Biochimica et Biophysica Acta 899 (1987) 205–212
Elsevier

BBA 73559

Different susceptibilities of platelet phospholipids to various phospholipases and modifications induced by thrombin. Possible evidence of rearrangement of lipid domains

Cheng-Teh Wang, Wei-Jern Tsai, Shee-Ming Chang, Young-Ji Shiao, and Chen-Chung Yang

Institute of Life Science, National Tsing Hua University, Hsinchu, Taiwan 300 (Taiwan, Republic of China)

(Received 17 September 1986)
(Revised manuscript received 27 January 1987)

Key words: Platelet membrane; Lipid domain; Phospholipase A_2; Susceptibility

On the membrane surface of the human platelet, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were hydrolyzed to different extents by the snake venom phospholipases A_2 of varying p*I* values. The susceptibility of platelet phospholipids to basic phospholipase A_2 of *Naja nigricollis* (p*I* 10.6) has been reported (Wang et al. (1986) Biochim. Biophys. Acta 856, 244–258). The susceptibilities of platelet phospholipids to acidic phospholipase A_2 of *Naja naja atra* (p*I* 5.2) and to neutral phospholipase A_2 of *Hemachatus haemachatus* (p*I* 7.3) were investigated in this study. In gel-filtered platelets, acidic phospholipase A_2 hydrolyzed 35% PC and 10% PE, while neutral phospholipase A_2 hydrolyzed 18% PC and 3% PE. In thrombin-induced shape-changed platelets, acidic phospholipase A_2 hydrolyzed 20% PC and 10% PE, while neutral phospholipase A_2 hydrolyzed 15% PC and 6% PE. In thrombin-activated platelets, acidic phospholipase A_2 hydrolyzed 25% PC and 7% PE, while neutral phospholipase A_2 hydrolyzed 25% PC and 10% PE. Sequential lipid hydrolysis experiments showed that basic phospholipase A_2 of *Naja nigricollis* could hydrolyze the remaining PC and PE in the membrane previously treated with the neutral enzyme. The results may mean that: (1) the PC and the PE domains exist on the platelet membrane surface; and (2) the lipid domains on the membrane surface of resting platelets are rearranged by thrombin.

Introduction

Membrane components are asymmetrically distributed in the lipid bilayer [1,2]. Lipid molecules may be organized nonhomogeneously in the lateral distribution to form domains. The site heterogeneity may have significance for the membrane structure-functional relationship [3]. Phospholipids are asymmetrically distributed in the platelet plasma membrane [4–9]. In the case of the resting platelet, Kannagi et al. [10] have suggested that a phosphatidylcholine (PC) domain surrounds the endogenous phospholipase A_2. Also, Karnovsky et al. [3] have indicated that a mixture of gel-liquid-like lipid domains exists in the platelet membrane.

Phospholipids in the platelet membrane outer leaflet are mainly PC, PE and sphingomyelin [4–9]. The existence of PC and PE domains on the membrane surface remains to be demonstrated.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid.

Correspondence: C.-T. Wang, Institute of Life Science, National Tsing Hua University, Hsinchu, Taiwan 300, Republic of China.

Shukla and Hanahan [11] have used the phospholipases A_2 with different p*I* values as probes to demonstrate the existence of PC domains in the erythrocyte membrane. We have employed the basic phospholipase A_2 of *Naja nigricollis* (p*I* 10.6) to estimate the asymmetric distribution of phospholipids in the platelet membrane [9]. In this study, another two phospholipases A_2 of different p*I* values were used to hydrolyze the human platelet membrane. These two phospholipases A_2 were acidic phospholipase A_2 of *Naja naja atra* (p*I* 5.2) and neutral phospholipase A_2 of *Hemachatus haemachatus* (p*I* 7.3) [12,13]. It has been reported that these two enzymes and the basic phospholipase A_2 have comparable substrate specificities to PC and PE [14,15]. Also, these two enzymes are known to be nonmembrane-lytic to erythrocytes and other tissues [14,15]. Platelets in three physiological states were prepared as the substrates. The states were the resting, the thrombin-induced shape-changed and the thrombin-activated states. The results showed the different susceptibilities of platelet phospholipids to these phospholipases. This may mean that both PC and PE domains existed on the membrane surface of resting platelet, and that thrombin rearranged the lipid domains. A preliminary report on this work has appeared elsewhere [16].

