Identification of Domains of Phosphatidylcholine in Human Erythrocyte Plasma Membranes

DIFFERENTIAL ACTION OF ACIDIC AND BASIC PHOSPHOLIPASES A2 FROM AGKISTRODON HALYS BLOMHOFFII

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Highly purified acidic (pl 4.9) and basic (pl 8.7) phospholipases A2 from snake (Agkistrodon halys blomhoffii) venom hydrolyzed approximately 20% and 60%, respectively, of the phosphatidylcholine (PC) of intact human erythrocytes prior to hemolysis. Sequential use of the acidic enzyme followed by the basic phospholipase A2 or vice versa manifested a characteristic PC hydrolysis pattern. For example, when acidic enzyme had hydrolyzed nearly 20% of this substrate, a subsequent treatment with the basic enzyme hydrolyzed only an additional 40% of the PC before hemolysis. On the other hand, in experiments where hydrolysis of about 20% of PC of erythrocytes was achieved by a short term incubation with the basic enzyme, then a further treatment of the same cells with the acidic enzyme caused only 10% additional PC hydrolysis before hemolysis. This demonstrated that the acidic enzyme hydrolyzed one domain of PC in the intact erythrocytes, whereas the basic enzyme hydrolyzed not only the same one but also another domain of PC in membranes. Analysis of fatty acids released by the action of these two phospholipases A2 on erythrocytes indicated further characteristic differences. In particular, the ratio of released saturated to unsaturated fatty acids was significantly higher with the acidic enzyme as compared with the basic phospholipase A2.

These results provide firm support to the conclusion that there are different domains of PC in human erythrocyte membranes and that the acidic and basic phospholipase A2 of A. halys blomhoffii can be used to identify them.

Phospholipases have been used extensively as probes for exploring the physical structure of the phospholipids in membranes, particularly the human erythrocyte plasma membrane (1, 2). While considerable progress has been made in our understanding of the arrangement of phospholipids in the plasma membrane using these enzymes, some problems have emerged. For example, the activities of phospholipases toward membranes depend on several factors, e.g. pH, [Ca²⁺], the age of the cells, and the ATP levels of the cells (1-5). Furthermore, phospholipases from different sources with varying physical characteristics do exhibit considerable differences in their activities toward the human erythrocyte (2, 5). It is evident that the phospholipase attack on this cell membrane is quite complex and that a systematic approach to its use as a probe is dictated. We have reported the isolation and purification of acidic (pl 4.9),¹ neutral (pl 6.9), and basic (pl 8.7) phospholipases A2 from the venom of Agkistrodon halys blomhoffii (6). These three phospholipases A2 have significantly different physicochemical properties and therefore have been considered of potential value in the study of membrane structure and function. To this end, recently, we presented preliminary evidence that the mode of attack of these three phospholipases A2 varied considerably on intact erythrocytes (7, 8). Specifically, the acidic and basic enzymes hydrolyzed approximately 20% and 60% of phosphatidylcholine, respectively, before hemolysis of erythrocytes occurred. This prompted us to investigate whether the interaction of these two phospholipases with erythrocyte membranes was such that there was recognition of different domains of phosphatidylcholine. The results of this investigation provide new insight into the mode of attack of these unique phospholipases A2 on membranes and also into the molecular arrangement of phosphatidylcholine in human erythrocyte membranes.

EXPERIMENTAL PROCEDURES

Materials

Crude lyophilized venom from A. halys blomhoffii was purchased from Miami Serpentarium (Miami, FL) and was separated into pure acidic (pl 4.9), neutral (pH 6.9), and basic (pl 8.7) phospholipases A2 by the method of Hanahan et al. (6). Standard fatty acid methyl esters were obtained from Supelco (Bellefonte, PA). Precoated thin layer chromatography plates (silica gel) were purchased from Analtech (Newark, DE). All other chemicals and solvents were of the highest analytical grade.

Blood was withdrawn from the antecubital vein of normal healthy adult volunteers (age range 20 to 35 years) in heparinized vacutainers (Becton-Dickinson, Rutherford, NJ). Erythrocytes were collected by centrifugation of this blood at 4°C for 10 min at 1000 × g and the plasma anduffy coat were carefully removed and discarded. The erythrocytes were washed twice by centrifugation as above with 5 volumes of isotonic NaCl-histidine (5 mM), pH 7.8, buffer and were used immediately.

