Effect of Procaine HCl on ATP: Calcium-dependent Alterations in Red Cell Shape and Deformability

By J. Palek, A. Liu, D. Liu, L. M. Snyder, N. L. Fortier, G. Njoku, F. Kiernan, D. Funk, and T. Crusberg

Procaine hydrochloric acid, a cationic anesthetic, although unable to prevent the effect of calcium ionophore A23187 on erythrocytes, inhibited the discocyte-echinocyte transformation, increased viscosity, and decreased filterability of red cells undergoing ATP depletion. The effects were abolished by washing ATP-depleted, procaine HCl-treated red cells prior to these determinations. Procaine HCl had no effects on volume, incubated osmotic fragility, or monovalent cation composition of ATP-depleted red cells. The drug increased 45Ca uptake by ATP-depleted red cells but did not change the fraction of membrane-bound calcium. Sodium dodecyl sulfate acrylamide gel electrophoresis of membrane proteins from ATP-depleted red cells revealed formation of high molecular weight protein complexes, which were not formed when biconcave shape and ATP content were maintained by incubation with adenine (0.54 mM) and inosine (12.7 mM). Formation of these complexes was not prevented when the biconcave shape was maintained by procaine HCl. It was concluded that the maintenance of the biconcave shape and normal deformability during ATP depletion by procaine HCl was not related to a displacement of membrane-bound calcium and inhibition of ATP-dependent rearrangement of red cell membrane proteins.

DEPLETION of red cell ATP and accumulation of calcium results in the discocyte-echinocyte transformation and a decrease in deformability, presumably due to alterations in physical properties of the membrane proteins actin and spectrin, which form a lattice at the red cell membrane cytosol interface.1

It has recently been observed that cationic anesthetics such as procaine hydrochloride prevent the discocyte-echinocyte transformation and improve red cell deformability in normal and sickled red cells undergoing ATP depletion.11 The present study was undertaken to correlate the effect of procaine hydrochloride on various red cell physical characteristics with simultaneous membrane protein rearrangement and calcium accumulation in ATP-depleted red cells.

MATERIALS AND METHODS

In Vitro Aging of Erythrocytes

Fresh, venous blood was collected in heparinized vacutainers, the plasma was separated, and the packed erythrocytes were washed three times in isotonic buffer containing 150 mM NaCl and 10 mM glycylglycine (pH 7.4). Washed cells were then incubated at 20°C, hematocrit under sterile conditions in a medium consisting of 50 mM glycylglycine (pH 7.4), 5 mM KCl, and NaCl up to a total osmolarity of 285 mOsm. In order to prevent bacterial growth, all incubation mixtures were treated with 100 μg/ml gentamicin.
contained 0.2 mg/ml streptomycin and 200 U/ml penicillin G. These suspensions were placed in sterile Nalgene Erlenmeyer flasks covered with parafilm and gently agitated in a water bath at 37°C for varying periods of time. After 4.6 hr of incubation, the cells were centrifuged and the pH was readjusted in the supernatant to pH 7.4 because the pH had dropped 0.1-0.3 units.

**Determination of Red Cell Shape**

Red cells were fixed in 0.75%, glutaraldehyde solution in Eagle's medium for 30 min and their shape was examined under phase contrast and scanning electron microscopy (Japan Electron Optics Laboratory, Model JSM-U3).

**Red Cell Viscosity and Filterability**

Viscosity of 75%, red cell suspension in saline was measured on a Wells Brookfield Cone Plate microviscometer (Model LVT) as previously described. Filterability was determined by measuring the time of flow of 5 ml of 0.04%, red cell suspensions in isotonic saline buffered by 20 mM glycylglycine (pH 7.4) across 3-μm Nuclepore filters mounted in an Amicon cell under a constant positive pressure of 10 mm of H2O at room temperature. Suspensions were made immediately before filtration by dilution of 20%, suspensions of red cells undergoing ATP depletion. The data were expressed as the ratio of flow times of red cell suspensions to that of a suspending fluid required for filtration of 1-5 ml of the suspensions.

**Cell Volume, Osmotic Fragility, Monovalent Cation Composition, and ATP Content**

Red cell volume was calculated from red cell counts and microhematocrits. Osmotic fragility was determined according to Carterwright. Sodium and potassium were determined in red cells twice washed in 0.11 M MgCl2 solution employing flame photometry. ATP was determined in perchloric acid extracts of red cell suspensions with hexokinase and glucose-6-phosphate dehydrogenase as previously described.

**Uptake of Calcium**

Washed red cell suspensions were incubated with 45Ca (New England Nuclear Co.; CaCl2 in 0.5 N HCl, specific activity ranging 6.1-14.5 mCi/mg); the uptake of 45Ca by red cells was determined as described elsewhere.

