Chlorpromazine Inhibits Vesiculation, Alters Phosphoinositide Turnover and Changes Deformability of ATP-Depleted RBCs

By Peter Bütikofer, Zeng Wen Lin, Frans A. Kuypers, Mark D. Scott, Caimin Xu, Gail M. Wagner, Daniel T.-Y. Chiu, and Bertram Lubin

To delineate further the underlying mechanism by which amphiphilic drugs can modulate vesicle release from human RBCs, we studied the effect of chlorpromazine on erythrocyte vesiculation induced by ATP depletion. This was correlated with turnover of the phosphoinositides as well as RBC deformability during the process since phosphoinositide metabolism may be involved in shape regulation of RBCs. Echinocytic shape transformation and subsequent vesiculation of RBCs, which commonly occur during ATP depletion, were inhibited by chlorpromazine. Furthermore, with a newly developed two-dimensional thin-layer chromatography separation of RBC membrane phospholipids, we showed that chlorpromazine significantly decreased the dephosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) in both ATP-depleted RBCs as well as in cells with partly maintained ATP levels. Concomitantly, there was a smaller increase in the relative amount of phosphatidylinositol. In addition, chlorpromazine also inhibited the decrease in RBC deformability as well as the shift of osmotic fragility that occurs during ATP depletion of erythrocytes.

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L O S S O F M E M B R A N E from human erythrocytes, in the form of spectrin-depleted microvesicles, occurs rapidly in normal RBCs after various in vitro manipulations that disrupt membrane protein/lipid interactions. Membrane vesiculation is also believed to occur slowly in vivo as erythrocytes age. In addition, membrane vesicles derived from RBCs are found in the circulating blood of patients with several hemolytic RBC disorders, suggesting that in vivo vesiculation may play a role in the premature destruction of such cells. In all vesiculation processes studied, RBCs undergo a shape transformation from discocytes to spherocytic cells before vesiculation. We recently showed that chemical reagents that alter RBC morphology are also potent modulators of RBC vesiculation. Specifically, membrane-penetrating agents, such as chlorpromazine, that partition in the inner half of the membrane bilayer inhibit echinocytic shape change and subsequent vesiculation. Conversely, agents that accumulate in the outer half of the bilayer facilitate both erythrocyte shape change and vesiculation.

Phosphoinositides within the RBC membrane may be involved in shape regulation of erythrocytes. When RBCs are ATP depleted, the induced echinocytosis coincides with and may be related to conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol (P1). To investigate the relationship between RBC shape change, vesiculation, and phosphoinositide turnover, we defined the effect of chlorpromazine on vesicle release from RBCs induced by ATP depletion as well its effect on the turnover of the phosphoinositides. The ektacytometer was used to follow changes in RBC deformability that accompanied this process.

MATERIALS AND METHODS

Erythrocytes. After informed consent was obtained, blood from normal adults was collected in heparinized tubes. Erythrocytes were pelleted by centrifugation for five minutes at 1,500 g and subsequently washed twice in isotonic saline and once in Tris-buffered saline (144 mmol/L NaCl, 10 mmol/L Tris, pH 7.4). After each centrifugation the buffy coat was carefully removed by aspiration.

ATP depletion of RBCs. Washed RBCs were incubated at 37°C in a hemocrit of 16% in 144 mmol/L NaCl, 10 mmol/L Tris, pH 7.4, containing 0.1 mg/mL streptomycin and 100 U/mL penicillin (buffer A) to deplete them of ATP. When required, chlorpromazine was added to the incubation buffer from a 100-mmol/L stock solution in water. When ATP levels were to be maintained during incubation, the incubation buffer was supplemented with 0.54 mmol/L adenine, 12.7 mmol/L inosine, and 11.1 mmol/L glucose (buffer B). Release of membrane vesicles from RBCs was monitored using acetylcholinesterase as a marker. The enzyme activity was measured according to the method of Ellman et al.

ATP measurement. ATP was extracted from RBCs with perchloric acid by the method of Beutler and was quantitated with commercially available luciferin luciferase assay (Sigma, St Louis).