Experimental procedures

Materials

Chemicals and organic solvents were purchased from E. Merck (Darmstadt, F.R.G.). All organic solvents were redistilled before use. The following reagents were obtained from Sigma (St. Louis, MO, U.S.A.): bovine thrombin, collagen (type III from calf skin), hirudin, and fatty acid free bovine serum albumin. Sepharose 2B came from Pharmacia (Piscataway, NJ, U.S.A.). The acidic, neutral and basic phospholipases A_2 were purified from the venom of *Naja naja atra* [12], *Hemachatus haemachatus* [13], and *Naja nigricollis* [17], respectively. The carrier-free [^{32}P]phosphate (2 mCi/ml, pH 7.4) was a generous gift from the Department of Radioisotope, National Tsing Hua University, Hsinchu, Taiwan, Republic of China.

Methods

Preparation of platelets in three different physiological states

9 vols. of fresh, healthy human blood were drawn into a plastic centrifuge tube containing 1 vol. of 0.11 M sodium citrate. The blood was centrifuged by swinging centrifugation at 250 *g* for 15 min at room temperature. The resting platelets were isolated from platelet-rich plasma by the gel-filtration according to the method of Lages et al. [18]. Unless otherwise specified, the buffer solution used in this study was the modified calcium free Tyrode's buffer containing 0.1% dextrose and 0.2% bovine serum albumin. The ^{32}P-labeled platelets were prepared in the same manner, except that 20 ml of platelet-rich plasma was incubated with 5 mCi of the carrier free [^{32}P]phosphate for 1 h prior to gel-filtration. The cell number was estimated in a hemacytometer by a phase contrast microscope (Type 104, Nikon, Tokyo, Japan).

The shape-changed platelets were prepared by a modification of the study of Holmsen et al. [19]. The gel-filtered platelets ($1.5 \cdot 10^8$ cells/ml) were incubated at 37°C for 5 min. Hirudin (1 U/ml) was then added. 1 min later thrombin (0.05 U/ml) was introduced. The suspension was then centrifuged. The pellet was resuspended in the buffer.

The thrombin-activated platelets were prepared as described previously [20]. In brief, the gel-filtered platelets ($1.5 \cdot 10^8$ cells/ml) were suspended in the buffer containing 2 mM EDTA (pH 7.4), and incubated at 37°C for 5 min. Thrombin (0.05 U/ml) was then added. After 2 min of incubation, the suspension was centrifuged. The pellet was resuspended in the buffer containing 2 mM EDTA.

Hydrolysis of platelet phospholipids by phospholipases A_2

For the hydrolysis of phospholipids in the gel-filtered platelets, the cells were washed with 2 mM EDTA once to prevent the contamination of phospholipids from plasma. The platelet suspension ($1.5 \cdot 10^8$ cells/ml) in each physiological state was incubated with 1 mM Ca^{2+} and 10 μM indomethacin at 37°C for 5 min. Lipid hydrolysis was initiated by adding 1 μg/ml of phospholipase

A_2. The reaction was stopped by introducing 2 mM EDTA, and chilled in ice. For studying the lipid hydrolysis of the activated platelets, the cells were incubated with 2 mM EDTA/10 μM indomethacin at 37°C for 5 min. Phospholipase A_2 (1 μg/ml) was then added, and 3 mM Ca^{2+} was immediately followed to initiate the reaction. The reaction was terminated by added 2 mM EDTA, and chilled in ice. The time-course of the hydrolysis of each lipid class was followed. A control experiment was performed by incubating platelets with the enzyme alone without adding Ca^{2+}. The experiment showed that these phospholipases A_2 could not hydrolyze lipid in the absence of Ca^{2+}, within a span of 3 h incubation.

