisms involving calmodulin or by contributing to the conversion of a membrane protein to a conformation that can accept Ca. Alternatively the erythrocytic changes produced by ATP depletion, i.e., echinocytosis, could have interfered with drug-induced 45Ca redistribution. These alternatives cannot be definitely resolved, but the first seems more likely in view of the results obtained with inhibitors of endocytosis.

Effect of Inhibitors of Endocytosis on Drug-Induced 45Ca Redistribution

The metabolic inhibitors, NaF and NEM, interfere with drug-induced endocytosis in intact erythrocytes. Therefore, these inhibitors were tested for their ability to block the 45Ca membrane-associated redistribution produced by endocytosis-inducing agents. NaF and NEM promptly blocked the ability of the drugs to produce 45Ca membrane association, and the block persisted over a 30-min incubation period (Fig. 1, Tables 4 and 5). Measurement of erythrocyte ATP content indicated that the blocked distribution of 45Ca occurred at times when there was only modest ATP depletion. Furthermore, there were no significant erythrocytic shape changes seen to account for the block in 45Ca redistribution (Fig. 1). Therefore, while an endocytosis inhibitor like NaF may act in part by producing ATP depletion, inhibitors like NEM may act by reacting with membrane sulfhydryls and thereby block the drug-induced redistribution of 45Ca from cytosol to membrane-associated sites.

The data presented show that under these specified in vitro conditions that lead to endocytosis, there is an exchange of 45Ca between cytosol and membrane-associated sites. There is no reason to assume that membrane-associated binding sites are of only one class subserving a single physiologic function. In fact, kinetic analyses show that there are three classes of cytosol Ca-binding sites. The movement of 45Ca between cytosol and membrane is enhanced by membrane-active drugs, and this enhancement is in turn blocked by the metabolic inhibitors and metabolic depletion that block endocytosis. Therefore, Ca movement can be considered to be important for erythrocytic endocytosis, a phenomenon that encompasses several important membrane functions. In addition, since Ca can stimulate an erythrocyte transglutaminase, which can bind proteases, induce membrane binding of proteases, and modify membrane properties, including the interaction of Mg-ATP with the membrane cytosol face, it can be proposed that careful control of the location and amount of membrane Ca might be an important mechanism by which the plastic properties of the erythrocyte are modulated.

REFERENCES

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SL Schrier, M Johnson, I Junga and J Krueger