Lipid extracts. RBC membrane lipids were extracted essentially according to the method of Dale. Three hundred microliters of packed RBCs were lysed in 35 mL cold 10 mmol/L Tris, pH 7.5, containing 2 mmol/L EDTA (lysis buffer). RBC membranes were pelleted at 17,000 g for ten minutes at 4°C and washed once with 35 mL lysis buffer. The resulting pink ghosts were taken up in 15 mL cold chloroform:methanol:6N HCl (25:50:1) and extracted for 45 minutes with frequent vortexing at room temperature. Subsequently, 5 mL water and 5 mL chloroform were added, and the suspension was vortexed vigorously. Separation of the two phases was obtained after centrifugation for five minutes at 300 g. The lower organic phase was collected, and the solvent was evaporated under a stream of nitrogen. The lipid was resuspended in a small volume of chloroform:methanol (2:1).

Thin-layer chromatography (TLC). RBC membrane lipids were separated on silica G 60 plates (Merck, Darmstadt, FRG), previously sprayed with oxalate [1% in methanol:water (3:2)], and activated for 15 minutes at 90°C. The plates were developed in the first direction in a solvent system composed of chloroform:methanol:acetone:water (65:25:4:4). The phosphatidylinositol content of the RBCs was determined by phosphorus analysis.

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of all phospholipid classes including P1, PIP, and PIP2.

Chloroform:methanol:acetone:acetic acid:water was used to separate the phospholipids. This procedure results in good separation of all phospholipid classes including P1, PIP, and PIP2.

Lipid measurement. Spots corresponding to the individual phospholipid classes were visualized by iodine vapor and identically recorded at a constant applied shear stress of 110:74:12:8 and in the second dimension in nol:ammonia:water.

Phospholipids (Sigma) identified by comparing migration of standard spots with those of the individual phospholipid classes were scraped from the plates and analyzed by the method of Bartlett.

Osmotic gradient ektacytometry. RBC deformability was measured with a Technicon Ektacytometer by a method described by Bessis and Ferrell and Huestis. This procedure results in good separation of all phospholipid classes including P1, PIP, and PIP2.

Erythrocytes were fixed in 3% glutaraldehyde and examined by light microscopy with Zeiss Nomarsky optics (Oberkochen, FRG). Morphologic assignments were based on Bessis nomenclature as outlined by Ferrell and Huestis.

Protein analysis. RBC membrane proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels by the method of Laemmli.

RESULTS

Effect of chlorpromazine on RBC vesiculation. When RBCs are depleted of their ATP over a period of 46 hours, they become spheroechinocytes and shed part of their membrane as microvesicles. This process can be monitored by using release of acetylcholinesterase as a marker. With the addition of chlorpromazine, an agent known to induce stomatocytosis in normal RBC membranes, the echinocytic shape transformation of erythrocytes during ATP depletion proceeded much slower (Table 1) and the release of acetylcholinesterase-containing vesicles was inhibited (Table 2). Both echinocytosis and vesiculation of RBCs were inhibited in a concentration-dependent manner by chlorpromazine. With increasing chlorpromazine concentrations from 0 to 100 μmol/L, release of membrane vesicles was inhibited from 0% to 100%. When concentrations were >100 μmol/L, hemolysis became apparent after prolonged incubation. When the incubation buffer was supplemented with adenine, inosine, and glucose, RBC ATP levels were partly maintained during the 46-hour incubation period and release of acetylcholinesterase-containing vesicles was inhibited (Table 2). Addition of chlorpromazine during incubation did not affect ATP levels.

Effect of chlorpromazine on phosphoinositide turnover in RBCs. To determine the relative amounts of the phosphoinositides (PI, PIP, and PIP2) during ATP depletion of RBCs, we developed a new two-dimensional TLC system that gives good separation of all the major phospholipid classes of the RBC membrane, including the phosphoinositides (Fig 1). This system circumvents use of radiolabeled phosphoinositides, which requires prelabeling procedures with 32P to analyze the small amounts of phosphoinositides present in human RBCs. Table 3 shows the relative phospholipid composition of native RBCs determined from five separate experiments using blood from different donors. Our values are in good agreement with those in the literature. The rather high SD in the percentages of the minor phospholipids (relative to actual values) should be noted; eg, the relative SD for PI, PIP, and PIP2 (calculated from the values in Table 3) were 36%, 20%, and 13%, respectively. This was largely due to variations in the values between cells from different individuals. The results of triplicate or quadruplicate determinations of the phospholipid composition of one individual showed much smaller relative SD of PI (15%), PIP (16%), and PIP2 (3%) (means of three experiments).