Sequential lipid hydrolysis by phospholipases A_2

Platelets ($1.5 \cdot 10^8$ cells/ml) of each physiological state were first hydrolyzed by 1 μg/ml neutral phospholipase A_2 for 10 min in a condition as described above. The reaction was stopped by adding 2 mM EDTA and centrifuged at $1000 \times g$ for 20 min, at room temperature. The neutral phospholipase A_2-treated platelets were resuspended in the buffer in a concentration of $1.5 \cdot 10^8$ cells/ml. The suspension was incubated with 1 mM Ca^{2+}/10 μM indomethacin at 37°C for 5 min. Then 1 μg/ml of either acidic or basic phospholipase A_2 was added to initiate the second hydrolysis. The reaction was stopped by adding 2 mM EDTA. In a control experiment, the readdition of 1 μg/ml neutral phospholipase A_2 was also performed as a control.

Analysis of lipid classes

Lipids were extracted from the hydrolytic reaction medium according to the method of Bligh and Dyer [21]. Each lipid class was identified by the two-dimensional thin-layer chromatography as described previously [9]. The thin-layer plate was prepared by the impregnation of silica gel 60H with 0.9% of magnesium acetate. The solvent systems were chloroform/methanol/25% ammonia/water (60 : 35 : 4 : 4, v/v) in the first dimension and chloroform/methanol/glacial acetic acid (60 : 27 : 8, v/v) in the second one. The chromatograms were visualized by iodine.

The concentration of phospholipid was determined by the estimation of phosphorus content as described by Rouser and Fleischer [22]. Each hydrolyzed phospholipid class was expressed as the percentage of its total amount in the platelet. The estimation of both hydrolyzed phosphatidylserine (PS) and phosphatidylinositol (PI) was calculated using sphingomyelin as an internal standard.

The hydrolysis of the ^{32}P-phosphate labeled platelets was performed as described above. In the control experiments, phospholipase A_2 was not added. Before thin-layer chromatography was performed, 10 μg of lipid phosphorus from the human erythrocyte lipid was added into the platelet lipid extract as an external standard. The radioactivity of each lipid class was determined by adding 5 ml of Econofluor (New England Nuclear) into the scraped sample, and counted in a scintillation counter (Beckman, Model LS-100C, U.S.A.).

Results

Hydrolysis of phospholipids of platelets in different physiological states by phospholipases A_2

Platelets in three different physiological states were prepared as the substrates of acidic and neutral phospholipases A_2. Table I presents the percentages of membrane surface lipids of the platelets in each physiological state hydrolyzed by phospholipases A_2 with different pI values. It shows that the substrate availabilities of these enzymes were different.

In gel-filtered platelets, acidic and neutral phospholipases A_2 accomplished their lipid hydrolyses of resting platelet phospholipids within 5 min (Figs. 1 and 2). No further hydrolysis was observed in a prolonged 2 h incubation. These two enzymes only hydrolyzed PC and PE. They did not hydrolyze either phosphatidylserine (PS) or phosphatidylinositol (PI). In the presence of 0.02% Triton X-100, these two enzymes completely hydrolyzed PC and PE (Figs. 1 and 2). This implies that these enzymes had a similar substrate specificity to the platelet PC and PE. The platelets, incubated with either acidic or neutral phospholipase A_2 for 2 h, exhibited the following properties (data not shown): (1) the cytoplasmic marker enzyme, lactate dehydrogenase, did not leak out of the cell; (2) the Ca^{2+}-uptake time-course profile of the cells was similar to that of the

TABLE I

HYDROLYSIS OF PHOSPHOLIPIDS ON THE MEMBRANE SURFACE OF HUMAN PLATELETS BY PHOSPHOLIPASES A_2 WITH DIFFERENT p*I* VALUES

Data (mean ± S.D.) were taken from at least ten independent experiments of the hydrolysis of each phospholipid class by the phospholipases A_2. The acidic (p*I* 5.2), neutral (p*I* 7.3), and basic (p*I* 10.6) phospholipases A_2 (PLA_2) were purified from the snake venom of *Naja naja atra* [12], *Hemachatus haemachatus* [13], and *Naja nigricollis* [15], respectively.