**Membrane Protein Analysis**

Red cell ghosts were prepared by hemolysis of 1 volume of washed red cells in 30 volumes of 10 mM Tris HCl, pH 7.4, followed by two washes in the same buffer. Ghost proteins were solubilized in the absence of dithiothreitol. Otherwise, conditions were as previously described. The gels were composites of 2.5%, acrylamide and 0.3%, agarose prepared according to Peacock and Dingman. Under such conditions, the high molecular weight spectrin polypeptides 1 and 2 were located in the middle third of the gel, allowing a separation of high molecular weight complexes of red cell membrane proteins.

**RESULTS**

**Shape, Viscosity, Filterability, Volume, and Monovalent Cation Composition**

The effect of procaine HCl on changes in shape of red cells undergoing ATP depletion is shown on Fig. 1. Procaine HCl at concentrations above 15 mM partially improved and at concentrations above 45 mM almost completely prevented the discocyte-echinocyte transformation associated with ATP depletion. Similar concentrations of procaine hydrochloride normalized the viscosity (Fig. 2) and filterability (Fig. 3), but had no effect on cell volume, osmotic fragility, or monovalent cation composition of cells undergoing ATP deple-
Scanning electron micrographs of fresh and ATP-depleted erythrocytes exposed to increasing concentrations of procaine HCl. Red cell suspensions (20%) in equal volumes of plasma and isotonic NaCl containing 50 mM glycylglycine (pH 7.4), 2 mM CaCl$_2$, MgCl$_2$, and 5 mM KCl were incubated for 24 hr at 37°C with increasing concentrations of procaine HCl. The drug effect was independent of the presence of external calcium or magnesium and could be abolished by washing the incubated red cells in isotonic saline solution three times. In addition, procaine HCl was able to transform the spheroechinocytic shape, characteristic of ATP-depleted cells, to their original discoidal shape despite the fact that ATP content remained unchanged (not shown). The inhibition of discocyte-echinocyte transformation by procaine HCl was pH dependent. Optimal inhibition was seen at pH 7.0–7.4.

**Fig. 2.** Viscosity of 75% suspensions of ATP-depleted red cells exposed to increasing concentrations of procaine HCl, as a function of shear rate. Red cells were incubated for 23 hr at 37°C with procaine HCl, as described in Fig. 1. Means of four experiments.
Fig. 3. Filterability of ATP-depleted red cells exposed to procaine HCl. Fresh and ATP-depleted red cells were incubated with and without procaine HCl (45 mM) as shown in Fig. 1. Red cell suspensions (0.04%) were passed through 3-μm Nuclepore filters under 10 cm H₂O positive pressure. Ratio of flow time of 0.04% red cell suspension to the flow time of the suspending medium is plotted against volume. Representative data from one of three similar experiments.

Changes in Red Cell ATP Content and Calcium Uptake

Procaine HCl had no effects on the rate of depletion of ATP, which at pH 7.4 decreased to less than 15% of preincubation values during 12-16 hr of incubation (not shown). The uptake of ⁴⁵Ca by cells undergoing ATP depletion expressed as a ratio of red cell disintegrations per minute (dpm) to external dpm (RBC ⁴⁵Ca/Ext ⁴⁵Ca) is shown in Fig. 5. In agreement with previous observations of others, ⁴⁵Ca uptake increased with an increase in the pH of

<table>
<thead>
<tr>
<th>Table 1. Effect of Procaine HCl on Volume, Osmotic Fragility, and Na⁺, K⁺ Content of Red Cells Undergoing ATP Depletion*</th>
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</thead>
<tbody>
<tr>
<td>1-hr Incubation</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>MCVt (μl)</td>
</tr>
<tr>
<td>p</td>
</tr>
<tr>
<td>Osmotic fragility (μl)</td>
</tr>
<tr>
<td>p</td>
</tr>
<tr>
<td>K⁺ (mEq/liter red cells)</td>
</tr>
<tr>
<td>p</td>
</tr>
<tr>
<td>Na⁺ (mEq/liter red cells)</td>
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<td>p</td>
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</tbody>
</table>

*Conditions as described in Fig. 1. Procaine HCl concentration was 45 mM. Means ± SD and statistical significance p of 7 experiments.
†Mean corpuscular volume calculated from red cell counts and microhematocrits.
‡NaCl concentration required for 50% hemolysis.
the incubation medium from 6.0 to 8.0. At each of the pH values tested, procaine HCl increased $^{45}$Ca uptake by the cells. This increase was particularly apparent after ATP dropped below a critical level (0.2 μmole/ml). This finding suggests that the procaine HCl dependent stimulation of $^{45}$Ca uptake was attributable to increased calcium influx followed by a net increase in calcium content of ATP-depleted red cells.

