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The lytic behavior of pure phospholipases A_2 and C towards osmotically swollen erythrocytes and resealed ghosts

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SUMMARY

1. Phospholipase C (phosphatidylcholine cholinephosphohydrolase, E.C. 3.1.4.3) from *Bacillus cereus* evoked hemolysis of intact human erythrocytes in hypotonic sucrose solutions at sucrose concentrations below 120 mM, whereas pancreatic phospholipase A_2 (phosphatide acyl-hydrolase, E.C. 3.1.1.4) became lytic below 100 mM sucrose. Treatment of intact cells with proteolytic enzymes prior to the incubations with phospholipases A_2 and C did not alter the lytic behavior of these phospholipases.

2. Resealed ghosts made in saline in the presence of 2 mM $CaCl_2$ retained 16–20% of their hemoglobin and were sensitive to lysis by phospholipase C, but not by phospholipase A_2; resealed ghosts made in saline in the presence of 1 mM EDTA retained only 1–2% of their hemoglobin and were lysed by both phospholipases A_2 and C. Resealed ghosts prepared in the presence of Ca^{2+} after increasing durations of lysis prior to the resealing procedure, showed a decrease in lysis due to phospholipase C, but no lysis due to phospholipase A_2.

3. Resealed ghosts made in sucrose in the presence of 2 mM $CaCl_2$ retained 24–30% of their hemoglobin and showed increasing lysis by phospholipase C with increase in the lysing tonicity prior to resealing. Phospholipase A_2 showed a minimum in the lysis of resealed ghosts, which were hemolysed at 20 mM sucrose prior to resealing. Treatment with subtilopeptidase A of resealed ghosts followed by treatment with phospholipase A_2 or C showed no increased lysis as compared with non-proteolysed cells.

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4. Alterations in membrane architecture occur during the process of ghost formation from intact erythrocytes by a hypotonic lysis procedure. During this process the polar headgroups of the phospholipids become more readily available for hydrolysis by phospholipase C than does the fatty acid–ester linkage for hydrolysis by pancreatic phospholipase A_2.

In a previous study[1] it was shown that pure pancreatic phospholipase A_2 (phosphatide acyl-hydrolase, E.C. 3.1.1.4) and pure phospholipase C (phosphatidylcholine cholinephosphohydrolase, E.C. 3.1.4.3) from *Bacillus cereus* were not lytic towards intact human erythrocytes in both isotonic and in 0.6% saline. Moreover, no significant hydrolysis of phospholipids could be established in these experiments. On the other hand, incubations of human red cell ghosts with either phospholipase A_2 or phospholipase C resulted in a complete degradation of the main phospholipid classes (except for sphingomyelin) comprising 70% of the total phospholipids. Most of our present-day knowledge of the erythrocyte membrane is based upon experiments with red cell ghosts. However, various investigators[1–7] using different techniques also have shown that great differences may exist between the membrane surrounding the intact cell and the isolated ghost. These results seem to support the thesis that the "stroma" from the erythrocytes must be considered at best as a first derivative of the original membrane[1,6].

In this study, the intact human erythrocyte is altered in a controlled manner so as to imitate the gradual transition to erythrocyte ghosts. The changes in accessability of the membrane phospholipids to phospholipases A_2 and C as a result of these alterations are studied in terms of the amount of hemolysis produced by these enzymes. In general, hypotonic sucrose solutions have been used in the studies with intact cells, since it has been shown[8] that the osmotic resistance curves in sucrose solutions provide a better approximation to the original osmotic properties of red blood cells than saline. The experiments with resealed ghosts have been carried out in both sucrose and NaCl solutions.

Freshly collected red cells (from acid–citrate–dextrose-treated blood; cells packed for 10 min at 3000 × *g*) were washed 4 times in isotonic sucrose–2mM $CaCl_2$, pH 7.5, with $NaHCO_3$. Aliquots of cells were incubated in sucrose solutions of varying tonicities in the presence of 2 mM $CaCl_2$ at pH 7.5 (with $NaHCO_3$) and tested for hematocrit and then for susceptibility to lysis by pure pancreatic phospholipase A_2[9] and pure phospholipase C[10] from *B. cereus*. The assay for hematocrit was as follows: 2 ml of packed red cells were suspended in 10 ml of the appropriate hypotonic sucrose solution and allowed to stand at room temperature for 2 h. The hematocrit was measured after centrifugation in a Hawksley micro hematocrit centrifuge. The cells were then washed twice in the same sucrose solutions and aliquots of 0.25 ml were taken for the hemolysis tests with 8–10 I.U. phospholipase A_2 and phospholipase C. The hemolysis with phospholipase A_2 and phospholipase C was measured after incubation for 1 h at 37°C as previously reported[1], with the exception that 5 ml of each sucrose solution (2 mM in $CaCl_2$, pH 7.5, with $NaHCO_3$) was used instead of 5-ml saline solutions.