Type of platelets [a]	Phospholipid class	% of total of each lipid class hydrolyzed		
		Acidic PLA_2	Neutral PLA_2	Basic PLA_2 [b]
GFP	PC	34.9 ± 1.0	18.2 ± 1.2	38.0 ± 1.2
	PE	10.4 ± 0.6	3.1 ± 0.1	30.5 ± 0.5
SCP	PC	20.4 ± 0.8	15.0 ± 0.6	38.0 ± 1.5
	PE	10.4 ± 0.6	6.4 ± 0.5	30.0 ± 1.5
TAP	PC	25.5 ± 1.1	25.4 ± 1.3	45.0 ± 0.8
	PE	7.3 ± 0.5	10.1 ± 0.7	30.0 ± 0.4

[a] The three physiological types of platelet were the resting gel-filtered (GFP), the thrombin induced shape-changed (SCP), and the thrombin-activated (TAP) platelets.

[b] The data were taken from the previous study [9].

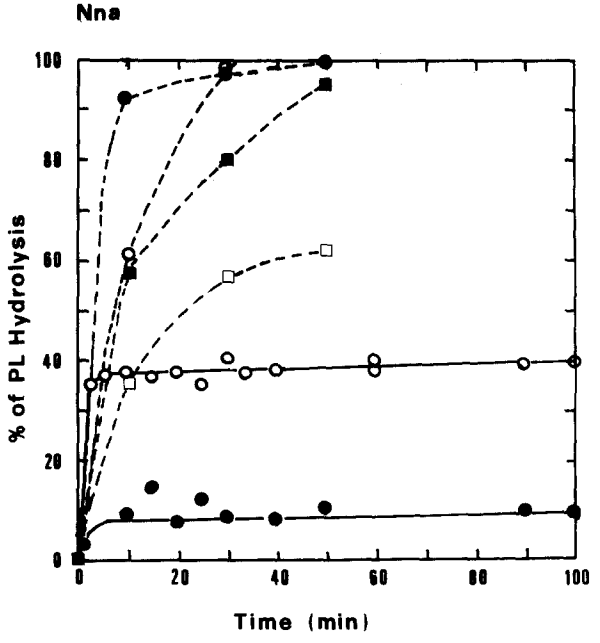

Fig. 1. Hydrolysis of phospholipids in resting platelets by the phospholipase A_2 of *Naja naja atra*. Details of the experiments are given in Experimental procedures. The phospholipid classes are PC (○), PE (●), PS (□), and PI (■). The dashed lines indicate the lipid hydrolysis found in the presence of 0.02% Triton X-100.

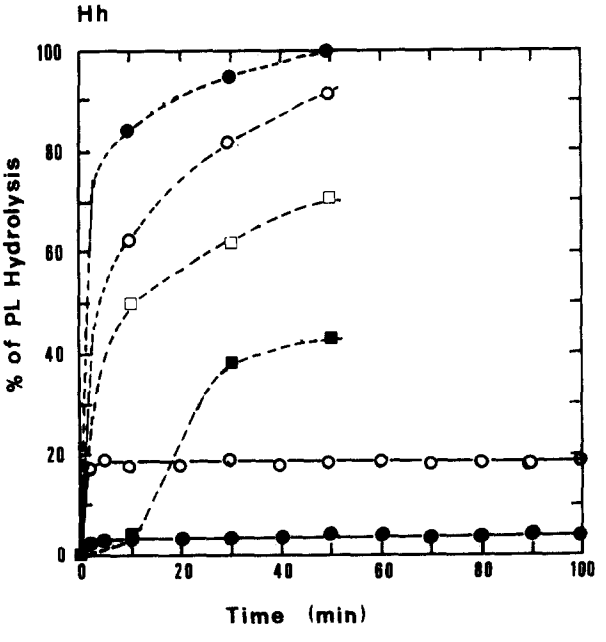

Fig. 2. Hydrolysis of phospholipids in resting platelets by the phospholipase A_2 of *Hemachatus haemachatus*. Details of the experiments are given in Experimental procedures. The phospholipid classes are PC (○), PE (●), PS (□), and PI (■). The dashed lines indicate the lipid hydrolysis found in the presence of 0.02% Triton X-100.