ATP depletion of RBCs was accompanied by a decrease in the relative amount of PIP2, with a concomitant increase in

<table>
<thead>
<tr>
<th>Table 1. Chlorpromazine Inhibits ATP Depletion-Induced Shape Change of RBCs</th>
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<tr>
<td>Morpphology</td>
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<tr>
<td>Stomatocytes</td>
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<td>Discocytes</td>
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<tr>
<td>Echinocytes I</td>
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<tr>
<td>Echinocytes II</td>
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<td>Spheroechinocytes</td>
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RBCs were incubated for 27 and 46 hours, respectively, in buffer A and subsequently fixed and analyzed under the light microscope. Values were obtained from a typical experiment by counting 5 x 100 RBCs (mean ± SD).

<table>
<thead>
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<th>Table 2. Chlorpromazine Inhibits ATP Depletion-Induced Vesiculation as Measured by Release of Acetylcholinesterase</th>
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<td>Incubation Time (h)</td>
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<td>2</td>
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<td>2</td>
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<td>22</td>
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<td>26</td>
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<tr>
<td>46</td>
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Acetylcholinesterase release is expressed as percentage of enzyme activity recovered in vesicle-containing supernatant after low-speed centrifugation (mean of two determinations). Total enzyme activity in the incubation mixture was taken as 100%. ATP levels are expressed as percentage of the initial value (1.1 mmol/L in packed RBCs) at the start of incubation. Details are given in the Materials and Methods section.
the relative amount of PI (Fig 2). No significant changes were observed for PIP and phosphatidic acid or for any other phospholipid class. These findings are similar to those reported by Ferrell and Huestis.9 When chlorpromazine (60 μmol/L, final concentration) was added during ATP depletion of RBCs, the conversion of PIP₂ to PI occurred to a lesser extent and a small but consistent increase in PIP was observed after prolonged incubation (Fig 2). When RBC ATP levels were partly maintained during incubation to prevent vesiculation, a substantially smaller change in the relative amounts of the phosphoinositides was observed than

### Table 3. Phospholipid Composition of Human RBCs

<table>
<thead>
<tr>
<th>Phospholipid Class</th>
<th>Relative Percentage</th>
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<tr>
<td>Phosphatidylcholine</td>
<td>27.14 ± 0.54</td>
<td>15</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>27.87 ± 1.75</td>
<td>10</td>
</tr>
<tr>
<td>Phosphatidylycerine</td>
<td>11.78 ± 1.69</td>
<td>15</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>22.37 ± 1.88</td>
<td>15</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>1.36 ± 0.49</td>
<td>15</td>
</tr>
<tr>
<td>Phosphatidylinositol-4-monophosphate</td>
<td>0.35 ± 0.07</td>
<td>15</td>
</tr>
<tr>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
<td>1.44 ± 0.19</td>
<td>15</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>3.40 ± 1.15</td>
<td>15</td>
</tr>
<tr>
<td>Lyso phosphatidylcholine</td>
<td>1.90 ± 0.43</td>
<td>15</td>
</tr>
<tr>
<td>Unidentified lipids + origin</td>
<td>2.67 ± 0.82</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>100.29</td>
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RBC phospholipids were extracted, subsequently separated by two-dimensional TLC, and lipid phosphorus was determined as outlined in the Materials and Methods section. Values are mean ± SD of n determinations (duplicates or triplicates from five independent experiments).
in ATP-depleted RBCs (Fig 2). Addition of chlorpromazine again resulted in a decrease in conversion of PIP$_2$ to PI during incubation. Indeed, dephosphorylation of PIP$_2$ was largely inhibited by addition of chlorpromazine. Regardless of the incubation condition, the sum of PI, PIP, and PIP$_2$ remained constant during incubation.

As a result of the variation in the percentages of the phosphoinositides between individuals, a comparison of the values obtained from different donors might lack statistical significance. This is particularly true of the data after 22-hour incubation, although they indicated the same trend as that observed after 46-hour incubation. The values after 46-hour incubation demonstrated statistically significant differences for the phosphoinositides between the samples incubated with or without chlorpromazine added.