Methods

Treatment with Phospholipases A2—The procedure for the treatment of erythrocytes with phospholipase A2 was similar to that described (7) where the incubation mixture (final volume 1 ml) contained the desired amount of the phospholipase A2 (acidic or basic), erythrocytes (final hematocrit 10%), and 90 mM Ca²⁺ in an

¹The abbreviations used are: pl, isoelectric point; PC, phosphatidylcholine; PE, phosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid.
isoionic NaCl/histidine (5 mM) buffer, pH 7.6. In experiments where erythrocytes were treated sequentially with two phospholipases, the cells were incubated with the first phospholipase A₂, and then mixed with 5 volumes of an ice-cold isoionic NaCl/histidine (5 mM) buffer, pH 7.6, containing 1 mM EDTA and centrifuged at 1000 × g at 4 °C for 10 min. This washing procedure was repeated twice and followed by a third wash with an EDTA-free isoionic saline solution. Washed erythrocytes were incubated in an isoionic NaCl/histidine (5 mM) buffer, pH 7.6 (final volume 1 ml) containing 30 mM Ca²⁺ and the second phospholipase A₂. The reaction was quenched by the addition of EDTA to a final concentration of 45 mM.

All incubations were conducted in duplicate or triplicate in plastic tubes. Hemolysis of the cells was determined by recording the absorbance 540 nm of the supernatant obtained by centrifuging the incubation mixture at 8000 × g for 2 min in an Eppendorf centrifuge 3290. A 100% value for hemolysis was obtained by lysing the cells either in water or by the addition of saponin (0.1 mg/ml) as described elsewhere (7). Lipids were extracted from erythrocytes and analyzed as described below.

**Lipid Extraction and Fatty Acid Analysis**—Phospholipids were extracted from erythrocytes and analyzed as reported before (7). Free fatty acids were separated from total lipids by thin layer chromatography on precoated Silica Gel G plates in a solvent system of petroleum ether/ether/acetic acid (80:20:1, v/v). Fatty acid areas were scraped into glass tubes (designed for methylation of fatty acids) and 1 ml of 0.5% (v/v) H₂SO₄ in methanol was added. The tubes were sealed and incubated at 70 °C for 1 h with intermittent mixing. At the end of the incubation, 0.1 ml of distilled water was added, and fatty acid methyl esters were extracted four times with 2 ml of n-hexane and dried under N₂.

Fatty acid methyl esters were analyzed by gas-liquid chromatography. Samples were injected into a column (GR 10% SP 2330 on 100/120 Chromosorb W, AW, Supelco) set at 170 °C, attached to a Varian Model 3700 gas chromatograph. Various mixtures of standard fatty acid methyl esters were run in parallel and the unknown fatty acids were identified by comparing the retention times. Values for the peak area of fatty acid were obtained either directly, from a programmable computer (Varian, CDS 111) attached to the Varian Model 3700 gas chromatograph or by calculating as described elsewhere (9). Both procedures gave identical results.

Lipid phosphorus was determined by the method of Bartlett (10) and protein was assayed by the Lowry technique (11).

**RESULTS AND DISCUSSION**

Throughout this investigation, our major goal was to examine the organization of phospholipids in human erythrocyte membranes, using purified acidic and basic phospholipases A₂ (A. halys blohmofii) under nonlytic conditions. The results of this approach are summarized below.

**Systematic Attack of Acidic and Basic Phospholipases A₂ on Intact Human Erythrocytes**—In a preliminary report (7), we showed that prior to hemolysis of erythrocytes the acidic and basic phospholipases A₂ hydrolyzed about 20% and 60%, respectively, of phosphatidylcholine in a highly characteristic manner. This demonstrated that these two enzymes behave differently toward human erythrocytes. One possibility was that these two phospholipases A₂ recognized different domains or populations of PC on the outer surface of the erythrocyte membrane. If this were the case, then a sequential action of the acidic enzyme followed by the basic enzyme or vice versa always should reveal two different patterns of hydrolysis under nonlytic conditions.