control; (3) the enzyme-treated cells still responded to the stimulation of agonists, such as thrombin and collagen; and (4) no cell lysis was observed by scanning electron microscopy. The enzyme-treated cells changed their shapes with pseudopod formation. The appearance was similar to that effected by the basic phospholipase A_2 in the first lipid hydrolytic phase [9]. The ^{32}P-phosphate-labeled platelets were used to investigate the physical aspects of lipid hydrolysis by acidic phospholipase A_2 (Fig. 3). The enzyme enhanced the ^{32}P-incorporation rates of PC and PI. However, it did not cause any increase in the radioactivity of either [^{32}P]lysoPC or [^{32}P]phosphatidic acid (PA). The neutral phospholipase A_2 displayed a similar to the acidic enzyme (data not shown). The results may indicate that these two enzymes did not induce the flip-flop of phospholipid in the membrane. These enzymes might only hydrolyze phospholipids on the outer leaflet of platelet membrane.

In shape-changed and inactivated platelets, these two enzymes hydrolyzed both PC and PE showing a biphasic time-course profile (Fig. 4). However, the biphasic hydrolysis patterns were different. In shape-changed platelets, the acidic phospholipase A_2 hydrolyzed 11% PC and 5% PE within 1 min. It hydrolyzed an additional 5% PC and 5% PE after a prolonged 20 min of incubation. Neutral phospholipase A_2 hydrolyzed 12% PC and 5% PE within 5 min, and it hydrolyzed another 3% PC after 30 min of incubation. In activated platelets, acidic and neutral phospholipases A_2 possessed similar time-course profiles in the hydrolysis of PC. The enzymes cleaved 15% PC in 5 min, then reached a plateau. After 30 min of incubation, the enzymes hydrolyzed another 10% PC. However, the biphasic hydrolysis patterns of PE for these two enzymes were different (Fig. 5). The acidic enzyme firstly hydrolyzed only 1% PE. It gradually hydrolyzed PE up to 7% in a prolonged 30 min incubation. Neutral phospholipase A_2 first hydrolyzed 5% PE, and then cleaved another 5% PE after 30 min of incubation.

In comparison (cf. Table I), the acidic phospholipase A_2 hydrolyzed more PC in resting platelets than in either shape-changed or activated platelets. Conversely, the neutral phospholipase A_2 hydrolyzed more phospholipids in activated platelets than in either resting or shape-changed platelets. This may mean that different lipid environments appeared on the membrane surfaces of platelets in the three physiological states.

Sequential lipid hydrolysis

In this study, phospholipases A_2 with different p*I* values behaved differently in hydrolyzing human platelet lipids (Table I). A possible explanation was that these three enzymes recognized different domains of phospholipids on the outer surface of platelet membrane. To test this, sequential lipid hydrolysis experiments were performed.

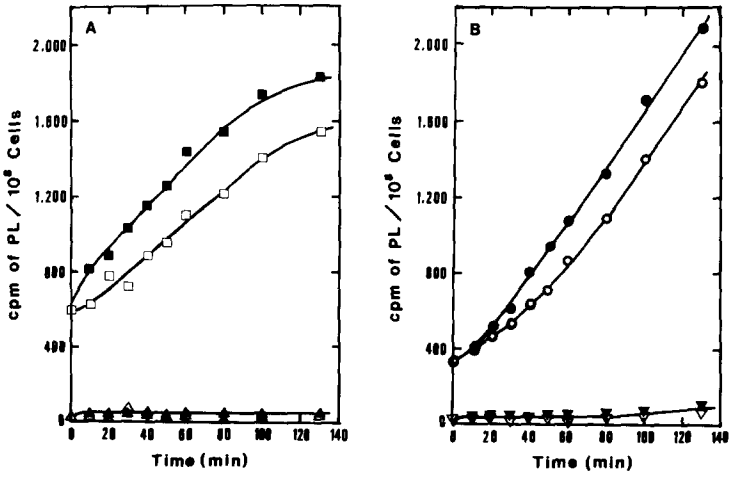

Fig. 3. The effect of acidic phospholipase A_2 on the incorporation of [^{32}P]phosphate gel-filtered platelet phospholipids. The solid symbols indicate the experiments in which acidic phospholipase A_2 was added. The open symbols represent the control experiments. Details of the experiments are given in the Experimental procedures. The hydrolysis of PI (□, ■), PA (△, ▲), PC (○, ●) and lysoPC (▽, ▼) are shown.

