SUSCEPTIBILITY OF MEMBRANE PHOSPHOLIPIDS IN ERYTHROCYTE GHOSTS TO PHOSPHOLIPASE C AND THEIR REFRACTIVENESS IN THE INTACT CELL

Yehudit LASTER, Esther SABBAN and Abraham LOYTER
Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

Received 8 December 1971

1. Introduction

Lately the erythrocyte membrane has served as a convenient model for studying the architecture of biological membranes. Hydrolysis of the erythrocyte membrane phospholipids by phospholipases from different sources has been used extensively as a tool for studying their organization within the membrane.

Condrea et al. [1] have shown that a fraction from cobra venom, which contained phospholipase A activity, was not able to release fatty acids from membranes of human erythrocytes. When another fraction of venom ("direct lytic factor") which on its own is weakly lytic was added to the phospholipase A fraction, a great increase in hemolysis occurred concomitantly with hydrolysis of membrane phospholipids. Thus it seems that phospholipase A could not attack membrane phospholipids unless the direct lytic factor was added.

Macfarlane [2], using phospholipase C preparations from different sources, has found a correlation between the hydrolysis of erythrocyte phospholipids and the amount of hemolysis obtained. She concluded that hemolysis is a consequence of hydrolysis of membrane phospholipids.

However, it is known that pure phospholipase C from *B. cereus* which is able to hydrolyze a wide spectrum of phospholipids, is not hemolytic [3]. Since this enzyme is available as a highly purified protein [4], its use seems advantageous in the study of phospholipid organization in the cell membrane.

In the present work the action of phospholipase C from *B. cereus* on human erythrocytes was studied. It is shown that although phospholipids of ghosts are readily hydrolyzed, phospholipids of intact erythrocytes are not available to hydrolysis by the enzyme. Implications of the findings for the architecture of the erythrocyte membrane are discussed.

2. Materials and methods

2.1. *Cells*

Human blood, type O, (3–6 wk old) which was suspended in citric acid dextrose was obtained from the blood bank of Hadassah Hospital, Jerusalem. After arrival in the laboratory, the blood was stored at 4° and used within a month.

The red cells were washed 4 times at 4° either with isotonic saline or with solution K, both buffered with 20 mM Tricine-NaOH pH 7.4. Unless otherwise noted, samples containing 3 ml packed washed cells were suspended in one of the above buffered salt solutions to give a final vol of 80 ml (final conc. of 3.7%, v/v).

2.2. *Media*

The following media were used throughout the work. A. Solution K which contained: 1×10^{-1} M KCl, 5.4×10^{-3} M NaCl and 1×10^{-3} M $MgSO_4$. B. Solution K′ identical to solution K except that the KCl concentration was 3×10^{-2} M. All media were buffered with 10 mM Tricine-NaOH at pH 7.4.

2.3. *Osmotic hemolysis, incubation with phospholipase C and phospholipid extraction*

A series of samples containing 3 ml of packed

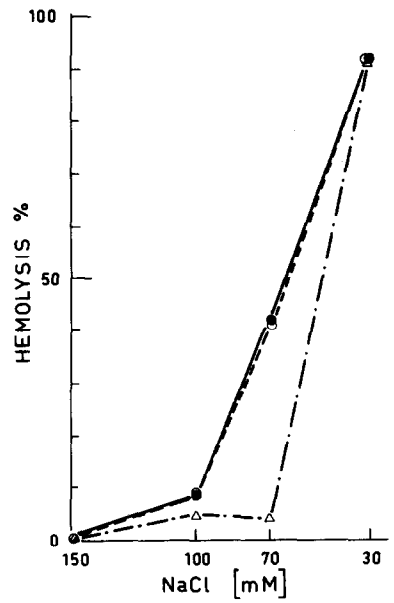

Fig. 1. Phospholipase C induced hemolysis: dependence on the osmolarity of the media. Cells were washed in isotonic media and suspended in decreasing concentrations of NaCl as described under Materials and methods. (△–△–△): Cells suspended in the absence of phospholipase C. (●–●–●): Cells suspended in the presence of 0.13 μg/ml of phospholipase C. (○–○–○): Cells suspended in the presence of 1.3 μg/ml of phospholipase C.

cells were suspended in solutions of NaCl which varied in concentration from 30 mM to 150 mM and contained 10 mM Tricine-NaOH at pH 7.4. To complete hemolysis samples were kept at room temp for 5 min and then the volume of the suspension was brought to 80 ml by the addition of 2.8 ml of solution K with or without 40 μg/ml of phospholipase C. The cell suspensions were then incubated for 30 min at 37° with gentle shaking (in a New Brunswick shaker at 100 rpm). At the end of the incubation period the cells were cooled and centrifuged for 40 min at 35,000 *g* (Spinco, Rotor 30). Samples of 2 ml were removed from the supernatant for hemolysis determination as previously described [5]. In experiments in which phospholipids were extracted, the pellets were washed twice more with the appropriate media and the lipids were extracted from the final sediment according to Burger et al. [6].

Table 1
Hydrolysis of human erythrocyte phospholipids at different degrees of hemolysis.

NaCl (mM)	Hemolysis (%)	Hydrolysis of phospholipids (%)
150	0	0
120	5	0
100	26	4
80	64	24
70	82	26
30	93	58

Total phosphorus in phospholipids extracted from control cells (intact erythrocytes) was found to be 9×10^{-15} g/cell.