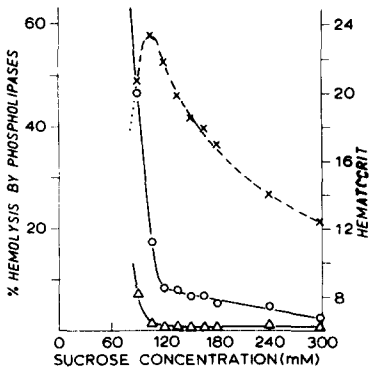
As expected, the hematocrit increased on lowering of the sucrose tonicity from isotonic to 105 mM (Fig. 1). The decrease in hematocrit between 105 and 90 mM sucrose is not caused by spontaneous hemolysis, but can probably be attributed to a drastic change in cell shape. Since spontaneous hemolysis (> 5%) occurs below 90 mM sucrose, the incubations of the cells with phospholipases below this point are not shown. The cells incubated with phospholipase C exhibited a very slow increase in hemolysis as the tonicity was decreased from isotonic to 120 mM sucrose, whereas below this point a sharp rise in hemolysis was observed. On the other hand, phospholipase A_2 did not become lytic before 100 mM sucrose★.

In order to establish whether or not the observed hemolysis was a genuine effect of enzymatic action, the entire hypotonic hemolysis scheme was carried out either in the presence of 2 mM phosphate (no Ca^{2+} added) to inhibit phospholipase A_2 or in the presence of 1 mM 1,10-phenanthroline to inhibit phospholipase C. In both cases no hemolysis above blank was observed. The lytic effect of phospholipase A_2 below 100 mM sucrose (Fig. 1) may be caused by the fact that 1–5% blank hemolysis was always observed in this region. This gives rise to the formation of ghosts which are easily degraded by phospholipase A_2[1], producing lyso-compounds which are potent lysins. By adding 0.25 mg of isolated red cell ghosts to the various incubation mixtures, a substantial increase in hemolysis with phospholipase A_2 was observed at tonicities lower than 135 mM, but no increase was found with phospholipase C.

In order to investigate whether or not there was any effect on the lytic behavior of phospholipase A_2 and phospholipase C (Fig. 1) after pretreatment with proteolytic enzymes, packed cells were first incubated for 1 h at 37°C with an equal volume of a 0.1% (w/v) solution in 120 mM sucrose of the following proteolytic enzymes: trypsin, chymotrypsin, pronase E, subtilisin, subtilopeptidase A, thermolysin and pilz proteinase. These treatments did not cause more than 4% lysis. The cells were subsequently washed 3 times with 120 mM sucrose and then incubated with phospholipase A_2 or phospholipase C as above. No significant difference in hemolysis with both phospholipases was observed between the proteolysed and the non-proteolysed cells.

Since there is a difference in the accessibility of the phospholipids to phospholipase A_2 and phospholipase C between the intact erythrocyte and its derived ghost[1,10], it is of interest to investigate the accessibility of the phospholipids to both phospholipases after the hemolysis procedure has been reversed in order to obtain resealed ghosts. Reconstituted ghosts were prepared as described by Hoffman[11] and Palek *et al.*[12], but several different lysing tonicities and durations of lysis before resealing were used. In the first experiments erythrocytes were hemolysed for 10 min at room temperature in 5 vol. of 10, 50 or 100 mosM NaCl, either in the presence of 2 mM $CaCl_2$ or in the presence

★Similar effects were observed with pure phospholipase A_2 from *Crotalus adamanteus*; pure phospholipase A_2 from bee venom, however, showed a lytic behavior similar to that observed with phospholipase C. These two enzymes were gifts from Professor G.H. de Haas who received them as generous gifts from Dr M.A. Wells and Professor C.A. Vernon, respectively.