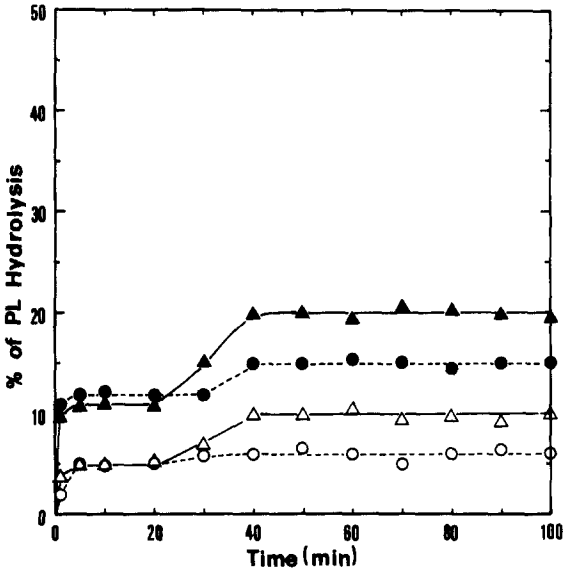

Fig. 4. Hydrolysis of PC (▲, ●) and PE (△, ○) in shape-changed platelets by the acidic (▲, △) and neutral (●, ○) phospholipases A_2 are shown. Details of the experiments are given in Experimental procedures.

The platelets were first treated with neutral phospholipase A_2 for 10 min and then washed. The neutral enzyme-pretreated platelets were then re-

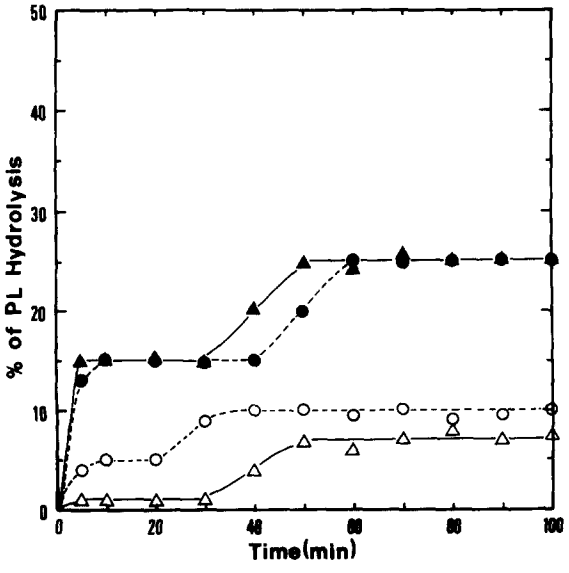

Fig. 5. Hydrolysis of PC (▲, ●) and PE (△, ○) in thrombin-activated platelets by the acidic (▲, △) and the neutral (●, ○) phospholipases A_2 are shown. Details of the experiments are given in Experimental procedures.

incubated with basic phospholipase A_2. The results for resting platelets showed that basic enzyme could hydrolyze lipids which were not susceptible to neutral phospholipase A_2 (Fig. 6).