Effect of chlorpromazine on RBC deformability. The effect of chlorpromazine on RBC deformability during ATP depletion was examined with an ektacytometer. The deformability profile for normal RBCs shows a maximal DI value at ~290 mOsm/kg. A decrease or increase in tonicity results in a lower deformability, with a minimum DI value in the hypotonic side at ~135 mOsm/kg. This value coincides with the osmolality at which 50% of the cells have lysed in a classical osmotic fragility test.$^{15}$ As shown in Fig 3A, the ektacytometric profile of RBCs incubated for ten minutes at 37°C was identical under all incubation conditions, regardless of the presence or absence of chlorpromazine or of the incubation buffer used.

After 22-hour incubation, the deformability profiles of the various samples showed considerable change (Fig 3B). The maximum DI value of ATP-depleted RBCs (<1% of the starting ATP level) decreased to 70% to 80% of the initial value (Fig 3B, curves 1 and 2). The maximum DI value of RBCs with 80% to 85% of the starting ATP levels was at 80% to 90% of its original value (Fig 3B, curves 3 and 4). Furthermore, ATP depletion resulted in a shift of the profile, indicating an increase in osmotic fragility due to a decreased ratio of surface area to volume. Addition of chlorpromazine during incubation led only to a slight change in the deformability profile. The slight shift of the profile of chlorpromazine-treated RBCs toward lower osmolality, as compared with RBCs incubated without chlorpromazine, may result from incorporation of chlorpromazine into the RBC membrane leading to an increase in the ratio of surface area to volume.

After 46-hour incubation, erythrocytes incubated with nutrients revealed a further decrease in the maximum DI value to 50% to 70% of the initial value (Fig 3C, curves 3 and 4). ATP-depleted RBCs, after shedding of vesicles, lost all deformability as measured by the ektacytometer (Fig 3C, curve 1). A similar observation was also made with RBCs undergoing vesiculation induced either by calcium loading of erythrocytes or by incubation at 51°C (results not shown) or incubation of RBCs at low pH.$^4$ Addition of chlorpromazine during ATP depletion clearly prevented this loss of deformability (Fig 3C, curve 2). The maximum deformability of these erythrocytes reached almost the same value as that observed with chlorpromazine-treated RBCs with 40% to 50% of the starting ATP levels (Fig 3C, curve 4). In addition, RBCs incubated for 22 or 46 hours, respectively, showed a slightly higher minimum DI value at low osmolality than untreated erythrocytes, indicating a more heterogenous RBC population.$^{15}$ (Fig 3A v B and C). This result is in accord with the broad distribution of RBC shapes observed under the light microscope (Table 1). The information obtained from the ektacytometric studies indicates that ATP depletion of RBCs increases the osmotic fragility of erythrocytes and concomitantly decreases RBC deformability, whereas incorporation of chlorpromazine enables the cell to compensate somewhat for these changes.

Effect of chlorpromazine on RBC membrane proteins. No difference was evident in the membrane protein pattern as analyzed by SDS-PAGE between RBCs incubated for 46 hours with or without chlorpromazine (results not shown).