In order to examine this latter point, intact cells were treated with acidic enzyme (100 μg/ml, 30 mM Ca²⁺, 30 min at 37 °C) wherein a maximum hydrolysis of PC (approximately 20%) was achieved without hemolysis (7). Cells then were washed twice by centrifugation at 4 °C with isoionic saline containing 1 mM EDTA (see "Experimental Procedures") and then reincubated with the basic enzyme (100 μg/ml, 30 mM Ca²⁺, 15 min at 37°C). The degree of hemolysis and PC hydrolysis was monitored and the results are shown in Fig. 1. It is important to mention here that the washing with isoionic saline containing EDTA removes all the phospholipase A₂ from the erythrocytes. This was checked by reincubating the erythrocytes, which were treated previously with phospholipase A₂, and then washed with the above solution, in a Ca²⁺-containing medium. Under these conditions, no further hemolysis or phospholipid hydrolysis was observed over a 3-h incubation period (results not shown), indicating a reversible interaction of phospholipases A₂ with these membranes. In this context, Jeng et al. (12) also have reported that phospholipase from Crotalus adamanteus does not bind to rabbit erythrocyte and ghost membranes, an observation with which we are in agreement. Such reversible binding of phospholipase A₂ on membranes indicates that a noncovalent interaction, hydrophobic and/or ionic, is involved.

It is clear from Fig. 1 that after the acidic enzyme had hydrolyzed 20% of PC of intact cells, addition of the basic enzyme caused hydrolysis of an additional 40% of the PC without causing hemolysis. Basic enzyme alone caused hydrolysis of 60% of PC under nonhemolytic conditions (Fig. 1 and Table I). In another experiment as described in Table I, hydrolysis of approximately 20% of PC was accomplished by incubation with basic phospholipase A₂ for 2 min. Cells were then washed and treated with acidic enzyme. As is evident from Table I, additional hydrolysis of PC amounted to about 10% prior to hemolysis. These results demonstrate that the acidic enzyme recognized and hydrolyzed one domain of PC in the intact erythrocytes, whereas the basic enzyme recognized and hydrolyzed not only the same one, but also another.

![Fig. 1](http://www.jbc.org/)

**TABLE I**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Hydrolysis of PC (%)</th>
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</thead>
<tbody>
<tr>
<td>(a) Acidic phospholipase A₂ only (30 min)</td>
<td>21.4 ± 1.3</td>
</tr>
<tr>
<td>(b) Acidic enzyme (30 min) followed by washing and basic enzyme (15 min)</td>
<td>63.2 ± 6.0</td>
</tr>
<tr>
<td>(c) Basic phospholipase A₂ only (15 min)</td>
<td>62.0 ± 3.0</td>
</tr>
<tr>
<td>(d) Basic phospholipase A₂ only (2 min)</td>
<td>19.7 ± 6.0</td>
</tr>
<tr>
<td>(e) Basic enzyme (2 min) followed by washing and acidic enzyme (30 min)</td>
<td>30.7 ± 3.6</td>
</tr>
</tbody>
</table>

**Acidic phospholipase A₂ or Basic phospholipase A₂**

**Sequential treatments of intact erythrocytes with acidic and basic phospholipase A₂**

Incubations of human erythrocytes with phospholipase A₂ (100 μg/ml, 30 mM Ca²⁺) and the procedure for washing were as described in the text. The period of the incubations varied as shown. Little or no hemolysis occurred under these conditions. Values are presented as mean ± S.D. for four different erythrocyte samples.
domain of PC which existed in a separate environment in the membrane. This conclusion thus is supported by two experimental observations: (a) acidic and basic enzymes separately hydrolyze 20% and 60% of PC, respectively, without hemolysis, and (b) the sequential treatments of intact cells with acidic followed by basic or vice versa always revealed distinct patterns of hydrolysis of PC by the two enzymes (see Fig. 1 and Table I).