The fraction of $^{45}$Ca retained by ghosts of ATP-depleted erythrocytes is
Effect of procaine HCl and pH on 45Ca uptake of red cells undergoing ATP depletion. Red cell suspensions (20%) were incubated as shown in Fig. 1 in the presence of 45Ca in the suspending solutions. RBC 45Ca/Ext 45Ca is plotted against time of incubation at various pHs. P, procaine-HCl (45 mM); C, control.

Both in control and procaine HCl treated samples, unwashed ghosts exhibited considerably lower radioactivity than corresponding red cells, indicating an absence of preferential calcium accumulation in red cell membranes as previously shown; this fraction decreased further after washing of the ghosts. The 45Ca of ghosts from procaine HCl-treated cells was higher than in control ghosts, but this could be attributed to the increased 45Ca loading of red cells in the presence of procaine HCl, as the fraction of red cell 45Ca retained by ghosts was the same as in control ghosts.

Effect of Calcium Ionophore A23187

Fresh red cells, following exposure to calcium ionophore A23187, accumulate calcium and undergo discocyte-echinocyte transformation. The effect

<table>
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<th>Table 2. Effect of Procaine HCl (45 mM) on the Recovery of 45Ca in Ghosts of ATP-depleted Erythrocytes*</th>
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<tbody>
<tr>
<td>45Ca</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Red cells (dpm/ml x 10^-2)</td>
</tr>
<tr>
<td>Ghosts unwashed (dpm/ml x 10^-2)</td>
</tr>
<tr>
<td>(percent of red cell 45Ca)†</td>
</tr>
<tr>
<td>Ghosts washed (dpm/ml x 10^-2)</td>
</tr>
<tr>
<td>(percent of red cell 45Ca)†</td>
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*Red cell suspensions were incubated for 22 hr with 45Ca (specific activity 0.2-0.3 mCi/mg external calcium), washed, and analyzed for 45Ca. In samples incubated with procaine HCl, the same concentration of the anesthetic was maintained during red cell washing. Washed red cells were hemolyzed (30 volumes of 10 mM Tris-HCl, pH 8.0), and ghosts were separated and analyzed for 45Ca. Part of the ghost pellet was washed two times in the above buffer before 45Ca determination. Duplicate determinations from one of four similar experiments.
†Calculated from dpm and particle counts (four) of red cell and ghost pellets.
Table 3. Effect of Procaine HCl on Discocyte–Echinocyte Transformation Induced by Calcium Ionophore A23187*

<table>
<thead>
<tr>
<th></th>
<th>Ca(^{2+}) (mM)</th>
<th>Mg(^{2+}) (mM)</th>
<th>Discocytes (%)</th>
<th>Echinocytes I, II (%)</th>
<th>Spheroechinocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.01</td>
<td>2.0</td>
<td>47 ± 20</td>
<td>39 ± 20</td>
<td>14 ± 10</td>
</tr>
<tr>
<td>Procaine HCl</td>
<td>0.01</td>
<td>2.0</td>
<td>56 ± 20</td>
<td>44 ± 20</td>
<td>—</td>
</tr>
<tr>
<td>Control</td>
<td>0.01</td>
<td>—</td>
<td>0</td>
<td>47 ± 5</td>
<td>53 ± 5</td>
</tr>
<tr>
<td>Procaine HCl</td>
<td>0.01</td>
<td>—</td>
<td>0</td>
<td>23 ± 7</td>
<td>77 ± 8</td>
</tr>
<tr>
<td>Control</td>
<td>0.1</td>
<td>2.0</td>
<td>0</td>
<td>33 ± 6</td>
<td>67 ± 5</td>
</tr>
<tr>
<td>Procaine HCl</td>
<td>0.1</td>
<td>2.0</td>
<td>0</td>
<td>25 ± 20</td>
<td>75 ± 20</td>
</tr>
</tbody>
</table>

*Red cell suspensions (10%) in isotonic NaCl, glycylglycine buffer (50 mM, pH 7.4) with KCl (5 mM), with or without MgCl\(_2\) (2 mM) and procaine HCl (45 mM) were incubated at 37°C. At 60 min, A23187 (final concentration 0.01 mM), and at 70 min, Ca\(^{2+}\) (final concentration 0.01–0.1 mM) were added; 45 min after Ca\(^{2+}\) addition, cells were fixed and their shapes classified according to Bessis. Means ± SD of six experiments. The differences were not statistically significant.

of procaine HCl on this phenomenon is shown in Table 3. Only at low external Ca\(^{2+}\) concentrations (0.01 mM) and in the presence of Mg\(^{2+}\) (2 mM) does procaine HCl exhibit a slight protective effect, which is not reproducible in all of the experiments and the difference is not statistically significant (p > 0.05). When Mg\(^{2+}\) in the incubation medium is omitted or Ca\(^{2+}\) concentration increased (in the presence of Mg\(^{2+}\)), the procaine HCl treated samples exhibit an even more pronounced spheroechinocytic change than samples made without this agent.