Discussion

Erythrocytes undergo shape transformation to spiculated cells (echinocytes) or cupped cells (stomatocytes) in response to various treatments, including exposure to amphiphilic
compounds. The action of an amphiphath on RBC morphology can largely be predicted by the bilayer couple hypothesis (ie, cationic drugs intercalate into the inner half of the membrane bilayer, thereby expanding it and causing the RBCs to adopt a cupped shape). Anionic compounds, on the other hand, insert in the outer half of the membrane bilayer and lead to spiculation of RBCs. Only a small relative expansion of either half of the membrane bilayer is required for these shape changes to occur. Echinocytosis caused by calcium loading as well as by ATP depletion of RBCs may also arise from changes in the bilayer balance. Under these conditions, shrinkage of the surface area of the inner half of the membrane bilayer may be caused by turnover of the phosphoinositides, namely the dephosphorylation of PIP2 to PI. Both calcium loading and ATP depletion of RBCs result in release of acetylcholinesterase-containing vesicles from RBC membranes. Because we previously demonstrated that the cationic compound chlorpromazine is a potent inhibitor of RBC vesiculation caused by dimyristoylphosphatidylincholine, we investigated the effect of chlorpromazine on vesicle release, phosphoinositide turnover, and RBC deformability during ATP depletion of RBCs. Our results demonstrate that chlorpromazine effectively inhibits ATP depletion-induced shape change and subsequent vesiculation of RBCs (Tables 1 and 2). In addition, we showed a significant decrease in dephosphorylation of PIP2 to PI during ATP depletion when chlorpromazine was added (Fig 2). A similar effect of chlorpromazine on phosphoinositide turnover was also observed when RBCs were incubated for 46 hours under conditions in which ATP levels were partly maintained (Fig 2). Because chlorpromazine did not affect ATP levels during incubation and we did not observe any changes in RBC membrane protein composition between samples incubated with and without chlorpromazine, we believe that chlorpromazine caused the observed effects solely by selective insertion into the inner leaflet of the membrane bilayer. Our observations suggest an alternative interpretation of the sequence of events associated with RBC ATP depletion as proposed by Ferrell and Huestis. They suggested that a decrease in ATP levels led to a decrease in the PIP2/PI ratio, which in turn caused a shape change of RBCs due to loss of inner membrane leaflet area relative to outer membrane leaflet area. Our experiments show that addition of chlorpromazine, independent of an effect on ATP, decreased the rate of dephosphorylation of PIP2. These data favor the hypothesis that RBC ATP depletion leads to a shape change which then causes a decrease in the PIP2/PI ratio. One can also argue that addition of chlorpromazine affected the enzymes involved in phosphoinositide turnover; these enzymes are located at or intercalated in the inner half of the membrane bilayer where the phosphoinositides are believed to be located. Because chlorpromazine accumulates on the inside of the membrane bilayer, its effect on the membrane may also modulate the activity of a membrane-associated enzyme involved in phosphoinositide turnover by changing its membrane environment or altering availability of the substrate(s). That the observed effect of chlorpromazine occurred through modulation of the bilayer balance was supported by the finding that addition of tetracaine, an agent which affects RBC morphology and vesiculation in a way similar to that of chlorpromazine, resulted in the same decrease in turnover of the phosphoinositides during ATP depletion (result not shown). Chlorpromazine had a significant effect on RBC deformability during ATP depletion of RBCs (Fig 3). Without chlorpromazine, ATP depletion and subsequent vesiculation led to a complete loss of RBCs deformability as measured by the ektacytometer. This observation was characteristic for all RBCs after release of vesicles, regardless of the method used to induce vesiculation. In addition, ATP-depleted RBCs revealed an increased osmotic fragility as compared with erythrocytes incubated with nutrients. Both RBC deformability as well as osmotic fragility were markedly improved by addition of chlorpromazine during incubation. Again, these results support our hypothesis that the mode of action of chlorpromazine on RBC vesiculation and phosphoinositide turnover occurs through a change in the bilayer balance. This change may also affect membrane characteristics and ultimately the state of cell hydration. The existence of a calcium-activated polyphosphoinositide phosphodiesterase (phospholipase C) in human erythrocyte membranes has been previously reported. Our results, however, do not indicate that a phospholipase C is involved in the changes in phosphoinositides during ATP depletion of RBCs since the sum of the relative amounts of the phosphoinositides remained constant during incubation. This observation is in accord with a similar conclusion by Ferrell and Huestis. In addition, although chlorpromazine was proposed to act on the phosphoinositide turnover through modulation of calmodulin in human platelets, recent reports have not confirmed such a relationship in human erythrocytes. In light of these reports, the effect of chlorpromazine on vesiculation and phosphoinositide turnover probably does not occur through modulation of calmodulin. In summary, our studies show that chlorpromazine can inhibit ATP depletion-induced shape change and subsequent vesiculation of RBCs. They also demonstrate that chlorpromazine can affect turnover of the phosphoinositides during ATP depletion. These changes are paralleled by an improvement of RBC deformability. We believe that the effects of chlorpromazine on ATP-depleted RBCs are mediated through its effect on the inner leaflet of the lipid bilayer. Our findings may provide a new approach to prevent membrane vesiculation and as such be applicable in circumstances such as blood storage.

ACKNOWLEDGMENT

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