There are several possible explanations to account for the proposal that acidic and basic enzymes recognize different PC in distinct environments in intact erythrocytes. For example, if we consider the charge characteristics of these enzymes as important in the attack, then probably the phospholipase A₂ interaction occurs with PC molecules which are located in a membrane area having a complementary charge property. One way to test this point was to examine the attack of the two enzymes by manipulating the pH of the medium (in the range from pH 5 to 8). However, no differences in the mode of attack of these enzymes were observed under the above experimental conditions. Also, the observed differences in PC hydrolysis by the acidic and basic enzymes cannot be explained by a product inhibition of the enzyme as reported for the action of *Crotalus atrox* on single bilayer vesicles (18). If lysis is allowed to occur, then a prolonged incubation with these enzymes with erythrocytes does cause a further hydrolysis of PC and PE. Another consideration is that the nature of lipid-protein, lipid-lipid interactions in these domains are different. In addition, an interesting explanation may lie in the differences in the composition of the 2-acyl chain of PC molecules which may exist in distinct domains. This latter point was explored.

**Characterization of Fatty Acids Released under the Non-lytic Action of Acidic and Basic Phospholipases A₂ on Intact Human Erythrocytes**—In order to gain further insight into the mode of attack and also on the recognition of discrete PC pools by these two phospholipases A₂, we have explored the fatty acid patterns, the ratio of saturated to unsaturated fatty acids released at different time intervals are presented. Values are given as mean ± S.D. (three to four experiments).

<table>
<thead>
<tr>
<th>Phospholipase A₂</th>
<th>Time of incubation (min)</th>
<th>Ratio of (16:0 + 18:0) to (18:1 + 18:2)</th>
<th>Ratio of 18:1/18:2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic</td>
<td>5</td>
<td>1.144 ± 0.5</td>
<td>0.846 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.439 ± 0.182</td>
<td>0.734 ± 0.294</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.205 ± 0.023</td>
<td>0.615 ± 0.113</td>
</tr>
<tr>
<td>Basic</td>
<td>2</td>
<td>0.114 ± 0.025</td>
<td>1.752 ± 0.858</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.124 ± 0.041</td>
<td>1.718 ± 0.331</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.148 ± 0.024</td>
<td>0.926 ± 0.223</td>
</tr>
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</table>

**Table II**

Patterns of fatty acids released by the action of acidic or basic phospholipase A₂ on human erythrocytes

Intact erythrocytes were treated with purified acidic or basic phospholipase A₂ (100 μg/ml, 10 μM Ca²⁺) for various time periods as described below. Fatty acids released by the phospholipase A₂ action were extracted together with lipids, separated by thin-layer chromatography, and then converted to their methyl esters before analyzing them by gas-liquid chromatography (see text for details). Ratios of the peak area of saturated (16:0 + 18:0) to unsaturated (18:1 + 18:2) fatty acids and of fatty acids 18:1 to 18:2 are presented. Values are given as mean ± S.D. (three to four experiments).

The data obtained in these studies provide strong evidence that PC in intact human erythrocyte membranes is arranged in distinct domains. This was established using physicochemically different pure phospholipases A₂ (acidic and basic) which attacked the membrane PC in a highly characteristic fashion prior to hemolysis. The evidence would also support the argument that the acidic and basic enzymes have different affinity sites on the membrane. When pure phospholipid dispersions are used as substrates, these enzymes do not exhibit such characteristic differences in their mode of attack as is evident on native membranes. The differences in the fatty acids released by the acidic and basic enzymes also support our conclusion that we are dealing with PC molecules which are located in separate domains. It is relevant to mention that separate pools or domains of phosphatidylglycerol in bacterial membranes (13), phosphatidylethanolamine in erythrocyte membranes (14), phosphatidylcholine in platelet membranes (15), and the existence of diacylglycerol in discrete regions in erythrocyte membranes (16) have been suggested. Furthermore, it is of considerable interest that recently, the existence of phosphatidylinositol-specific phospholipase C, phosphatidylinositol was shown to exist in discrete populations in rat hepatocytes (17). It is therefore conceivable that occurrence of individual phospholipids in pools or domains may be a general feature of membranes. The nature of these domains is not known. Whether they consist of protein-free lipid regions or protein-containing lipid regions, or in any other form, remains to be ascertained. Obviously, the knowledge of molecular details of these domains and their importance in membrane function would be topics of considerable research interest.

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**REFERENCES**


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