![Fig. 6. SDS polyacrylamide gels of solubilized membrane proteins. 1, fresh red cells; 2, ATP depleted; 3, ATP depleted + procaine HCl (45 mM); 4, ATP maintained with adenine (0.54 mM) and inosine (12.7 mM). Conditions for ATP depletion were exactly as described in Fig. 1. Arrows indicate the principal membrane protein aggregates. The major membrane polypeptides are indicated by numbers according to Fairbanks et al. Hb, hemoglobin; T.D., tracking dye.](image-url)
Analysis of Red Cell Membrane Polypeptides

The analysis of red cell membrane polypeptides employing sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis of solubilized ghost proteins (Fig. 6) revealed that ATP depletion was associated with a formation of new components of molecular weights ranging from 258,000 to >1,000,000 daltons (indicated by arrows). We have recently shown that these complexes consist of spectrin and other membrane and cytoplasmic proteins linked by intermolecular disulfide bonds.23 Figure 6 shows that procaine HCl (45 mM), which prevented discocyte-spheroechinocyte transformation, failed to prevent the formation of these complexes. In contrast they were completely prevented by maintenance of red cell ATP and a biconcave shape with adenine (0.54 mM), inosine (12.7 mM), and glucose (2 mg/ml), both in the absence and the presence of procaine HCl (45 mM).

DISCUSSION

Procaine HCl and other cationic local anesthetics have been shown to exhibit multiple effects on biologic membranes, including expansion of membrane lipids, modification of monovalent cation movements, displacement of membrane-bound calcium, and alteration of membrane proteins presumably involved in regulation of cellular shape.24,25 In the present report, we have demonstrated that procaine HCl, while maintaining discoidal shape and normal deformability, had no effect on monovalent cation composition, osmotic fragility, volume, and ATP content of red cells undergoing ATP depletion in vitro. Although the drug increased the uptake of calcium, it failed to displace calcium which accumulated in ghosts of ATP-depleted erythrocytes. Furthermore, the drug failed to prevent the formation of high molecular weight complexes of membrane proteins (Fig. 5) principally composed of spectrin, smaller amounts of actin, certain integral membrane polypeptides, and unidentified cytoplasmic proteins.23 This finding was of considerable interest, as changes in the physical state of spectrin at the cytosol membrane interface were suggested to be of significance in regulation of cellular deformability and shape.14,24,3,10

The mechanism whereby procaine HCl maintains shape and deformability despite its inability to displace calcium or prevent spectrin rearrangement remains unknown. However, we speculate that this effect can be explained on the basis of the “bilayer couple hypothesis” recently proposed by Sheetz and Singer.26 These authors propose that the two halves of a membrane bilayer which differ in protein and lipid composition27 can respond differently to perturbation. Thus, permeable cationic drugs such as procaine HCl intercalate mainly into the lipid of the cytoplasmic half of the membrane, expanding the layer relative to the exterior half, and thereby causing the formation of stomatocytes. This differential distribution of the drug is attributed to its interaction with the phosphatidyl serine concentrated in the cytoplasmic half of the membrane.

We speculate that the echinocytic shape of ATP-depleted cells results from an asymmetrical “contraction” of the inner half of the membrane bilayer, presumably due to spectrin aggregation, and that procaine HCl prevents this shape change by expanding this layer. This hypothetical model is indirectly supported
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by our observation that the drug maintains biconcave shape without changing membrane protein arrangement and that its effect disappears after washing of cells, presumably due to its removal from low-affinity membrane-binding sites. In addition, it is of interest that the maintenance of the biconcave shape of ATP-depleted cells by procaine HCl is associated with a marked improvement in viscosity and filterability. These observations suggest that cellular shape per se rather than the physical changes of protein on the cytosol-membrane interface is of primary importance in the regulation of cell deformability.

It should be emphasized, however, that the model is strictly hypothetical and that a bilayer couple hypothesis is only one of several possible explanations of red cell shape, which include an inequality in surface tension, membrane thickness, elasticity, and charge distribution. In addition, it does not explain the lack of procaine HCl effects on shape transformation induced by A23187, which, however, may be related to different mechanisms of echinocytogenesis in such cells.

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