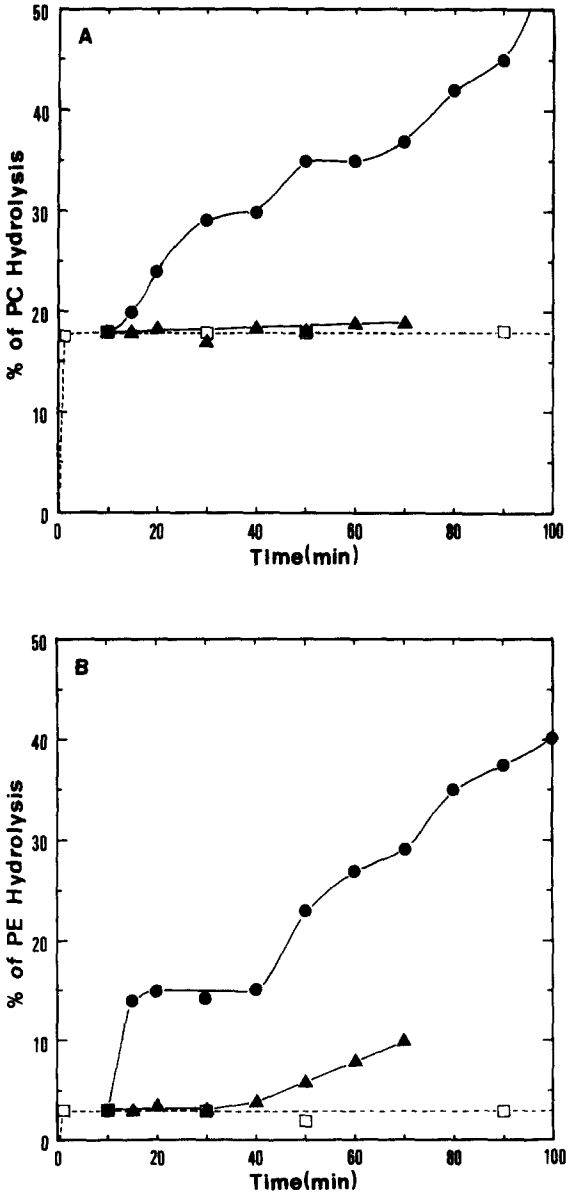

Fig. 6. The sequential hydrolysis of PC (A) and PE (B) of resting platelets by treated with the neutral phospholipase A_2 first and followed by either the basic (●) or the acidic (▲) phospholipases A_2. Details of experiments are given in Experimental procedures. Controls (□) represent both PC (A) and PE (B) in the neutral enzyme-treated gel-filtered platelets hydrolyzed by the neutral phospholipase A_2 again.

Control experiments, in which either calcium ion alone or neutral phospholipase A_2 was readded into the washed neutral phospholipase A_2-pretreated platelets, showed no further lipid hydrolysis Acidic phospholipase A_2 did not hydrolyze the remaining PC on neutral phospholipase A_2-pretreated platelets. However, it hydrolyzed 5% more of PE. This may indicate that the neutral phospholipase A_2 had changed the membrane surface environment which was unfavorable for the hydrolysis of PC by the acidic enzyme.

In shape-changed and in activated platelets, the sequential lipid hydrolysis also showed that lipids remained in neutral enzyme-pretreated platelets could be gradually hydrolyzed by the basic phospholipase A_2 (data not shown). The hydrolytic time-course profiles were similar to those with the basic enzyme alone [9]. Control experiments for shape-changed platelets showed that readdition of neutral phospholipase A_2 into the enzyme-pretreated platelets could further hydrolyze 3% more PC, while the control experiments for activated platelets showed that lipids remaining in the enzyme-pretreated platelets could be further hydrolyzed up to 25% PC and 10% PE. These results may mean that the PC and the PE domains appeared on the surface of resting platelets, and that thrombin induced the rearrangement of lipid domains on the membrane surface of platelets at the shape-changed state.

Discussion

The acidic and the neutral phospholipases A_2 employed in this study hydrolyze only a limited amount of phospholipids in human platelet (Figs. 1, 2, 5 and 6). These two enzymes are non-lytic toward human platelets. The enzymes may only attack phospholipids in the outer leaflet of platelet membrane.