2.4. *Incubation of ghosts and reconstituted ghosts with phospholipase C*

Human erythrocyte ghosts were prepared by suspending the final sediments of the washed cells in 1 mM $MgSO_4$ or in solution K′, both buffered with 10 mM Tricine-NaOH at pH 7.4, to give final conc. of 10% (v/v). The volume of the ghost suspension was then brought to 77.2 ml with either of the above media.

Reconstitution was carried out essentially according to Redman [7]. Samples (30 ml) of suspensions of ghosts (10% v/v) were readjusted to isotonic conditions by adding 1.7 ml of a solution containing 3 M KCl and 1.6×10^{-2} M $MgSO_4$. The volume of the suspension was brought to 77.2 ml by the addition of solution K. Both the ghosts and the reconstituted ghosts were incubated for 30 min at 37° with gentle shaking and then 2.8 ml of phospholipase C (*B. cereus*) (40 μg/ml dissolved in solution K) were added. At the end of the incubation period, the ghosts were washed by centrifugation and lipids were extracted as described above.

Total phosphate was determined according to Ames [8]. Glucose-1-phosphate served as a standard. During phospholipase C action water soluble phosphoryl choline is released. On that basis percent hydrolysis of phospholipids was calculated as follows:

a = μg total phosphorus in phospholipids extracted from intact erythrocytes

b = μg total phosphorus in phospholipids extracted from treated samples

Table 2
The effect of phospholipase C (*B. cereus*) on human erythrocyte ghosts.

	System	Hemolysis (%)	Hydrolysis of phospholipids (%)
Exp. I			
	Intact erythrocytes	0	0
	Ghosts	96	85
	Reconstituted ghosts	83	23
	Reconstituted ghosts (phospholipase C was added before reconstitution)	83	61
Exp. II			
	Ghosts without incubation at 37°		79
	Ghosts incubated for 30 min at 37°		30
	Ghosts incubated for 60 min at 37°		29
	Reconstituted ghosts		17

Exp. I: Intact erythrocytes were washed and suspended in solution K. Ghosts were prepared by suspending the cells in 1 mM $MgSO_4$ buffered with 10 mM Tricine-NaOH at pH 7.4.
Exp. II: Ghosts were prepared by suspending the washed erythrocyte pellets in solution K' buffered with 10 mM Tricine-NaOH at pH 7.4 and were incubated for 0, 30 and 60 min at 37° before phospholipase C was added. Total phospholipid phosphorus was 9.4×10^{-15} g/cell in intact erythrocytes and 9.0×10^{-15} g/cell in ghosts.

$$\% \text{ hydrolysis} = \frac{a-b}{a} \times 100$$

Protein was determined according to Lowry et al. [9]. Highly purified phospholipase C (*B. cereus*) was obtained from Makor Chemicals Ltd., Jerusalem, Israel.

3. Results and discussion

Phospholipase C from *B. cereus* which is highly active in hydrolyzing micelles of phospholipids could not hemolyze red blood cells [3]. Substantial hemolysis was, however, induced by the enzyme when the cells were suspended in hypotonic media. It can be seen in fig. 1 that a negligible amount of hemoglobin is released from erythrocytes suspended in 70 mM NaCl in the absence of enzyme while about 50% hemolysis took place when the cells were incubated in the presence of phospholipase C.

In isotonic media, where phospholipase C failed to hemolyze erythrocytes, no hydrolysis of membrane phospholipids was observed (table 1). Hydrolysis of phospholipids occurred only in hypotonic media, in which the erythrocytes undergo partial hemolysis. As can be seen in table 1 about 60% of membrane phospholipids were hydrolyzed in cells suspended in 30 mM NaCl.

Interestingly, when ghosts were reclosed by transferring them back to isotonic media, the amount of hydrolysis was greatly decreased (table 2). Apparently, the membrane phospholipids again became unavailable to attack by phospholipase C. When ghosts were incubated with phospholipase C, as much as 80% of the membrane phospholipids were hydrolyzed as compared to only 23% or 17% in reconstituted ghosts (table 2, Exps. I and II). When the enzyme was added before the ghosts were reconstituted, no marked change in the extent of hydrolysis could be noted as compared to unreconstituted ghosts (table 1, Exp. I). Prolonged incubation of ghosts in hypotonic media at 37° is known to cause partial resealing of its membrane although the ghost remains expanded [10]. When phospholipase C was added to ghosts resealed in this way there was a decrease in the amount of hydrolysis from 79% in open ghosts to 30% or to 29% in ghosts incubated at 37° for 30 min or 60 min, respectively.

The present work clearly shows that phospholipid molecules of the intact human erythrocyte membrane cannot be attacked by phospholipase C (*B. cereus*). In order to expose them to attack by phospholipase C, a certain amount of hemolysis is required.

These results can be explained by at least two hypotheses. In the first case, due to asymmetric disposition of the membrane proteins, as suggested by Steck et al. [11], phospholipase C may be able to attack membrane phospholipids from inside the cell. When hemolysis takes place the phospholipase C possibly enters the cells and thus attacks the phospholipids from within. Alternatively, the phospholipids in intact erythrocytes are masked, possibly by glycoproteins, or buried within the membrane. During hemolysis the phospholipids are exposed and can be attacked.

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