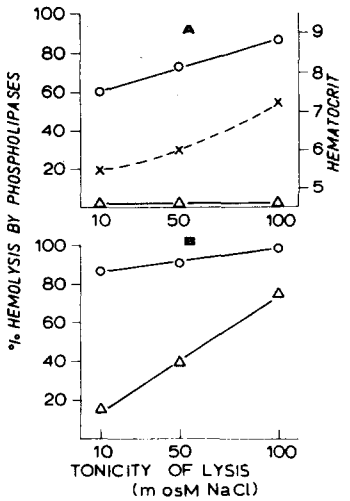


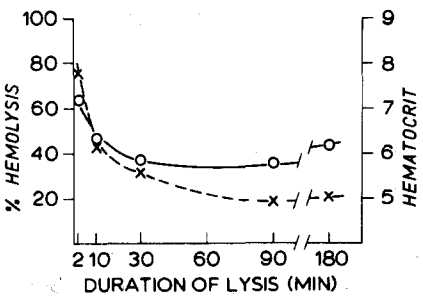
Fig. 1. Hematocrits and hemolysis by phospholipases of erythrocytes in hypotonic sucrose solutions. X– –X, hematocrit (arbitrary units); o——o, hemolysis by phospholipase C; △——△, hemolysis by phospholipase A_2.

Fig. 2. Hematocrits and hemolysis by phospholipases of resealed ghosts in isotonic NaCl–2 mM $CaCl_2$. (A) Resealed ghost from cells originally lysed in hypotonic NaCl (abscissa) in the presence of 2 mM $CaCl_2$. (B) Resealed ghost from cells originally lysed in hypotonic NaCl (abscissa) in the presence of 1 mM EDTA. X– –X, hematocrit (arbitrary units); o——o, hemolysis by phospholipase C; △——△, hemolysis by phospholipase A_2.

of 1 mM EDTA. The ghosts were resealed at room temperature by restoring isotonicity with the addition of 1.5 M NaCl, containing 2 mM $CaCl_2$ or 1 mM EDTA, respectively. Hematocrits were measured and the amount of hemoglobin trapped inside was determined[12]. It was not possible to obtain accurate hematocrits on the EDTA-resealed ghosts and they trapped only 1–2% of the initially present hemoglobin. The Ca^{2+} resealed ghosts trapped 16–20% hemoglobin and had a hematocrit of 40–60% of that of the original cells. Both the Ca^{2+} and the EDTA ghosts were then centrifuged and washed twice with isotonic NaCl–2 mM $CaCl_2$, pH 7.5 ($NaHCO_3$). The precaution was always taken to remove the dark red button that appeared under the ghosts after centrifugation, assuming that they were unlysed, osmotically resistant cells. Aliquots were subsequently incubated with phospholipase A_2 and phospholipase C in isotonic saline in the same manner as with intact cells. The results are shown in Fig. 2. The ghosts made in the presence of Ca^{2+} are much more contracted than those made in EDTA[12]; this shows up in the greater susceptibility to lysis of the EDTA ghosts. There is also an effect of the lysing tonicity, *i.e.* the higher the lysing tonicity the less osmotic gradient across the cell when restored to isotonicity. This causes less shrinkage than would occur with a cell lysed at lower tonicity. A prerequisite for this effect is that the selective permeability has been largely restored before the restoration of isotonicity. This has been shown to be true by Palek *et al.*[12] and by Seeman[13]. It is obvious that phospholipase C caused more hemolysis the larger the resealed

ghost, whereas phospholipase A_2 caused almost no hemolysis of the Ca^{2+} resealed ghosts but showed increasing hemolysis with the EDTA ghosts.

The next experiments were designed to determine if any changes in duration of lysis produced any changes in susceptibility to phospholipase A_2 and phospholipase C. Red cells were lysed in 50 mosM NaCl–2 mM $CaCl_2$ (pH 7.5 with $NaHCO_3$) and resealed at room temperature by restoring isotonicity with 1.5 M $NaCl_2$–2 mM $CaCl_2$ after 2, 10, 30, 90, and 180 min. The hematocrits and percent hemoglobin retention were measured, the ghosts washed twice in isotonic NaCl–2 mM $CaCl_2$ (pH 7.5 with $NaHCO_3$), and aliquots were taken for testing with phospholipase A_2 and phospholipase C. The results are shown in Fig. 3. There was a decrease in hemoglobin trapping as well as in hematocrit with increasing duration of lysis; this indicates that the ghosts before resealing were somewhat permeable to both small ions and larger molecules. With increasing shrinkage a decrease in accessibility of the membrane phospholipids to phospholipase C was observed, whereas phospholipase A_2 appeared not to be lytic.