This study and the previous report [9] show the different susceptibilities of human platelet phospholipids to three kinds of phospholipase A_2 of different p*I* values (Table I). This phenomenon can be explained in several ways. However, two of the considerations may be excluded. One is that the substrate specificities of these three enzymes toward human platelets may be different. We have observed that these enzymes exhibit the similar substrate specificities toward PC and PE of platelets (Figs. 1 and 2 and Ref. 9). Also, Condrea et al. [14,15] have reported that these enzymes have the similar specificities toward liposomes prepared from mixture of purified PC and PE. The second consideration that may be excluded is that of steric and charge hindrances of the cell surface in the action of the enzymes. The enzymes employed in this study have the same molecular size and conformation [23], and we have also observed that the enzymes exhibit the same mode of action in lipid hydrolysis by manipulating the pH of the medium (from pH 6 to 8.5) (data not shown). The possible explanations for this phenomenon may be the heterogeneity of platelet population, or the fact that these enzymes have different sites of affinity to the platelet membrane. The sequential lipid hydrolysis experiments may support the latter (Figs. 4 and 5). It is conceivable that PC and PE domains exist on the platelet membrane surfaces. Phospholipids of platelets in three physiological states are hydrolyzed to different extents by these three enzymes (Table I). It is conceivable that the distribution of existing lipid domains on the membrane surface of the resting platelet is changed in the case of the shape-changed as well as in the case of the activated platelets. Dynamic motion of the membrane components may be important in the platelet activation [24].

In summary, this report shows the different substrate susceptibilities of platelet phospholipids to the phospholipases A_2 of varying p*I* values. It may mean that lipid domains do exist on the platelet membrane surface. Also, the domains may be rearranged by thrombin in the shape-changed platelets.

Acknowledgment

The research was supported by a grant from the National Science Council of the Republic of China.

References

1 Rothman, J.E. and Lenard, J. (1977) Science 195, 743–753
2 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71
3 Karnovsky, M.R., Kleinfled, A.M., Hoover, R.L. and Klausner, R.D. (1982) J. Cell Biol. 94, 1–6

4 Otnaess, A.B. and Holm, T. (1976) J. Clin. Invest. 57, 1419–1425
5 Chap, H.J., Zwaal, R.F.A. and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 467, 146–164
6 Perret, B., Chap, H.J. and Douste-Blazy, L. (1976) Biochim. Biophys. Acta 556, 434–446
7 Schick, P.K., Kurica, K.B. and Chacko, G.K. (1976) J. Clin. Invest. 57, 1221–1226
8 Bevers, E.M., Comfurius, P. and Zwaal, R.F.A. (1983) Biochim. Biophys. Acta 736, 57–66
9 Wang, C.T., Shiao, Y.J., Chen, J.C., Tsai, W.J. and Yang, C.C. (1986) Biochim. Biophys. Acta 856, 244–258
10 Kannagi, R., Koizumi, K. and Masuda, T. (1981) J. Biol. Chem. 256, 1177–1184
11 Shukla, S.D. and Hanahan, D.J. (1982) J. Biol. Chem. 257, 2908–2911
12 Yang, C.C., King, K. and Sun, T.P. (1981) Toxicon 19, 645–659
13 Yang, C.C. and King, K. (1980) Toxicon 18, 529–547
14 Condrea, E., Yang, C.C. and Rosenberg, P. (1980) Biochem. Pharmacol. 29, 1555–1563
15 Condera, E., Flecher, J.E., Rapuano, B.E., Yang, C.C. and Rosenberg, P. (1981) Toxicon 19, 61–71
16 Wang, C.T., Chang, S.M., Tsai, W.J., Hsieh, R.C. and Yang, C.C. (1983) Thromb. Haemoctas. 50, 338
17 Yang, C.C. and King, K. (1980) Biochim. Biophys. Acta 614, 373–388
18 Lages, B., Scrutton, M.C. and Holmsen, H. (1975) J. Lab. Clin. Med. 85, 811–825
19 Holmsen, H., Dangelmaier, C.A. and Holmsen, H.K. (1981) J. Biol. Chem. 256, 9393–9396
20 Wang, C.T. and Schick, P.K. (1981) J. Biol. Chem. 256, 752–756
21 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917
22 Rouser, G. and Fleischer, S. (1967) Methods Enzymol. 10, 385–406
23 Dufton, M.J., Eaker, D. and Hider, R.C. (1983) Eur. J. Biochem. 137, 537–544
24 Tandon, N., Harmon, J.T., Robard, D. and Jamieson, G.A. (1983) J. Biol. Chem. 258, 11840–11845