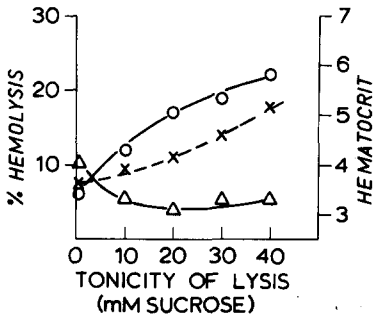


Fig. 3. Hematocrits and hemolysis by phospholipase C of resealed ghosts in isotonic saline–2 mM $CaCl_2$ prepared after lysis for different times in 50 mosM NaCl–2 mM $CaCl_2$. X– –X, hematocrit (arbitrary units); o——o, hemolysis by phospholipase C.

Fig. 4. Hematocrits and hemolysis by phospholipases of resealed ghosts in isotonic sucrose–2 mM $CaCl_2$. The abscissa represents the sucrose concentrations at which the ghosts have been produced prior to resealing. X– –X, hematocrit (arbitrary units); o——o, hemolysis by phospholipase C; △——△, hemolysis by phospholipase A_2.

Resealed ghosts made in sucrose solutions from red cells washed in isotonic sucrose showed similar results as the resealed ghosts in NaCl. Cells were lysed with 5 vol. of 0, 10, 20, 30, or 40 mM sucrose–2 mM $CaCl_2$ (pH 7.5 with $NaHCO_3$) and allowed to stand for 30 min at room temperature. They were then resealed at room temperature with 1.5 M sucrose–2 mM $CaCl_2$, pH 7.5, during which they trapped 24–30% hemoglobin, and

after which they had a hematocrit of 40–60% of that of the original cells. The resealed ghosts were then washed twice in isotonic sucrose–2 mM $CaCl_2$, pH 7.5, and aliquots were taken for incubations with phospholipase A_2 and phospholipase C. No experiments have been carried out with resealed ghosts prepared in sucrose–EDTA, since the density of these ghosts was so close to that of the medium that they were difficult to sediment by low speed centrifugations. As shown in Fig. 4, phospholipase C showed a similar behavior as with resealed ghosts in NaCl; that is, it produced increasing lysis with increasing ghost size. However, phospholipase A_2 showed a slightly anomalous behavior as compared with NaCl ghosts. Treatment of sucrose resealed ghosts, originally lysed in 2 mM $CaCl_2$, with subtilopeptidase A prior to incubations with phospholipase A_2 and phospholipase C did not cause any increase in hemolysis with these phospholipases, as was shown in Fig. 4 (0 mM sucrose).

The results of all these experiments suggest that there is a correlation between the ability of phospholipase A_2 and phospholipase C to cause hemolysis and the state of expansion of the cell membrane. It is obvious that hemolysis is a reflection of lipid hydrolysis by these phospholipases. However, when the residual intact cells (free from ghosts) after partial hemolysis of the total cell population by phospholipase A_2 or phospholipase C were analysed for phospholipids[14], no significant degradation of any phospholipid class could be detected. On the other hand, if the total incubation mixture was analysed, a considerable proportion of the phospholipid classes was degraded; this is obviously a reflection of phospholipid hydrolysis in ghosts produced by lysis. These results imply that degradation of an indetectable amount ($< 5\%$) of phospholipids in the intact cells causes hemolysis.

The ghosts resealed in the presence of EDTA are much more susceptible to phospholipases than ghosts resealed in the presence of Ca^{2+}. This may be caused by either the increased cell volume of the EDTA ghosts[12], or by an alteration of the membrane structure induced by the absence of divalent cations within the cell.

It is apparent that during the process of ghost formation from intact erythrocytes by a hypotonic lysis procedure, the polar headgroups of the phospholipids become more readily available to phospholipase C than the fatty acid–ester linkage at C-2 does to pancreatic phospholipase A_2. This indicates that at least part of the phospholipid is present in the vicinity of the outer surface of the membrane surrounding the intact erythrocyte. It is not clear, however, whether at decreasing tonicities the increase in accessibility of these phospholipids for phospholipases is caused by a stretching of the lipid layer or by an alteration in the tertiary structure of those outer membrane proteins, which are covering the lipids and are not digested by proteolytic enzyme treatments of intact cells and resealed ghosts. Apparently, the changes which occur in the membrane during ghost formation cannot be completely reversed by a resealing procedure. Although a vast number of investigations on isolated red cell ghosts have certainly contributed to a better understanding of the structure and function of the erythrocyte membrane, one has to be aware of the limitations of this approach, especially in those studies from which membrane models have been designed.

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